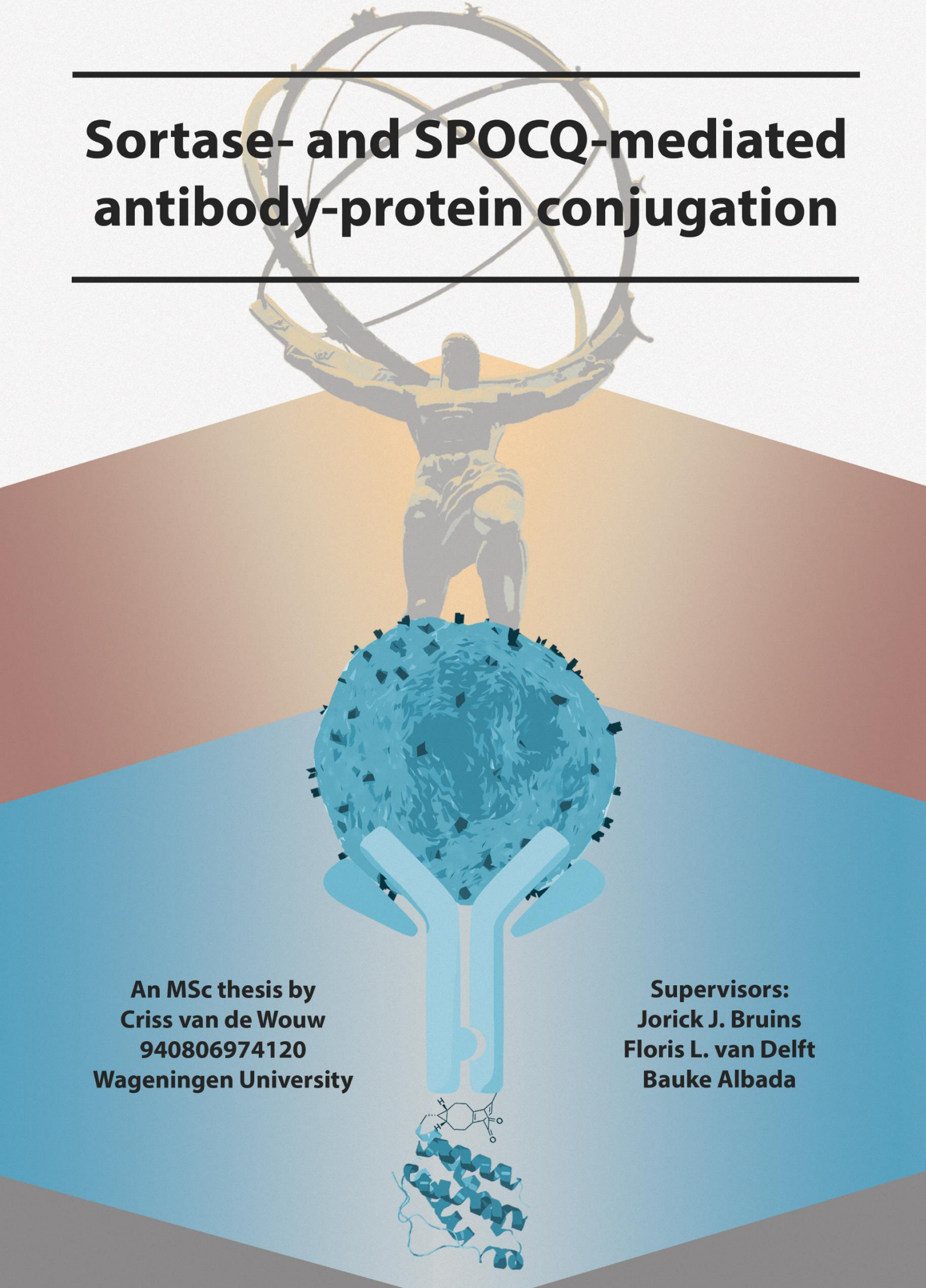
Sortase- and SPOCQ-mediated antibody-protein conjugation



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On the cover: *An analogy is made between the Atlas, as found in Rockefeller plaza in New York, and an antibody. In ancient Greek mythology the Atlas prevents the sky from crashing onto the earth, effectively forming a bridge between the ground and an imminent threat to humanity. In a similar fashion, an antibody can bridge a specific compound with a cancer cell, a grave threat to the safety of human individuals. At the base of the antibody an interleukin-2 molecule can be found, attached using strain-promoted oxidation-controlled ortho-quinone cycloaddition between a bicyclo[6.1.0]nonyne-moiety and a quinone.*

It is well understood that antibodies do not bind to epitopes the way it is displayed on the cover, but then again the Atlas is clearly displaying poor form by lifting with his back, not just his legs, so Lee Lawrie (the sculptor) understood that artistic freedom is more important than technical accuracy.

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Common abbreviations	**Meaning**
BCN	Bicyclo[6.1.0]nonyne
cpTCO	Cyclopropanated *trans*-cyclooctene
HHC	Hole-heavy chain
HPLC	High performance liquid chromatography
IL2	Interleukin 2
LCMS	Liquid chromatography mass spectrometry
Lis	Lissamine Rhodamine B
MeTz	Methyltetrazine
mTyr	Mushroom tyrosinase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPAAC	Strain-promoted alkyne-azide cycloaddition
SPIEDAC	Strain-promoted inverse electron-demand Diels-Alder cycloaddition
SPOCQ	Strain-promoted oxidation-controlled *ortho*-quinone cycloaddition
TCO	*Trans*-cyclooctene
UCHT1	Anti-human CD3 single chain variable unit

Abstract

Antibody-drug conjugates are potent biomolecules that combine specific targeting with an effector molecule, for example a cytotoxic compound or an immunomodulating molecule. Synthesis of these conjugates is often done by means of post-translational modification utilizing various biochemical strategies. One of these strategies is strain-promoted oxidation-controlled *ortho*-quinone cycloaddition (SPOCQ), a bio-orthogonal click reaction between an *ortho*-quinone and a strained-alkene or –alkyne. The quinone is generated from an exposed C-terminal tyrosine moiety using mushroom tyrosinase, allowing site-selective modification of the antibody. However the possibility of creating antibody-protein conjugates using this method was previously left unexplored. Here we show a two-step method to synthesize an antibody-protein conjugate by first introducing a tetra-glycyltyrosine onto trastuzumab via sortase ligation and subsequently performing SPOCQ with a bicyclo[6.1.0]nonyne-protein probe. The SPOCQ reaction was finished within 1 hour and the conjugation rate over both reactions was ~85%. Starting with a knob-in-hole antibody containing a sortase tag on the hole-heavy chain, a large variety of tags were installed using sortase ligation. This allows for conjugation of proteins onto antibodies using strain-promoted inverse electron-demand Diels-Alder cycloaddition (SPIEDAC), SPOCQ and strain-promoted alkyne-azide cycloaddition (SPAAC). This results in a toolbox capable of performing various click-reactions on antibodies.

Introduction

Modified antibodies are an important tool for identifying or combatting diseases in medicine.[1,2] By attaching tracer molecules to a specific antibody a diseased area, for instance a tumor, can be marked.[3] Alternatively, an antibody can be conjugated with a cytotoxic compound, delivering its payload specifically to its target cells.[4] The specificity is highly important to decrease adverse effects of systemic drug release. Similar to non-protein ligands on antibodies, antibody-protein conjugates have many applications. Attaching a fluorophore, such as green-fluorescent-protein, allows visualization, while enzyme conjugation can be used for immunoblotting.[1] Another protein candidate are tumoricidal agents, which are often plant-based toxins.[5] Together with an antibody they create a highly potent immunotoxin, which showed some clinical promise.[6] However, due to fast clearing from the blood stream and low-dosage requirements means medical applications are unlikely. A promising subcategory of proteins is those with an immunoregulatory function. Interleukin-2 (IL2) is a protein that promotes proliferation of $CD4^+$ and $CD8^+$ T-cells.[7,8] These cells can then specifically target and subsequently destroy other cells, for example cancer cells.

