# Tyrosine-Based Bioconjugations

## Strain-Promoted Cycloadditions for Site-Specific Generation of Protein Conjugates

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This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition, and Health Sciences)

# Tyrosine-Based Bioconjugations

## Strain-Promoted Cycloadditions for Site-Specific Generation of Protein Conjugates

**Jorick J. Bruins**

**Thesis**
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 21 February 2020
at 4 p.m. in the Aula.

Jorick J. Bruins
Tyrosine-Based Bioconjugations – Strain-Promoted Cycloadditions for Site-Specific Generation of Protein Conjugates,
142 Pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2019)
With references, with summary in English

ISBN: 978-94-6395-242-2
DOI: https://doi.org/10.18174/509054

# Table of Contents

# Preface

## Summary for non-scientists

The most asked questions to us by non-scientists are along the lines of: “What do you research?” and “What purpose does your research serve?”. These are very important questions, and every scientist should be able to explain their research and importance in understandable terms.

I would like to do the same, and to do that I would like to explain the title ‘*Tyrosine-Based Bioconjugations*’, as the title often gives a very clear explanation of the contents. Let’s start with ‘*bioconjugations*’; this is a term used for any chemical technique that connects two (or more) molecules together, with at least one of these molecules has to be a “biological molecule”, such as proteins, sugars or DNA. In this thesis, we focus only on proteins.

Proteins are constructed by any combination of 20 different building blocks called ‘amino acids’. Differences between proteins can occur from different amounts of total amino acids, as well as differences in the order of building blocks. For example: haemoglobin, the protein that is present in the blood and allows us to bind the oxygen that we breathe. This crucial protein has 135 amino acids in a very specific sequence allowing us to, well.. live. Another protein, alcohol dehydrogenase, ensures that alcohol is broken down in the liver. This is an enzyme, which means it perform reactions by itself via catalysis, has 375 amino acids in a very different order to fulfil a completely different purpose.

About the amino acids. One of these amino acids is tyrosine, which is the key focus in our research. We were able to install extra tyrosine ‘blocks’ in proteins without them losing their function. Then, we used these tyrosine residues to do the bioconjugation, where we attached molecules to our protein of choice, like adding fluorescent groups!

Why were we doing this? Well, we used to connect to proteins called ‘antibodies’. These nifty proteins are comparable to a ‘heat-seeking missile’; each different antibody has a different target to which they bind, meaning they will stick to a specific target. Some of these antibodies can bind to cancer cells (for example Trastuzumab, also called Herceptin), whilst ignoring other cells. That is why we used our developed chemistry to bind very toxic molecules, used in chemotherapies or radiotherapies, to these antibodies. This created a system that can bring the chemotherapy straight to the cancer cells, whilst ignoring the healthy cells. This results in a lot less adverse effects.

These constructs are called antibody−drug conjugates, or targeted therapies. This field has been growing a lot over the past year. This thesis describes a new and promising approach to creating these medicines.

## Samenvatting voor niet-wetenschappers

De meest gestelde vraag door niet-wetenschappers aan ons is in de trant van: “Wat onderzoek je?” en “Waarvoor onderzoek je het?”. Dit zijn zeer belangrijke vragen en iedere wetenschapper zou zijn onderzoek en belang daarvan moeten kunnen uitleggen.

Ik zou graag hetzelfde willen doen, daarvoor wil ik graag verwijzen naar de titel ‘*Tyrosine-Based Bioconjugations*’, wat vertaald naar ‘*Tyrosine-gebaseerde bioconjugaties*’. De term ‘bioconjugatie’ verwijst naar een chemische techniek die twee (of meer) moleculen aan elkaar koppelt, waarvan op zijn minst één een biologisch molecuul is, zoals eiwitten, suikers, of DNA. In dit proefschrift focussen we op eiwitten.

Eiwitten zijn opgebouwd uit een combinatie van 20 verschillende bouwstenen genaamd ‘aminozuren’. Het verschil tussen eiwitten kan ontstaan uit de hoeveelheid aminozuren, maar ook uit de verschillende volgordes waarin deze bouwstenen aanwezig zijn. Bijvoorbeeld Hemoglobine; dit is een eiwit dat voorkomt in bloed en zuurstof bindt. Dit cruciale eiwit heeft 135 aminozuren in een zeer specifieke volgorde dat ons toestaat om.. nou, te leven. Een ander eiwit, alcohol dehydrogenase, zorgt ervoor dat alcohol in de lever afgebroken kan worden. Dit is een enzym, wat inhoudt dat het een eiwit is die reacties uitvoert via catalyse, heeft 375 aminozuren in een compleet andere volgorde om een ander doel te realizeren.

