Highly Efficient Mono-Functionalization of Knob-in-Hole Antibodies with Strain-Promoted Click Chemistry

Jorick J. Bruins,† Criss van de Wouw,‡ Koen Wagner,† Lina Bartels,‡ Bauke Albada,*‡ and Floris L. van Delft*†

†Laboratory of Organic Chemistry, Wageningen University & Research, Stippeneng 4, 6708 WE Wageningen, The Netherlands
‡AIMM Therapeutics, Meibergdreef 59, 1105 BA Amsterdam, The Netherlands

Supporting Information

ABSTRACT: Knob-in-hole antibodies can be utilized to introduce a single tag for chemo-enzymatic functionalization. By either introducing a single C-terminal sortase tag (sortase-tag expressed protein ligation) or tyrosine tag (G4Y), mono-functionalization of the monoclonal antibody trastuzumab was achieved rapidly and in high yields. This method was applied to selectively and efficiently introduce a single fluorescent tag, cytokine or single-chain variable fragment, as well as produce clean homo dimers of trastuzumab.

1. INTRODUCTION

Chemical conjugation of functional molecules to monoclonal antibodies is widely applied in fields spanning from fundamental biology research to targeted therapy. These applications include, but are not limited to, cellular imaging,1,2 antibody–drug conjugates,3,4 and diagnostics.5 Common conjugation strategies, based on side-chain modification of lysine or cysteine, are straightforward and efficient but provide poor control of regioselectivity and stoichiometry.6,7 With the expansion of the genetic code to incorporate noncanonical amino acids, chemically orthogonal handles can be introduced for chemoselective oxime ligation10 or click chemistry.11 In addition, a wide arsenal of methods is available for site-specific protein modification with varying degrees of versatility.12

Besides chemical strategies, enzymatic conjugations have emerged recently, as they enable highly controlled modification of antibodies through specific peptide tags.13 Examples of enzymatic processes include sortase ligation,18,15 phosphopantetheinyl transferase,16 and transglutaminase.17 Recently, we demonstrated that selective labeling of monoclonal antibodies can be ensured in a single step based on the introduction of a C-terminal tetra-glycyltyrosine tag (G4Y, Figure 1A).18,19 Specifically, oxidation of the phenol moiety of the tyrosine in the G4Y tag to an ortho-quinone by mushroom tyrosinase (mTyR) and in situ Diels–Alder reaction with bicyclo[6.1.0]-nonyne (BCN) leads to selective labeling in high yields and under mild conditions, with reaction rates exceeding those of the strain-promoted alkyne–azide cycloaddition by at least a factor of 500. This strain-promoted oxidation-controlled ortho-quinone cycloaddition (SPOCQ) was subsequently shown to also proceed with cyclopropenes, albeit significantly slower,20 or could be further accelerated by employing cyclopropanated trans-cyclooctene.21

Given the symmetrical nature of antibody, the site-specific methods described above lead to dual labeling of the protein. However, in some cases, forming a 2:1 antibody format (i.e., two antigen binding sites and one conjugated molecule of interest) may be more desirable, for example, in the generation of an antibody–drug conjugate with an extremely potent cytotoxin such as a pyrrolobenzodiazepine dimer22 or by radiolabeling with α-emitters such as thorium-227.23 While these formats have been reported, generation of these conjugates relies on approaches with poor control of site and stoichiometry like random lysine conjugation followed by isolation of the mono-functionalized conjugate,24 or required the rearrangement of several disulfide bridges between the light and heavy chains (HCs), resulting in a significant loss of binding activity and stability.25 Similarly, various antibody conjugates with a 2:1 format to enhance the therapeutic window, e.g., with IL-225 and α-CD3,26 have been reported. The latter conjugates were obtained by the fusion of an immunocytokine or T-cell engager to a single chain of an asymmetric antibody format, known as knob-in-hole (KiH) antibodies.