Antibody-single chain variable domain (scFv) conjugates, which are a subclass of bispecific antibodies, have found clinical use by recruiting and activating immune cells, multiple-receptor interference and forced protein complex association.[9] Research on bispecific antibodies is focused on cancer therapy and inflammatory diseases. An example of such an scFv is UCHT1, a ~25 kDa fragment that is anti-human CD3, which is an antigen present on T-cells.[10,11]

Knob-in-hole antibodies are antibodies produced by, in this case, a chinese-hamster-ovary cell. Site-directed mutagenesis and co-transfection into the cell line allows for production of two distinct heavy chain products; one with a hydrophobic bulge at the C_H3 domain (knob), and one with a hydrophobic pocket at the C_H3 domain (hole).[12,13] This promotes heterodimerization of the two heavy chain halves. By including a C-terminal modification tag on the hole-heavy chain, the resulting asymmetrical product allows for selective modification of only one of the heavy chains. This is especially important when attempting to create homodimers of these antibodies since having a singular modification-handle prevents polymerization. Furthermore, when attempting to conjugate two proteins to a symmetrical antibody with two heavy chain C-terminal handles, the crowded environment that arises after the first protein conjugation could sterically hinder the conjugation of the second protein. This problem can be circumvented when using knob-in-hole antibodies, resulting in a higher yield of well-defined product.

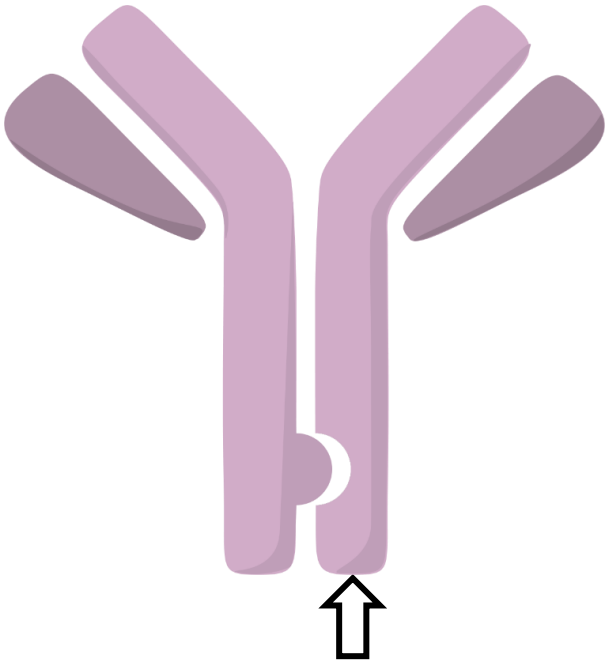

Figure 1. Schematic representation of a knob-in-hole antibody, displaying the heavy chain heterodimer and location of the C-terminal modification handle.

Early work on protein modification using amino moieties of the side-chain of lysine residues, or thiol groups of the side-chain of cysteine residues was fruitful but lacked site specificity.[14] This approach utilizes small molecules as reagents, modifying all cysteines, making it an inherently non-specific approach. For a chemically well-defined product, site specific conjugation methods have been developed.[15–17] These methods rely on the inclusion of non-canonical amino-acids or genetic modification to include a specific peptide tag, for example a *p*-acetylphenylanine. However this approach is restricted to recombinant cell lines and depends on permeability of the non-natural amino acid substrate. Tyrosine modification has also been demonstrated, using small molecules, and as previously explained was hardly site-selective.[18] However, enzymatic modification of exposed tyrosines was shown to be possible, making it site-selective towards these tyrosines.[19]

To expand the biochemical toolkit, a novel conjugation method was developed; strain-promoted oxidation-controlled *ortho*-quinone cycloaddition (SPOCQ).[20,21] This method relies on the activity of mushroom tyrosinase (mTyr). Using molecular oxygen in water, this enzyme oxidizes tyrosine to DOPA, and subsequently to an *ortho*-quinone. The quinone is then able to react with bicyclo[6.1.0]nonyne (BCN), due to the heavy strain on the BCN-moiety, forming the structure depicted in figure 2. Due to the natural bulk of the tyrosinase enzyme only exposed tyrosines can be efficiently accessed by the catalytic center.[22] This means the SPOCQ reaction is site-specific for peripheral tyrosines, for example a C-terminal, spaced, tyrosine.

Besides BCN, quinones react readily with *trans*-cyclooctene (TCO) and cyclopropanated *trans*-cyclooctene (cpTCO).[23,24] SPOCQ reaction rate constants of *ortho*-quinone with TCO was reported to be approximately 100-fold slower when compared with BCN, while cpTCO was reported to be approximately 2.5-fold faster. BCN, TCO and cpTCO can also undergo strain-promoted inverse electron-demand Diels-Alder cycloaddition (SPIEDAC) with tetrazines, which has even higher reported reaction rates.[25,26] This reaction will be used in this thesis as a baseline, as the high specificity and rapid reaction rates ensure a high level of orthogonality.

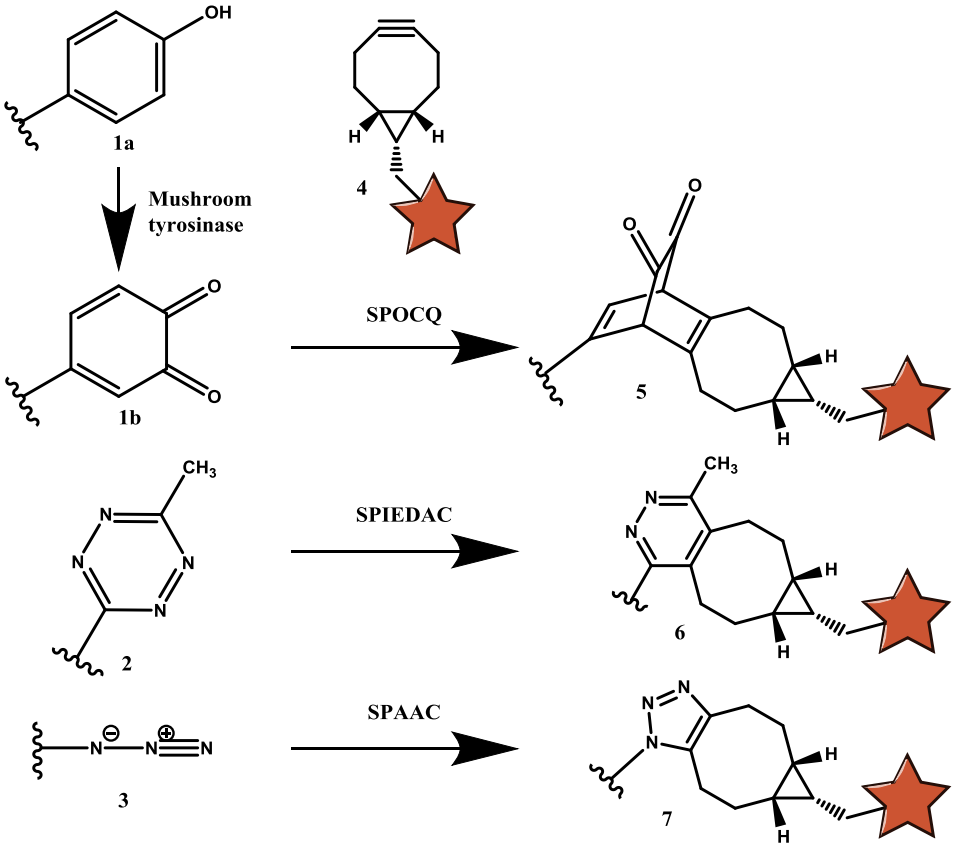

Figure 2. Schematic representation of the click reactions possible between the modification handles tyrosine **1a** when converted to *ortho*-quinone **1b**, methyltetrazine **2** or azide **3** with *endo*-BCN **4** yielding the SPOCQ **5**, SPIEDAC **6** or SPAAC product **7** respectively.