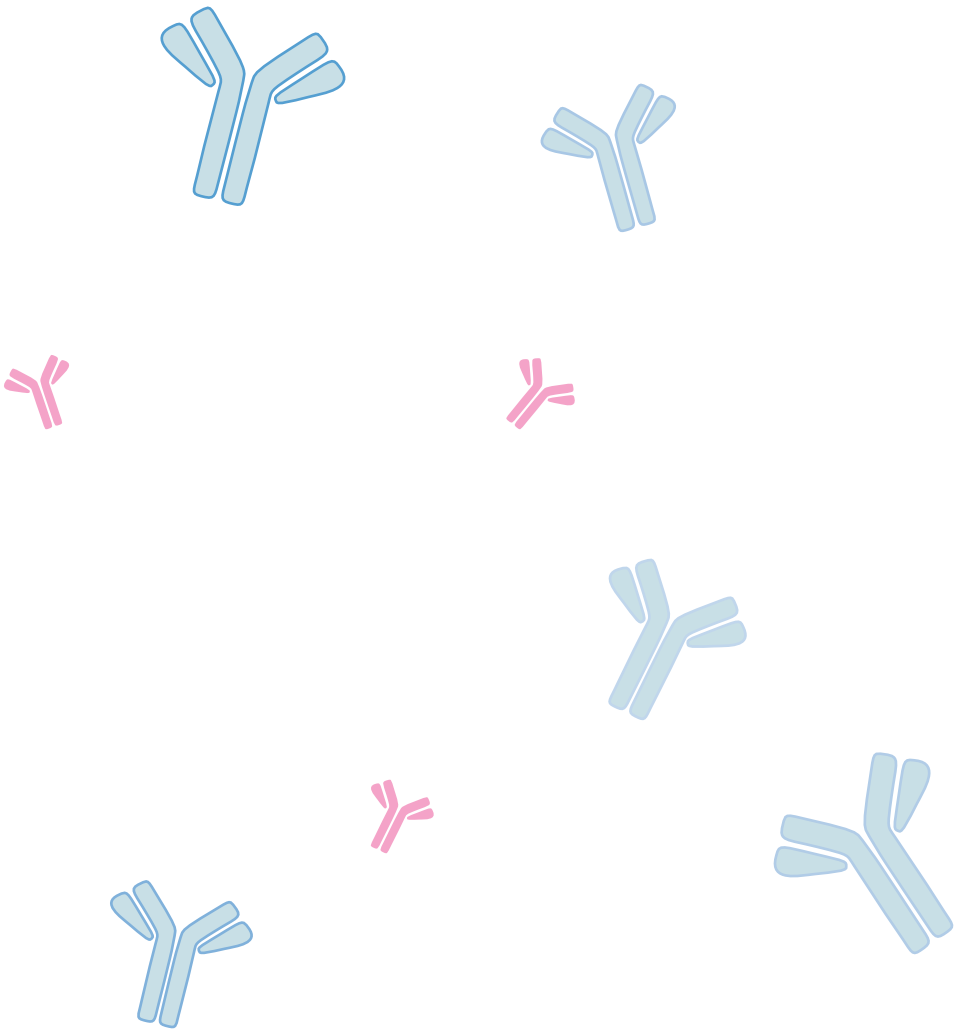
Zoals ik hierboven al schreef zijn eiwitten opgebouwd uit aminozuren. Een van de aminozuren is tyrosine, wat een hoofdrol speelt in ons onderzoek. Wij kunnen extra tyrosine ‘bouwsteentjes’ toevoegen aan eiwitten zonder dat deze hun functie verliezen. Vervolgens kunnen wij, aan de hand van deze tyrosine blokjes, dan bioconjugatie doen aan deze eiwitten, zoals het toevoegen van fluorescente groepen!

De reden waarom wij dit willen doen is het volgende: we koppelden moleculen aan eiwitten genaamd ‘antilichamen’. Deze slimme eiwitten zijn vergelijkbaar met ‘hittezoekende raketten’; elk verschillend antilichaam heeft een ander doelwit waaraan hij bindt, wat inhoudt dat ze aan verschillende doelen blijven plakken. Sommige van deze antilichamen kunnen specifiek binden aan kankercellen (zoals Trastuzumab, ook Herceptin genoemd), terwijl ze andere cellen negeren. Daarom gebruikten wij onze techniek om zéér giftige stoffen (die gebruikt worden in chemotherapiën en radiotherapiën) aan antilichamen te koppelen. Dit resulteerde in een systeem dat chemotherapiën direct naar de kankercellen kan brengen, terwijl ze de gezonde cellen negeren. Dit resulteert in veel minder bijwerkingen.