By changing only a few key amino acids in the CH3 domain that are pivotal to the pairing of the heavy chains, i.e., replacing a small side chain with a large side chain on one heavy chain (knob) and vice versa for the other heavy chain (hole), heterodimerization can be promoted.27,28 While the KiH technology has been applied to create immunocytokines based

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on genetic encoding of a C-terminal fusion protein on one of the heavy chains,25 a modular approach derived from a single engineered antibody scaffold would allow for easy access to a wide range of mono-functional conjugates.

Here, we show that the knob-in-hole technology can be readily applied for the generation of 2:1 antibody conjugates by selective strain-promoted cycloaddition of a single heavy chain (HC) based on ortho-quinone chemistry or tetrazine ligation (Figure 1B). We found that this strategy is suitable for the formation of antibody–small molecule, antibody–protein, and antibody–antibody conjugates.

2. RESULTS AND DISCUSSION

2.1. Two-Stage Antibody Conjugation Based on Tetrazine Ligation. A knob-in-hole variant of trastuzumab was generated by the introduction of a single mutation in the “knob” heavy chain (T366W; knob-HC) and three mutations in the “hole” heavy chain (T366S, L368A, and Y407V; hole-HC).31 For conjugation, the hole-HC contained a C-terminal sortase LPETG-tag (Supporting Information (SI)).14,31 After transient expression and subsequent purification by protein A affinity chromatography, analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), high-performance liquid chromatography (HPLC), and liquid chromatography-mass spectrometry (LC-MS) confirmed the existence of two distinct heavy chains (Figure 2A, SI). To facilitate analysis of the Fc region of the antibody by LC-MS, it was deglycosylated by endo S2 and digested below the hinge region by IdeS to generate the Fc/2 and F(ab’)2 fragments.22,32 Following these steps, one of the heavy chains was found to have a molecular weight of 25 565 Da, corresponding to the hole-HC containing the sortase tag (calc.: 25 564 Da). The second peak, corresponding to the knob-HC, was found to have a molecular weight of 24 220 Da (calc.: 24 221 Da).

The KiH trastuzumab containing the sortase tag (abbreviated as Tras[KiH-HC]ST) was subjected to sortase-mediated ligation to introduce trans-cyclooctene (Tras[KiH-HC]TCO) or a methyltetrazine moiety (Tras[KiH-HC]MeTz) for conjugation via inverse-electron demand Diels–Alder (IEDDA), also known as tetrazine ligation.33 The products resulting from sortagging were purified using protein A affinity column chromatography, after which the HPLC analysis showed full conversion of the starting material to the anticipated product with a single light chain and two distinct heavy chains (Figure 2B,C). In the obtained chromatograms,
the unmodified light chain and knob-HC eluted at 6.47–6.50 and 7.66–7.67 min, respectively, while elution of the hole-HC shifts from 7.51 min (sortase tag, A) to 7.83 min (TCO-group, B) or 7.77 min (MeTz-group, C). The LC-MS analysis confirmed the formation of the anticipated products, with no observed sortase-mediated hydrolysis (SI).

With the mono-functionalized antibodies in hand, Tras[KiH-HC]TCO was first reacted with a methyltetrazine-labeled fluorophore (MeTz-TAMRA). As expected, SDS-PAGE analysis showed a slight upward shift for the band corresponding to the hole-HC (Figure 3, lane 2), and the formation of a single fluorescently labeled band could be identified (SI). The HPLC analysis displayed an efficiency exceeding 90%, and LC-MS confirmed the formation of the desired hole-HC IEDDA product, while the knob-HC remained unmodified (SI).

Next, we pursued the preparation of monovalent antibody–protein conjugates based on this strategy. In particular, we considered the strategy for application in cancer immunotherapy by combining the targeting power of a monoclonal antibody with cytokine IL-2 or an α-CD3 T-cell engager. To this end, a MeTz moiety was attached to the C-terminus of either the cytokine interleukin 2 (IL-2) or to the short-chain variable fragment to UCHT1 (α-CD3 scFv) or using sortase. Subsequently, Tras[KiH-HC]TCO was incubated with an excess MeTz–IL-2 (Figure 3, lane 3) or MeTz–UCHT1 (Figure 3, lane 5), resulting in either MeTz-functionalized protein reacting with TCO-functionalized Tras[KiH-HC]TCO antibody. To be precise, MeTz–IL-2 showed a clean labeling of the hole-HC, resulting in the formation of a new band with the expected molecular weight of ~70 kDa, i.e., 50 kDa for the HC and 18 kDa for MeTz–IL-2. The HPLC analysis showed full conversion of the TCO-labeled hole-HC, which was confirmed by the LC-MS analysis (SI). Similarly, the reaction of Tras[KiH-HC]TCO with MeTz–UCHT1 showed a near full conversion to the expected 80 kDa band, with HPLC and LC-MS confirming the efficient formation of the expected bifunctional antibody via this method.