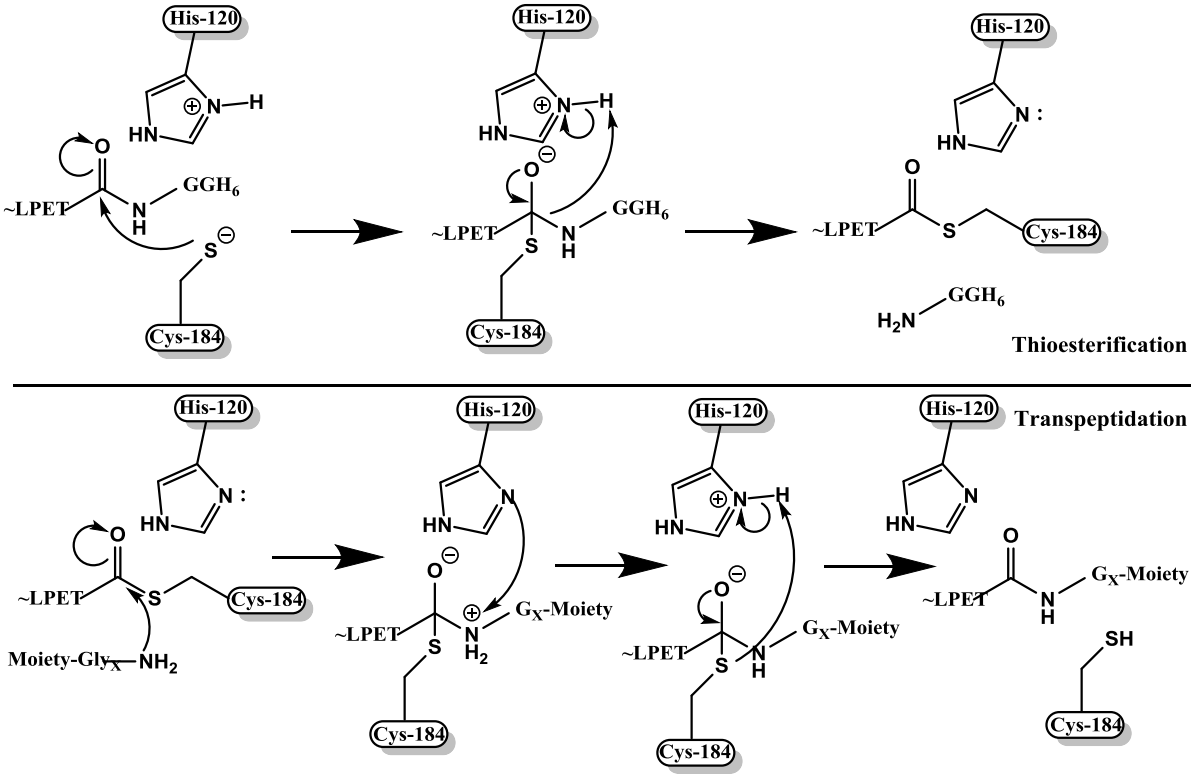

Figure 3. Schematic representation of the thioesterification and subsequent transpeptidation reactions that occur in the catalytic center of sortase A.

One important application of SPOCQ has been left unexplored; is it possible to make a protein-protein conjugate using SPOCQ. The goal of this thesis is to verify the viability to use SPOCQ in protein-protein coupling. To do this, knob-in-hole antibodies are used with a sortase-tag on the C-terminus. This sortase tag is recognized by the sortase A enzyme, a transpeptidase found in *Staphylococcus aureus* (see figure 3). This enzyme cleaves between the threonine and the glycine in the LPETG-tag, ligating instead an oligo-glycine presenting an exposed tyrosine to the C-terminus.[27–29] The exposed tyrosine is then able to be converted into a quinone by mTyr, meaning it can react with a BCN-scFv or BCN-IL2 creating an antibody-scFv or antibody-IL2 conjugate respectively. In this thesis a trastuzumab-knob-in-hole (Tras-KIH) with a sortase tag on the hole-heavy chain is used (Tras[HHC]LPETGGH$_6$).

The setup of this system allows for incredible modularity. Starting with trastuzumab containing the sortase tag on the hole-heavy chain, a wide array of moieties can be ligated onto it. For example, ligating a tetraglycyltyrosine (G$_4$Y), a triglycylmethyltetrazine (G$_3$MeTz) or a tetraglycylazidolysine (G$_4$K(N$_3$)) moiety to Tras-KIH using sortase A, allows for SPOCQ, SPIEDAC or SPAAC respectively (see figure 2). This results in the option to tailor the type of click reaction to the requirements of the application.

Materials and methods

Chemicals

2-Mercaptoethanol (BME), calcium chloride, dithiothreitol (DTT), sodium sulfite and tetramethylenediamine (TEMED) were obtained from Acros. Coomassie brilliant blue G250 was obtained from Amresco. Glycine was obtained from Applichem. Precision Plus Protein Dual Color Standards were obtained from BioRad. Acetic acid, acetonitrile, formic acid and trifluoroacetic acid were obtained from Biosolve. TAMRA-PEG4-Methyltetrazine (TAMRA-MeTz) was obtained from BroadPharm. Sodium chloride was obtained from Chem-Lab NV. Trastuzumab knob-in-hole with sortase tag (Tras[HHC]LPETGGH$_6$) antibodies were obtained from Evitria. Ammonium persulfate and butanol were obtained from Fisher Scientific. FabRICATOR and GlycINATOR were obtained from Genovis. MilliQ was obtained using a milliQ integral 3 system from Millipore. Methanol was obtained from Rathburn. Hydrochloric acid was obtained from Scharlau. Acrylamide was obtained from Serva. Dimethylsulfoxide (DMSO), mushroom tyrosinase enzyme (mTyr), sodium azide, streptavidin-maleimide, tris(hydroxymethyl)amino-methane (Tris) and tyrosine were obtained from Sigma-Aldrich. BCN-PEG2-lissamine (BCN-lis) was obtained from Synnafix (see figure 4A for structure). Sodium dodecyl sulfate and sodium phosphate monobasic were obtained from VWR. cpTCO-PEG2-lissamine (cpTCO-lis) was synthesized by and obtained from Jorick Bruins as reported.[23] cpTCO-PEG2000 and cpTCO-PEG5000 were obtained from Jorick Bruins. BCN-interleukin-2 (BCN-IL2), BCN-anti-human-CD3-antibody-fragment (BCN-UCHT1), G$_3$-Biotin, G$_3$-MeTz, G$_3$-TCO, G$_4$Y and the Sortase A enzyme were graciously donated by AIMM therapeutics. G$_4$K(N$_3$) was obtained from GenScript.

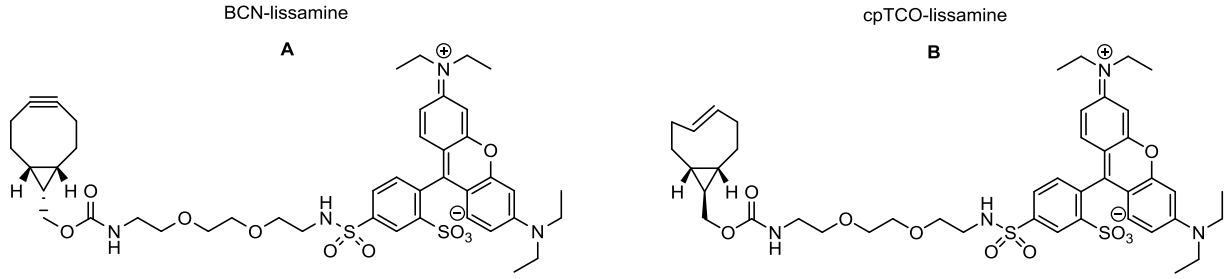

Figure 4. (A) chemical structure of BCN-Lissamine. (B) Chemical structure of cpTCO-Lissamine.

Sortase ligation

Prior to the sortase labelling reaction, the buffer of Tras[HHC]LPETGGH$_6$ was rebuffered to 25 mM Tris pH 6.8 + 10 mM CaCl2 + 150 mM NaCl (Tris pH 6.8) or 25 mM Tris pH 8.0 + 10 mM CaCl2 + 150 mM NaCl (Tris pH 8.0) using 50 kDa Amicon Ultra-0.5 spin filters. To any amount of Tras[HHC]LPETGGH$_6$ 350 µL Tris pH 6.8 or Tris pH 8.0 is added and spun down for 10 minutes at 14.000 RPM using an Eppendorf centrifuge 5424 R, after which the flow-through was discarded. This step was repeated 2 more times for a total of 3 times. The filter tube is reversed in a new collection tube and spun for 2 minutes at 2.000 RPM. The remaining liquid is collected and diluted to its original concentration using its respective buffer.

30 µM Tras[HHC]LPETGGH$_6$ in Tris pH 6.8 or Tris pH 8.0 is mixed with target moiety. After this 30 µM Sortase A is added and the mixture is incubated at 37°C for 3 hours. An example of Tras[HHC]LPETGGH$_6$ sortase reaction is illustrated below (see table 1).

Table 1. Example of a pipetting scheme for the Sortase labelling reaction

Compound	**Volume**	**Concentration**
Tris pH 6.8 or Tris pH 8.0	117.6 µL	-
Tras[HHC]LPETGGH$_6$ Tris (184 µM)	23.7 µL	3.0E-5 M
G$_4$Y (50 mM)	3.0 µL	1.0E-3 M
Sortase A (790 µM)	5.7 µL	3.0E-5 M
	150.0 µL	

After sortase labelling, Trastuzumab was purified using a NAb Protein A Plus Spin Column 0.2 mL from Thermo Fisher, following the complementary protocol described by Thermo Scientific.[30] At the end of this protocol three fractions were collected and their protein contents assessed using a NanoDrop 2000 Protein A280 protocol. Fractions containing high protein concentration (≥0.025 mg/mL) are combined into a final sample and used for further purification. After purification on protein A spin column, the final sample was concentrated and the buffer was exchanged to PBS pH 5.5 (50 mM phosphate + 150 mM NaCl) using the method described above.

SPIEDAC mediated labelling

In general, to 5 µL of 4.5 mg/mL Tras[HHC]MeTz in PBS pH 5.5, 5-10 equivalents of cpTCO-label/TCO-label/BCN-label in DMSO was added and incubated for 1 hour at room temperature. An example of Tras[HHC]MeTz SPIEDAC with cpTCO-lis is illustrated below (see table 2).

Table 2. Example of a pipetting scheme for the SPIEDAC labelling reaction

Compound	Volume	Concentration
PBS pH 5.5	4.75 µL	-
Tras[HHC]MeTz (4.5 mg/mL)	5.00 µL	15 µM
cpTCO-Lis (5.0 mg/mL)	0.25 µL	145 µM
	10.00 µL	

SPOCQ mediated labelling

In general, to 5 µL of 4.5 mg/mL Tras[HHC]G_4Y in PBS pH 5.5, 5-10 equivalents of cpTCO-label/TCO-label/BCN-label in DMSO was added, after which mTyr in phosphate buffer pH 5.5 was added and the mixture was incubated for 3 hours at 4 °C. Samples that contain a protein label were shaken at 300 RPM in an Eppendorf Thermomixer C. An example of Tras[HHC]G_4Y SPOCQ with BCN-lis is illustrated below (see table 3).

Table 3. Example of a pipetting scheme for the SPOCQ labelling reaction

Compound	Volume	Concentration
PBS pH 5.5	4.25 µL	-
Tras[HHC]G_4Y (4.5 mg/mL)	5.00 µL	15 µM
BCN-lis (5.0 mg/mL)	0.25 µL	145 µM
mTyr (10.0 mg/mL)	0.50 µL	4 µM
	10.00 µL	

For time series experiments, the reaction was stopped by quickly doing the HPLC pre-treatment as described below, and subsequently freezing the samples at -20°C until the series had resolved and all samples were ready for HPLC analysis.

SPAAC mediated labelling

In general, to 5 µL of 4.5 mg/mL Tras[HHC]G_4K(N_3) in PBS pH 5.5, 5-10 equivalents of BCN-label in DMSO was added and incubated overnight at room temperature. An example of Tras[HHC]G_4K(N_3) SPAAC with BCN-lis is illustrated below (see table 4).

Table 4. Example of a pipetting scheme for the SPAAC labelling reaction

Compound	Volume	Concentration
PBS pH 5.5	6.55 µL	-
Tras[HHC]G_4K(N_3)(7.0 mg/mL)	3.20 µL	15 µM
BCN-Lis (5.0 mg/mL)	0.25 µL	145 µM
	10.00 µL	

SDS-PAGE analysis

Prior to analysis the samples were reduced by adding 5 µL sample buffer (containing 5% β-mercaptoethanol) to 5 µL reaction mixture and incubating for 5 minutes at 95°C. After this the samples were loaded onto a 12% acrylamide gel, using Precision Plus Protein Dual Color Standards as a reference ladder. After electrophoresis using a BioRad mini-PROTEAN tetra system operating at 100 V, the gel was imaged using a BioRad ChemiDoc XRS+. After fluorescence imaging the gel was stained using coomassie brilliant blue for 30 minutes, destained with 10% acetic acid, 50% methanol, 40% water for 30 minutes, then destained further in water overnight.

For the non-reducing gel the same protocol was followed, except the samples were denatured in sample buffer without β-mercaptoethanol, and loaded on a 7.5% acrylamide gel.

HPLC analysis

To 5 µL reaction mixture 2.5 µL 0.2 M DTT in 0.1 M Tris pH 8.0 (2.5 µL for each 10 µg of antibody) was added and incubated for 15 minutes at 37°C. After this 30 µL MMF (49% Acetonitrile, 49% MilliQ and 2% formic acid) was added. MMF is added to protonate any thiols that may be regenerated at the column temperature of 80 °C after DTT treatment. Reverse phase HPLC was performed using a MAbPac RP 4 µm 3x50 mm column on a Agilent Technologies 1220 Infinity LC, operating at 0.6 mL/min at 80 °C using a 10 minute linear gradient from 20% buffer B to 45% buffer B, staying at this ratio for 1 minute, then using a 2 minute linear gradient from 45% buffer B to 20% buffer B and finally staying at this ratio for 4 minutes, making for a total runtime of 18 minutes (buffer A: 95% MilliQ, 5% acetonitrile and 0.1% trifluoroacetic acid. Buffer B: 5% MilliQ, 95% acetonitrile and 0.1% trifluoroacetic acid).[31]

Sortase reaction conversion rate was determined using the following equation:

$$X_{sortase} = \frac{A_{SPOCQ\ HHC}}{A_{SPOCQ\ LC} * X_{SPOCQ}} * \frac{A_{Ref\ HHC}}{A_{Ref\ LC}} \quad (1)$$