Having successfully demonstrated the suitability of tetrAzine ligation for the generation of 2:1 antibody–protein conjugates, we also explored the inverse tetrAzine ligation strategy. Thus, Tras[KiH-HC]MeTz was subjected to conjugation with either small-molecule fluorophore BCN–lissamine (Figure 3, lane 8 and the SI), TCO–IL-2 (Figure 3, lane 9), or TCO–UCHT1 (Figure 3, lane 11). In all cases, the desired mono-
functionalized antibody was successfully conjugated on the desired hole-HC, and the formation of the fluorophore-, IL-2-, and UCHT1-conjugates on Tras[KiH-HC]MeTz were confirmed by LC-MS (SI). Interestingly, SDS-PAGE shows that the conversion is significantly cleaner when compared to the Tras[KiH-HC]TCO MeTz–protein combination. A potential explanation for the lower conjugation efficiency of the TCO-modified antibody may be due to in situ isomerization of the trans-alkene in TCO caused by the cysteine thiol in the active site of sortase A. In this light, having the MeTz on the antibody and the TCO/BCN on the reactive protein partner is clearly the preferred strategy for antibody–protein conjugates.

Finally, we explored the potential of the two-stage protocol of sortagging–tetrazine ligation for the controlled formation of functionally homogeneous antibody dimers. To this end, Tras[KiH-HC]MeTz was incubated with Tras[KiH-HC]TCO in a 1.5:1 ratio. The rationale for the latter strategy was based on our finding that excess tetrazine-modified antibody could be conveniently removed by capturing with TCO-modified agarose beads, leading to clean antibody dimers (SI). Utilization of the modularity of these antibodies allows for the straightforward production of dimeric antibodies with good conversion and high purity, and offers a viable chemical alternative for known methods that require the expression of fusion proteins or are based on chemical approaches yielding highly heterogeneous antibody dimers.

2.2. Direct Conjugation Based on Ortho-Quinone Chemistry. Besides the two-step method that combines sortagging with MeTz or TCO followed by tetrazine ligation, the possibility of direct labeling of knob-in-hole antibodies with SPOCQ chemistry was investigated next. SPOCQ (strain-promoted oxidation-controlled ortho-quinone cycloaddition) was originally developed based on the chemical generation of ortho-quinones. Recently, we showed it could be conveniently applied in a one-pot process involving the oxidation of a tyrosine side chain to an ortho-quinone by the action of mushroom tyrosinase (mTyr), followed by in situ reaction with a BCN-bearing probe by (4 + 2) cycloaddition to form a stable product (Figure 4B). Earlier we described that SPOCQ on the symmetrical antibody Tras[HC]G4Y converts to 95% of the desired bisfunctionalized adducts when small fluorophores were conjugated. However, this level of conversion could not be achieved when protein–protein conjugation was attempted on symmetrical G4Y-tagged heavy-chain antibodies (SI). Presumably, the second ligation is sterically hindered, leading to intramolecular side reactions of the in situ generated, highly reactive ortho-quinone intermediate with nucleophilic side chains of amino acids such as lysine and histidine. However, we anticipated that a single SPOCQ conjugation event on a KiH antibody would not suffer from incomplete conjugation. In addition, direct SPOCQ would offer the opportunity to eliminate the sortase-mediated step, as an exposed tyrosine moiety can be readily engineered at the C-terminus of any protein.