Here *A* is the area of the respective peaks, with A_{SPOCQ} being Tras[HHC]cpTCO-Lis and its respective hole-heavy chain or light chain peaks, and A_{Ref} being Tras[HHC]LPETGGH$_6$ and its respective hole-heavy chain or light chain peaks. X_{SPOCQ} is the yield of the SPOCQ reaction between cpTCO-lis and *ortho*-quinone, which is set at 0.95.[23]

SPIEDAC and SPOCQ reaction conversion rate was determined using the following equations:

$$X_{SPIEDAC} = \frac{A_{Ref\ LC}}{A_{Ref\ LC} + A_{Ref\ HC}} / \frac{A_{SPIEDAC\ LC}}{A_{SPIEDAC\ LC} + A_{SPIEDAC\ KHC}} \quad (2)$$

$$X_{SPOCQ} = \frac{A_{Ref\ LC}}{A_{Ref\ LC} + A_{Ref\ HC}} / \frac{A_{SPOCQ\ LC}}{A_{SPOCQ\ LC} + A_{SPOCQ\ KHC}} \quad (3)$$

Here *A* is the area of the respective peaks, with A_{Ref} being Tras[HHC]G$_3$MeTz for SPIEDAC and Tras[HHC]G$_4$Y for SPOCQ and their respective hole-heavy chain or light chain peaks, $A_{SPIEDAC}$ being the SPIEDAC mediated product and its respective knob-heavy chain or light chain peaks, and A_{SPOCQ} being the SPOCQ mediated product and its respective knob-heavy chain or light chain peaks.

LCMS analysis

Prior to MS analysis samples had their buffers exchanged to PBS pH 7.2 using the method described above. The samples then had their glycan-variety reduced by adding 1 unit of GlycINATOR for each 1 µg of antibody and incubating for 1 hour at 37 °C.[32] After this the samples were cleaved by adding 1 unit of FabRICATOR for each 1 µg of antibody and incubating for 1 hour at 37 °C.[33] To this mixture 20 µL MMF was added and the samples were analyzed on a Thermo-Fisher Q-Exactive Focus Orbitrap LC-MS using the same operating conditions as with HPLC analysis but substituting 0.1% TFA with 0.1% FA in the eluents. Spectra deconvolution was done using UniDec.[34]

Results and discussion

Optimizing sortase reaction

One of the goals of this thesis was to set up a protocol with which Tras[HHC]LPETGGH$_6$ can be functionalized with a polyglycylmoiety (*e.g.* G$_4$Y), utilizing sortase A and the C-terminal LPXTG-tag on the hole-heavy chain (HHC). Initial experiments regarding the sortase ligation were performed at pH 6.8.[35] Exchanging the buffer to Tris is important, as PBS precipitates the calcium ions required for sortase A functionality, in the form of calcium phosphate.[36] Sortase ligation of G$_4$Y onto Tras[HHC]LPETGGH$_6$ is mediated by sortase A cleaving between threonine and glycine in the LPETG-tag. Subsequently, sortase A ligates G$_4$Y onto the hole-heavy chain yielding the Tras[HCC]G$_4$Y product.

Prior to any follow-up experiment the reaction mixture is purified using a protein A spin column, followed by spin filtration to concentrate the product in the correct buffer. The reasoning behind this is that sortase A contains a free thiol in its catalytic center, making for a potent thiol-yne click reaction candidate with a BCN-moiety.[37,38] Furthermore, peripheral tyrosines can be converted into *ortho*-quinones by mTyr, allowing for SPOCQ on sortase A (see figure S1), as well as instigating reactivity between the quinones and cysteines. Therefore it is paramount that sortase A is removed prior to further experiments. Native IgG antibodies do not contain free thiols, since every cysteine contributes to its tertiary and quaternary structures with disulfide bonds.[39,40] The yield of this purification step was measured to be approximately 95-100% and sortase A was absent entirely (see figure S2).

HPLC was used to estimate the sortase ligation conversion, and Figure 5A shows a clear separation between the hole-heavy chain and the knob-heavy chain.[41] After sortase ligation it was observed that the Tras[HHC]G$_4$Y peak overlaps with the Tras[KHC] peak (see figure 5B). To separate Tras[HHC]G$_4$Y more clearly, SPOCQ was performed on the exposed tyrosine using cpTCO-lis. This resulted in a delay in retention time of approximately 30 seconds, separating it entirely from the Tras[KHC] peak (see figure 5C). The peak area of Tras[HHC]lis was used to calculate sortase conversion. By using unmodified Tras[HHC]LPETGGH$_6$ to determine the ratio between the hole-heavy chain and the light chain, the light chain can be used as an internal standard in the other samples. Using this method, a rough estimation can be given to the yield of the sortase reaction. The conversion was calculated to be around 50%. This was not sufficient to continue on to follow-up experiments, therefore this protocol needed to be improved.

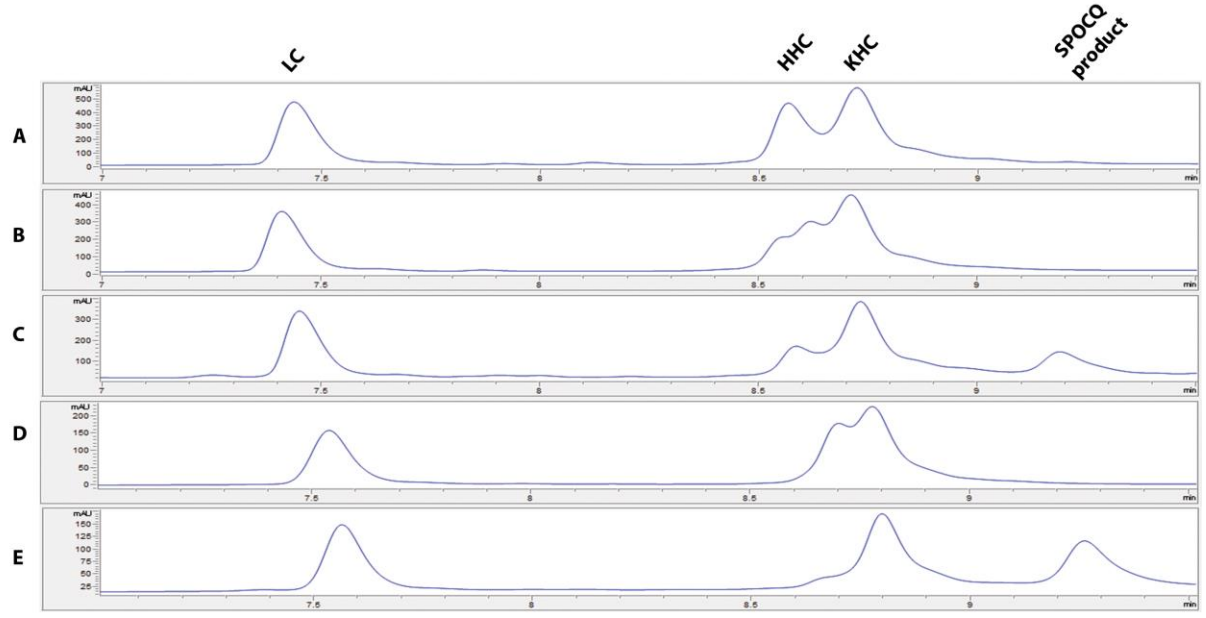

Figure 5. HPLC spectra of (A) Tras[HHC]LPETGGH$_6$, (B) Tras[HHC]G$_4$Y synthesized at pH 6.8, (C) Tras[HHC]G$_4$Y synthesized at pH 6.8 + SPOCQ with cpTCO-lis, (D) Tras[HHC]G$_4$Y synthesized at pH 8.0, (E) Tras[HHC]G$_4$Y synthesized at pH 8.0 + SPOCQ with cpTCO-lis.

Based on literature the optimal pH for sortase A was not at pH 6.8, but at pH 8.0.[42] It was speculated that this pH was favorable for the thioesterification step involving the thiol group of C184, which activates the enzyme.[28] This in turn results in a higher population of sortase A in its active form. Therefore, the experiment was repeated at pH 8.0. Even before the SPOCQ reaction, it was observed by the shape of the peaks that the conversion was already much improved; the starting material peak had greatly diminished compared to pH 6.8 leading to the subsequent increase of the Tras[HHC]G_4Y peak (see figure 5D-E). The new conversion was calculated to be around 85-90%, which is in line with yields reported in literature, a bit higher even. It was therefore decided that no further optimization was needed, since other variables, such as incubation temperature and time, were already close to optimal according to literature.

Proof of concept: protein-protein coupling using SPIEDAC

To verify whether protein-protein coupling could work on a sortase modified trastuzumab, a system was first tested based on the SPIEDAC click reaction. The inverse electron demand Diels-Alder reaction between tetrazines and *trans*-cyclooctenes is renowned for its exceptional reaction rates and bio-orthogonality.[25] Therefore this ensures site-selectivity and high yields.

To this end, two antibody products were made; one with a methyltetrazine moiety (Tras[HHC]G_3MeTz) and one with a *trans*-cyclooctene moiety (Tras[HHC]G_3TCO) using the optimized sortase protocol, netting an estimated >90% conversion rate for both reactions, measured using HPLC. Both samples were clear of the sortase A enzyme after protein A purification and subsequent concentration and buffer exchange (see figure 6, lanes 7 and 8). Upon addition of BCN-lis to Tras[HHC]G_3MeTz the SPIEDAC click reaction should readily occur, since the former is a relatively small molecule. This was indeed observed on both gel and HPLC. In lane 9 a small mass increase can be observed when compared to lane 7. Successful conjugation is readily apparent on the fluorescent image of the gel, which shows a clear signal just above 50 kDa, the mass of the hole-heavy chain after denaturation (see figure S1). Seeing no fluorescence signal in Tras[HHC]LPETGGH_6 samples indicates that the MeTz-moiety is needed for successful conjugation (see figure S3). To prove that site-selective antibody-protein conjugation is possible after sortase mediated ligation, TCO-UCHT1 was added to Tras[HHC]G_3MeTz. TCO-UCHT1 has a theoretical mass of 28 kDa, and the result in lane 10 shows a very clear mass shift of the hole-heavy chain to 75 kDa, which matches the expected mass after conjugation. The strong reduction in intensity of the hole-heavy chain band indicates a high conversion, which is backed up by the HPLC data (see figure S4-9). Next, Tras[HHC]G_3MeTz and Tras[HHC]G_3TCO were added together 1:1, which should result in a monofunctional bispecific antibody conjugate with a mass around 100 kDa after reduction. This is exactly what was observed in lane 11, with a surprisingly high conversion rate with regards to the stoichiometry.

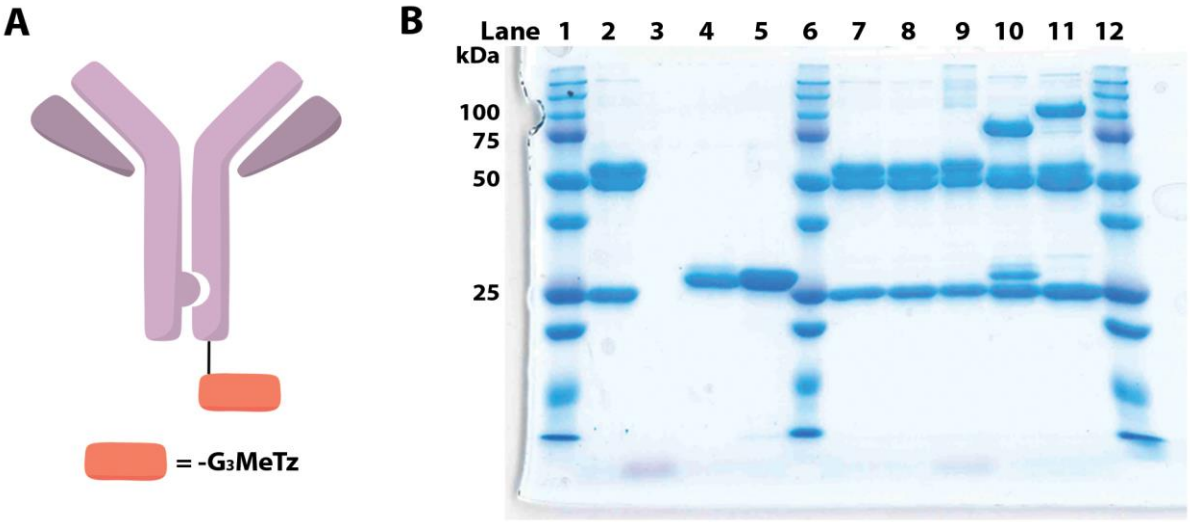

Figure 6. Schematic representation of Tras[HHC]G3MeTz. (B) SDS-PAGE analysis of SPIEDAC on Tras[HHC]G3MeTz. Lane contents; 2: Tras[HHC]LPETGGH_6, 3: BCN-lis, 4: TCO-UCHT1, 5: Sortase A, 7: Tras[HHC]G3MeTz, 8: Tras[HHC]G3TCO, 9: Tras[HHC]G3MeTz + BCN-lis, 10: Tras[HHC]G3MeTz + TCO-UCHT1, 11: Tras[HHC]G3MeTz + Tras[HHC]G3TCO.

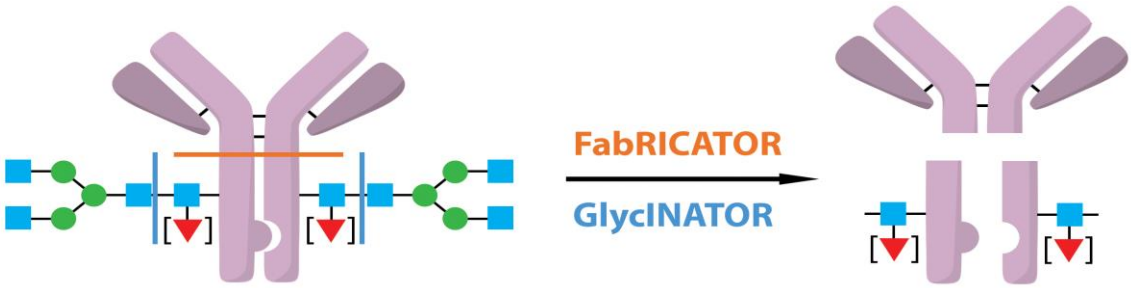

Figure 7. Schematic representation of FabRICATOR and GlycINATOR treatment on trastuzumab.