Thus, a knob-in-hole antibody with the same four mutations as described above was expressed bearing a single C-terminal G4Y tag (Tras[KiH-HC]G4Y; Figure 4A). The antibody showed three distinct peaks on HPLC corresponding to the two different heavy chains and the light chain. Similar to our earlier approach, SPOCQ was first performed on Tras[KiH-HC]G4Y with BCN–lissamine, and subsequent HPLC analysis showed >95% conversion of G4Y-HC within 20 min (Figure 4C), significantly faster than 90 min required for the dual labeling of symmetrical Tras[HC]G4Y as reported earlier. Analogously to Tras[KiH-HC]ST, the knob-HC was found to have a molecular weight of 24 221 Da (calc. Mw: 24 221 Da) and 24 349 Da for the hole-HC containing the G4Y moiety (calc. Mw: 24 350 Da) (Figure 4D). After SPOCQ, the molecular weight of the hole-HC increased from 879 to 25 229 Da (calc. Mw: 25 229 Da). This corresponds to the addition of 14 Da during the oxidation by mTyr and addition of 865 Da by BCN–lissamine.

Finally, we explored the suitability of Tras[KiH-HC]G4Y for the generation of protein–protein conjugates. SDS-page (SI) and LC-MS analysis showed the formation of trastuzumab–UCHT1 conjugates by the SPOCQ conjugation of Tras[KiH-HC]G4Y (Figure 4E). Two masses were found by LC-MS: the first being 52 351 Da (calc. Mw: 52 351 Da), corresponding to the hole-HC plus 14 Da for oxidation by mTyr and the addition of BCN–UCHT1 (calc. Mw: 27 987.0 Da), and the second peak is 53 448 Da, corresponding to the same conjugate with incomplete deglycosylation by endo S2, corresponding to a G0F glycan (calc. Mw: 53 446 Da).

3. CONCLUSIONS

We have successfully developed a modular approach to site-selectively mono-functionalize one heavy chain of an antibody. Using asymmetrical knob-in-hole antibodies, a single peptide tag was readily introduced for chemo-enzymatic functionalization of one heavy chain by an indirect approach using sortase or by direct labeling with SPOCQ. Subsequently, the method was shown to allow for the rapid and selective introduction of a single fluorophore, a cytokine, or an scFv on the antibody. Additionally, highly efficient dimerization of monoclonal antibodies was achieved, which would allow for the production of clean bispecific antibodies in a modular fashion and with high conversions. The interest in these bispecific antibodies has increased significantly over the last years, since bispecific antibodies can directly target immune cells to tumors, with many of them in clinical trials. With SPOCQ, near-quantitative conversions are obtained after only 20 min of reaction time. We envision this modular approach may open up possibilities for new and potent therapeutic agents. Comparison of our technology with the established fusion formats for making such bioconjugates should reveal if our current method is, indeed, a viable chemical alternative to the more established biological methods.

4. EXPERIMENTAL SECTION

4.1. Materials. Unless stated otherwise, all chemicals and solvents were obtained from Sigma-Aldrich or Fisher Scientific and used as received. Dimethyl sulfoxide (DMSO) (molecular biology grade) and mushroom tyrosinase enzyme was obtained from Sigma-Aldrich. Dithiothreitol (DTT) was obtained from Fisher Scientific. 2-Mercaptoethanol (BME) and tetramethylenediamine were obtained from Acros. Sodium dodecyl sulfate (SDS), bromophenol blue, coomassie brilliant blue G250, sodium phosphate, sodium chloride, and Tris were obtained from VWR. Precision Plus Protein Dual Color Standards was obtained from Bio-Rad. Glycine was obtained from AppliChem. BCN-POE3-NH–lissamine rhodamine B conjugate was obtained from SynAffix. MeTz-TAMRA was obtained from BroadPharm. Trifluoroacetic acid (TFA), formic acid (FA), and MeCN were obtained from BioSolve.
Trastuzumab antibodies were ordered from Evitria AG and purified by Hitrap protein A HP (ProtA) column (5 mL) on an Agilent 1260 Preparative HPLC with diode-array detector (Section 4.6). The antibody, present in sterile filtered CHO media samples, were applied to the ProtA column without any pretreatment.

Sortase A, UCHT1 variants, and IL-2 variants were obtained and purified as reported.

4.2. Buffer Constitutions. Fifty millimolar phosphate was prepared from sodium phosphate monobasic. Phosphate-buffered saline (PBS) was 50 mM phosphate and 150 mM NaCl and was prepared from sodium phosphate monobasic. Tris-buffered saline (TBS) was 25 mM Tris and 150 mM NaCl and was prepared from Tris base. DTT was 200 mM DTT in 0.1 M Tris pH 8.0. Mycophenolate mofetil (MMF, MQ, and was prepared from Tris base. DTT was 200 mM DTT in 0.1 M Tris pH 8.0.

4.3. Sortase Reaction. Generally, Tras[KIH-HC]ST was constituted in a 10 mg/mL TBS pH 8.0. Typical reaction conditions were as follows: 100 μL of 10 mg/mL Tras[KIH-HC]ST (7.35 mg/mL final concentration), 10 μL of 17 mg/mL sortase A in TBS pH 8.0 (1 equiv), 13.6 μL of 100 mM CaCl2 in TBS pH 8.0 (10 mM final concentration), 4 μL of 50 mM sortase tag (G3-TCO, G3MeTz, G4Y) in DMSO (30 equiv), and 9.6 μL of DMSO (10% final concentration). The sample was incubated overnight at 37 °C and then purified using protein A affinity chromatography.

4.4. SPOCQ Reaction. In a typical experiment, Tras[KIH-HC]G4Y was constituted in PBS buffer (pH 5.5). The BCN-label BCN-lissamine in DMSO or BCN-UCHT1 in PBS was added in excess of 3–10 times and cooled to 4 °C. Finally, mTyr (10.0 mg/mL) in phosphate buffer pH 6.0 (50 mM phosphate) was added to obtain an antibody concentration of 5–6 mg/mL. The sample was set to react for 30 min.

4.5. IDEDA Reaction. In a typical experiment, Tras[KIH-HC]MeTz (2.0–4.0 mg/mL) was constituted in the PBS buffer (pH 7.2) and 2–5 equiv of label (e.g., BCN-lissamine in DMSO, TCO–UCHT1 in PBS pH 7.2) was added. The sample was set to react for 30 min.

4.6. Protein A Affinity Chromatography. For >1 mg of antibody, the antibody was purified by Hitrap protein A HP (ProtA) column (5 mL, run at 5 mL/min) on an Agilent 1260 Preparative HPLC. The ProtA column was pre-equilibrated with 20 mM sodium phosphate pH 7.0; the antibody was applied to the column and washed with 10 column volumes of 20 mM sodium phosphate pH 7.0. The product, which was eluted with 5 column volumes of 0.1 M citric acid pH 3.0, was obtained in 5 mL fractions and diluted with 1 mL of 1.0 Tris buffer of pH 9.0 each. The product fractions were concentrated and the buffer exchanged to the specified buffer. For <1 mg of antibody, NAB Protein A Plus Spin Columns (0.2 mL) were used for the purification of the antibody. Purification was performed exactly as stated in the manual. After the purification, the product fractions were concentrated and buffer exchanged with Amicon ultrafiltration units (50 kDa MWCO) to PBS. The concentration was determined using Thermo Fisher Scientific NanoDrop 2000.

4.7. SDS-PAGE Gel Preparation. Twelve percent acrylamide gels were prepared according to bio-rad bulletin 6201 protocol. Afterward, the gels were stained using coomassie brilliant blue (0.1% Coomassie Blue R250 in 10% acetic acid, 50% methanol, and 40% demineralized water) for 0.5 h and destained with destaining solution (10% acetic acid, 50% methanol, and 40% demineralized water) for 1 h. Afterward, the destaining solvent was replaced with water and shaken gently overnight at room temperature.