To further investigate whether the antibody-protein conjugation was successful, mass analysis was performed. Tras[HHC]G_3MeTz conjugates with BCN-lis, BCN-IL2, TCO-IL2 and TCO-UCHT1 were measured using LCMS. Before measuring the masses, the samples were reduced by incubating with GlycINATOR and FabRICATOR (see figure 7). GlycINATOR is an endoglycosidase that hydrolyzes all different glycoforms on the Fc-glycosylation site of IgG. This enzyme leaves only the base *N*-acetylglucosamine (GlcNAc) with a possible fucosylation modification, reducing the variation from different glycoforms to two.[32] Subsequent incubation with FabRICATOR reduces the antibody to a fraction of its former size, by cleaving specifically below the hinge of the heavy chains at the...CPPCPAPELLG/GPSVF... position, yielding an Fc fragment of the hole-heavy chain with a known mass.[33] Unfortunately, the supplier of the IL2 and UCHT1 moieties did not provide the exact mass, and in-house mass determination showed that the samples were heterogeneous. However knowing the approximate masses, based on literature and gel results, allowed successful deduction of the masses of BCN-IL2 (~17846 Da and ~17190 Da) and TCO-IL2 (~17548 Da and ~16892 Da) from the LCMS data. The different masses are either different glycoforms or native IL2 lacking the respective BCN- or TCO-handles. The mass of TCO-UCHT1 was not so easily deduced, therefore nothing could be concluded regarding the conjugation product. Despite knowing the amino acid sequence and the FabRICATOR cleavage site, the measured mass of the hole-heavy chain did not match the calculated mass (see table S2). This then of course also applied to any of the conjugates. It is currently unknown why the theoretical masses differ so greatly from the measured masses.

All in all, these results were promising enough to continue on to similar experiments using SPOCQ to create antibody-protein conjugates.

Protein-protein coupling using SPOCQ

The initial goal of this thesis was to create an antibody-protein conjugate using SPOCQ. To this end, a tetra-glycyltyrosine was ligated onto Tras[HHC]LPETGGH_6 using the optimized sortase A protocol, yielding Tras[HHC]G_4Y of ~90% purity as determined by HPLC (see figure S16-17). Sortase A and excess G_4Y were removed by protein A purification and subsequent concentration and buffer exchange. It was previously shown that the presence of mTyr converting the exposed tyrosine into a quinone is a prerequisite for labelling with a BCN- or cpTCO-moiety,[21] which was reconfirmed in this thesis (see figure S1). While quinones can react with the sidechains of cysteine, histidine and lysine, the extremely fast reaction rates with BCN or cpTCO favor the SPOCQ reaction over the Michael addition with these amino acids.[24,43] This results in site-selectivity and means that, in the case of the Tras-KIH-based system, a singular modification should be made.

To test the possibility of site-selective antibody-protein conjugation with SPOCQ, Tras[HHC]G_4Y was mixed with BCN-lis as a positive control, BCN-IL2 and BCN-UCHT1, after which mTyr was added to facilitate the reaction. Fluorescence analysis of the gel on which these samples were loaded showed that the SPOCQ reaction was successful for the BCN-lis sample, showing a clear signal at the hole-heavy chain just above the 50 kDa mark (see figure S22). Also here a small mass shift was observed in the stained gel when compared to the starting material (see figure 8, lane 12). With BCN-IL2 having a theoretical mass of ~16 kDa, this put the corresponding conjugate at around 66 kDa, which was where a band was observed in lane 13. This signified a singular, and therefore site-selective, modification of Tras[HHC]G_4Y.

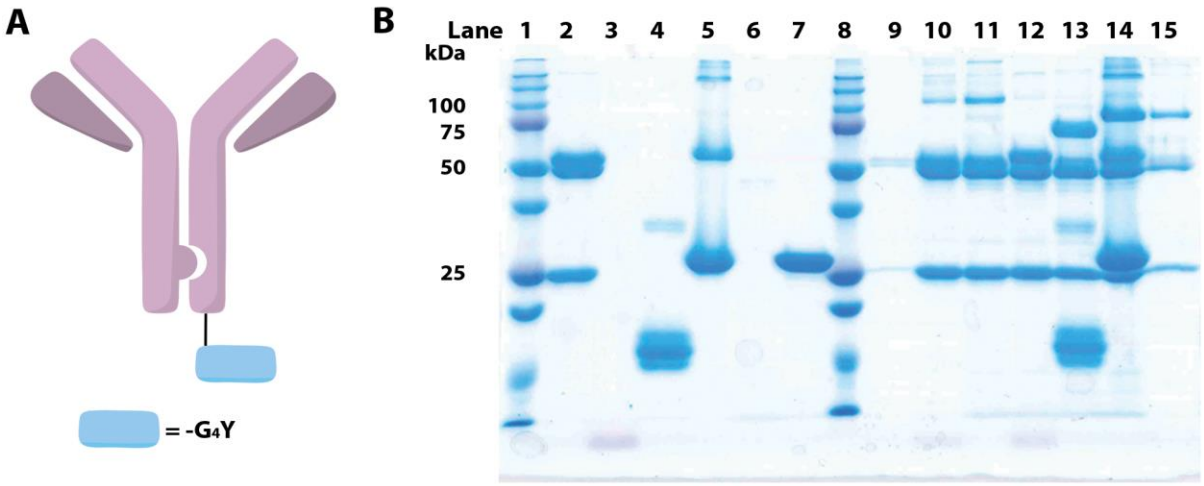

Figure 8. Schematic representation of Tras[HHC]G_4Y. (B) SDS-PAGE analysis of SPOCQ on Tras[HHC]G_4Y. Lane contents; 2: Tras[HHC]LPETGGH_6, 3: BCN-lis, 4: BCN-IL2, 5: BCN-UCHT1, 6: mTyr, 7: Sortase A, 9: Tras[HHC]G_4Y, 10: Tras[HHC]G_4Y + BCN-lis, 11: Tras[HHC]G_4Y + mTyr, 12: Tras[HHC]G_4Y + BCN-lis + mTyr, 13: Tras[HHC]G_4Y + BCN-IL2 + mTyr, 14: Tras[HHC]G_4Y + BCN-UCHT1 + mTyr, 15: Tras[HHC]G_4Y + BCN-UCHT1 + mTyr after protein A purification.

The theoretical mass of the BCN-UCHT1 probe was around 25 kDa, but the amount of impurities present made it hard to ascertain with certainty that it was indeed the 25 kDa mass that was functionalized with the BCN-moiety (see lane 5). Especially the fact that the ~60 kDa impurity covered the heavy-chain region made it hard to get a good readout of the Tras[HHC]G_4Y + BCN-UCHT1 conjugate sample in lane 14, due to the fact that the unreacted hole-heavy chain was not visible. To clean up the sample, the contents of this reaction mixture were subjected to the protein A purification step with subsequent concentration. This resulted in the sample in lane 15, where the shift of the hole-heavy chain to ~80 kDa was readily observed. In all cases the HPLC spectra corroborated the successful conjugation of the probes onto Tras[HHC]G_4Y, which showed a decrease in intensity where the hole-heavy chain would be when compared with native Tras[HHC]G_4Y (see figure S19-21). Upon further investigation it appeared that, based on a time series, the SPOCQ reaction between Tras[HHC]G_4Y and BCN-IL2 had reached its maximum potential between 30 minutes and 1 hour, yielding an approximate 80% conversion (see figure S24-30). This was compared to an experiment that used an antibody which contained a tyrosine moiety on both heavy chains, where it was observed that mostly a singular conjugation event occurs (see figure S31). This alluded to a sterically crowded environment that blocked the second conjugation event and, compared with the results in this thesis and a 3D model of IgG and UCHT1 (see S41-43), indicated a clear advantage to using a single modification handle on antibodies.

Also here mass analysis was done to provide insight into the produced conjugates by matching a theoretical mass to a mass measured by LCMS. As mentioned before, unfortunately the masses of BCN-UCHT1 and BCN-IL2 were not known by the supplier, and since both samples are quite heterogeneous no accurate mass could be determined using LCMS (see figure 8 lanes 4 and 5). However, for the Tras[HHC]G_4Y-BCN-Lis construct a mass of ~25898 Da was found, which matches the calculated mass within a 2 Da difference (see table S5).

Protein-protein coupling using SPAAC

Being able to incorporate a handle upon which a strain-promoted alkyne-azide cycloaddition (SPAAC) can be performed gives further bio-orthogonality with regards to SPOCQ. Since azides react very slowly with cpTCO and the quinone has to be generated using mTyr, both strategies can be applied on the same protein, or as a mixture in the same pot.[23]

To expand on the toolbox that was set up here, an antibody was functionalized with an tetra-glycylazidolysine using the optimized sortase protocol, yielding Tras[HHC]$G_4K(N_3)$. Experiments using BCN-lis and BCN-IL2 were performed to assess conjugation efficiency. Unfortunately results were mixed. Based on the gel results, some SPAAC did occur (see figure 9, lane 4). The fluorescence imaging showed

significant lissamine signal on the hole-heavy chain, with some random labelling observed on the light chain (see figure S34). However when looking at the antibody-protein conjugation experiment the conversion rate was low.

Further investigation using HPLC showed strange results; although the heavy chain peaks showed the expected behavior after the sortase mediated ligation, the light chain peak had a very unusual shape and the BCN-lis conjugate showed a messy spectrum altogether (see figure S36). The BCN-IL2 sample showed almost no difference when compared to the Tras[HHC]$G_4K(N_3)$ spectrum, corroborating the gel observations.

Further investigation into the starting materials on this reaction showed that the Tras[HHC]$LPETGGH_6$ showed the same peak tailing on the light chain (see figure S38). This stock had been sitting at pH 8.0 at 4°C for a few weeks, which in hindsight might have adversely affected the quality. As to why the antibody-protein conjugation yielded much a much lower conversion when compared to the fluorophore conjugation experiment, it was suspected that the BCN-moiety on the interleukin-2 might have degraded over time while being stored at 4°C, while the BCN-moiety on the lissamine had not at -20°C. Due to time constraints further research into this particular topic was suspended.

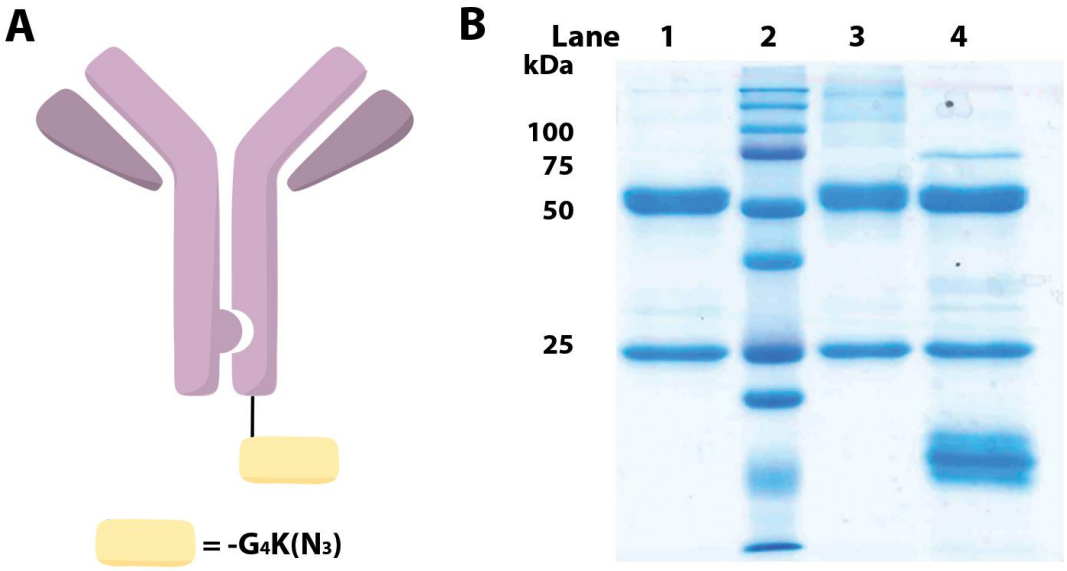

Figure 9. Schematic representation of Tras[HHC]$G_4K(N_3)$. (B) SDS-PAGE analysis of SPAAC on Tras[HHC]$G_4K(N_3)$. Lane contents; 1: Tras[HHC]$G_4K(N_3)$, 3: Tras[HHC]$G_4K(N_3)$ + BCN-lis, 4: Tras[HHC]$G_4K(N_3)$ + BCN-IL2.

Conclusions

A workflow for synthesis of antibody-protein conjugates was established, which allowed for site-specific conjugation of various protein-based moieties onto the C-terminus of trastuzumab, using SPIEDAC, SPOCQ and to some extent SPAAC. This toolbox started with Tras[HHC]LPETGGH$_6$ with a sortase tag which was expanded to a MeTz-, TCO-, tyrosine- or azide-handle using sortase A mediated transpeptidation. A protocol for sortase A mediated ligation was set up successfully. Yields were similar to those reported in literature, across all moieties, namely ~85-90%. Protein A purification work-up proved to be essential in reducing side products by removing the sortase A enzyme before continuing to follow-up experiments. The SPIEDAC mediated antibody-protein conjugation was performed successfully, which yielded a site-specific conjugate, with a 75% conversion rate. Production of a monofunctional bispecific antibody conjugate was successfully performed as well, with a 81% conversion rate. The SPOCQ mediated antibody-protein conjugation was achieved within 1 hour at a ~85% conversion rate. The SPAAC reaction was concluded to have potential, but that it needs to be optimized before it can be considered viable.

Recommendations

After this thesis there are still some questions left unanswered and opportunities left unexplored. For my successor I will leave a short summary of recommendations and notes after reflecting on my work on this system.

Based on the conclusions it would be beneficial if the system's reliance on BCN-moieties were replaced with cpTCO-moieties. This results in less random labelling, higher SPOCQ reaction rates and enhanced bio-orthogonality. Except lack of commercial availability of cpTCO-moieties there are no drawbacks in replacing the one for the other. It is worth noting that random labelling is most strongly observed on SDS-PAGE, which could indicate that the sample work-up exacerbates the occurrence. A simple filtration step to remove excess BCN-probes before doing the gel work-up could provide more insight to whether this is the case.

Collaboration with AIMM therapeutics has resulted in the need for a relatively large quantity of Tras[HHC]UCHT1 to be produced using SPOCQ (±150 µg). Starting with Tras[HHC]LPETGGH$_6$, to obtaining the purified product is quite the laborious process, but the larger volume should pose no difference in workflow. With the purified Tras[HHC]UCHT1 AIMM therapeutics wants to do a live-cell assay, the details of which are to be determined and ultimately not relevant right now.

Not a lot of variation with stoichiometry was done. Especially when doing antibody-protein coupling it became evident that stoichiometry optimization can be quite useful. The UCHT1 and IL2 stocks dwindled rapidly upon repeated experiments, therefore it is in everyone's best interest to minimize the amount of material that is used. With industrial applications in mind this becomes even more important, therefore this part of the up-stream processes should be looked into.

Accurate mass determination is key in elucidating trastuzumab-protein conjugates, resulting in a well-defined product. While gel results give a good indication of antibody-protein product formation, matching an exact calculated mass to a measured mass is much stronger proof. Therefore more effort should be put into LCMS analysis of trastuzumab and the respective conjugation moieties. Using clean and well known proteins helps tremendously in matching the masses.

If size determines clearing from blood, it could be useful to observe clearance of scFv-conjugates, see if it increases the lifetime of scFv.[44,45] Renal filtration sets a size threshold which should exclude the mAb-scFv conjugate, therefore decreasing systemic clearance when compared to lone scFv-fragments. However, this inherently means an increase in size and thus a decrease in penetration efficiency. Again, depending on the specific application the size could be tuned to meet the specifications. This is not necessarily a subject in which active research must be done, but more something to take into consideration when designing antibody-protein conjugates for medical applications.

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