4.8. SDS-PAGE Sample Preparation. After the indicated reaction time had expired, 2.5 μg of antibody in 2.5 μL of PBS buffer was incubated with 2.5 μL of SDS-PAGE sample buffer (2×) including 5% BME for 5 min at 95 °C. The denatured sample was then applied for SDS-PAGE analysis (12% acrylamide gel).

Precision Plus Protein Dual Color Standards was used as a reference protein ladder.

4.9. HPLC Sample Preparation. Prior to HPLC analysis, the samples were diluted with 0.1 M Tris pH 8.0 to contain 10 μg of antibody in 10 μL of solution. Afterward, 5 μL of 0.2 M DTT in 0.1 M Tris pH 8.0 was added and the sample was incubated for 30 min at 37 °C. Next, 30 μL of MMF was added to the mixture.

Reverse-phase HPLC was performed on a Agilent 1220 HPLC Infinity using a Thermo Fisher Scientific MAbPac reversed phase (RP) column (3.0 × 50 mm2, 4 μm) column run at 0.6 mL/min at 80 °C using a 10 min linear gradient from 20 to 45% buffer B (with buffer A = 95% Milli-Q, 5% MeCN, 0.1% TFA and buffer B = 95% MeCN, 5% Milli-Q, 0.1% TFA).

4.10. LC-MS Sample Preparation. As reported earlier, trastuzumab can be denatured with DTT or FabRICATOR (Ides) before analyzing by LC-MS. DTT would denature all thios, resulting in light and heavy chains. However, FabRICATOR hydrolyzes the antibody just below the hinge region (...CAPPELLG/GPSVF...) without reducing the disulfide bridges, resulting in 2 Fc fragments of about 25 kDa containing the glycan chain and a F(ab′)2 region of about 96 kDa. For LC-MS, the samples were treated with GlycINATOR and subsequently FabRICATOR, unless stated otherwise.

The samples were analyzed on a Thermo Finningan Surveyor HPLC system with a ThermoScientific MAbPac reversed phase (RP) (3.0 × 50 mm2, 4 μm) column, coupled to a Thermo Fisher Scientific Q-Exactive Focus Orbitrap. Analysis was performed with a flow rate of 0.6 mL/min at 80 °C using a 10 min linear gradient from 20 to 45% buffer B (with buffer A = 95% Milli-Q, 5% acetonitrile, 0.1% formic acid (FA) and buffer B = 95% acetonitrile, 5% Milli-Q, 0.1% FA). If IL-2 was present in the mixture, the gradient was altered to a 10 min linear gradient from 20 to 45% buffer B, after which a 6 min linear gradient from 45 to 100% buffer B was applied.

Afterward, the chromatograms were analyzed using a Thermo Xcalibur Qual browser, exported to text files, and deconvoluted using UniDec software.

4.11. DTT. Prior to HPLC analysis, 0.2 M DTT solution in 0.1 M Tris pH 8.0 was freshly prepared. Antibody samples were diluted to 1 μg/μL antibody and 2.5 μL of DTT stock solution was added per 10 μg of antibody. The mixture was incubated for 30 min at 37 °C to reduce the interchain disulfide bonds, after which the reaction was quenched by adding 30 μL of a mixture of 49:49:2 (v/v/v) Milli-Q/McCN/FA per 10 μg of antibody.

4.12. GlycINATOR (endo S2). Trastuzumab was reduced by incubating a solution of 20 μg of antibody in PBS pH 7.2 with 10 μL of 2 U/μL GlycINATOR for 1 h at 37 °C.
4.13. FabRICATOR (IdeS). Trastuzumab was reduced by incubating a solution of 20 μg of antibody in PBS pH 7.2 with 1 μL of 20 U/μL FabRICATOR for 1 h at 37 °C.

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**Supporting Information**

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Methods and materials; protein structures; HPLC spectra; and LC-MS spectra (PDF)

**AUTHOR INFORMATION**

**Corresponding Authors**

*E-mail: Bauke.albada@wur.nl (B.A.).
E-mail: Floris.vandelft@wur.nl (F.L.v.D.).

**ORCID**

| Jorick J. Bruins: 0000-0003-1470-5557 |
| Bauke Albada: 0000-0003-3659-2434 |

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