Deze constructen heten antilichaam-drug conjugaten (in engels: Antibody-Drug Conugates, of ADCs). Dit veld is aanzienlijk aan het groeien in de laatste jaren. In dit proefschrift beschrijven wij een nieuwe en veelbelovende methode om deze medicijnen te maken.

# Chapter 1

# General Introduction



**Abstract:**

Here, we introduce topics related to the research discussed in this thesis. The first part will discuss antibodies, and touch upon their structure and function (section 1.1.), bispecific and trispecific antibodies (section 1.1.2.), and antibody conjugates (section 1.1.3.). Subsequently, several types of antibody conjugation strategies are discussed (section 1.2.), which is followed up by a brief introduction of the enzyme mushroom tyrosinase (section 1.3.). This is followed up by a review regarding quinones and derivatives thereof (section 1.4.), and is finalized by an overview of the SPOCQ reaction (section 1.5.) and the aim of using the SPOCQ reaction in this thesis (section 1.6.).

**Part of this work was published as:**

Jorick J. Bruins, Bauke Albada, Floris L. van Delft, *Chem. Eur. J.* **2018**, *24*, 4749−4756.

## 1.1. Role and anatomy of monoclonal antibodies

Antibodies, or immunoglobulins, are Y-shaped proteins released by B cells of the adaptive immune system to neutralize pathogens.[1] Five main isotypes of immunoglobulins (Ig) are known, immunoglobulin A (IgA), immunoglobulin D (IgD), immunoglobulin E (IgE), immunoglobulin G (IgG), immunoglobulin M (IgM), each with different structural properties (*i.e.* glycosylation patterns, oligomeric complexes) and functions (*i.e.* toxin neutralization, pathogen binding).[1] The full extent of antibodies and their functions are well reviewed and beyond the scope of this thesis.[2, 3] Here, we will only focus on a single isotype, immunoglobulin G1 (or IgG1), which is the most common antibody found in blood circulation[1] and in pharmaceutical applications as biological drugs. We will also only focus on monoclonal antibodies, which are antibodies that come from a single B cell lineage and bind a unique epitope on a specific target antigen, versus polyclonal antibodies that can bind multiple epitopes on the same target.[4] For clarity, we will refer to monoclonal IgG1 as antibodies (or mAbs).

The structure of an IgG1 antibody consist of two identical light chains of about 25 kDa, and two identical heavy chains of about 50 kDa (Figure 1A, C).[4] There are four interchain disulfide bridges that connect the light and heavy chains together (Figure 1A, B, depicted in red), as well as 12 intrachain disulfide bridges that assist in stabilizing the structure (not depicted).[5] The overall structure can also be divided into six domains, two variable domains ($V_L$ and $V_H$) and four constant domains ($C_L$ and $C_H1$, $C_H2$, and $C_H3$). Whereas the constant domains remain largely identical between antibodies, the variable domains contain the binding sites of the target, or antigen, and vary significantly from antibody to antibody depending on the target antigen. More specifically, several strands of β-loops in both the $V_L$ and $V_H$ domains are responsible for the antigen-binding site (also called epitope), these strands are called the complementary determining regions (CDR).[1, 4] Finally, the antibody can also be divided into three 50 kDa fragments; the two Fab regions (fragment, antigen-binding) consisting of the $V_L$, $V_H$, $C_L$, and $C_H1$ domains, and the Fc region (fragment, crystallizable) consisting of the $C_H2$ and $C_H3$ domains. The Fab regions have the antigen-binding site, whereas the Fc regions that can interact with cell surface receptors (Fc receptors) that can activate the immune system[1] and have a conserved glycan-residue at N297 essential for Fc receptor-mediated activity.[6]

While different glycans do not have any effect on antigen binding, they do have an effect on biological mechanisms.[6] For example: the absence of a fucose on the penultimate GlcNAc improves binding to human FcγRIII, which translates into improved antibody-dependent cellular toxicity (ADCC).[7] Similarly, addition or removal of sugars such as sialic acid, or even the removal of the total carbohydrate can have profound effects on functional activities.[8] This is even true between the two most common glycoforms, $G_0F$ and $G_1F$ (Figure 1D), where a single galactose can increase the complement-dependent cytotoxicity by a factor two.[6] These facts illustrate the importance of understanding the importance of detailed analysis of the antibody structure.

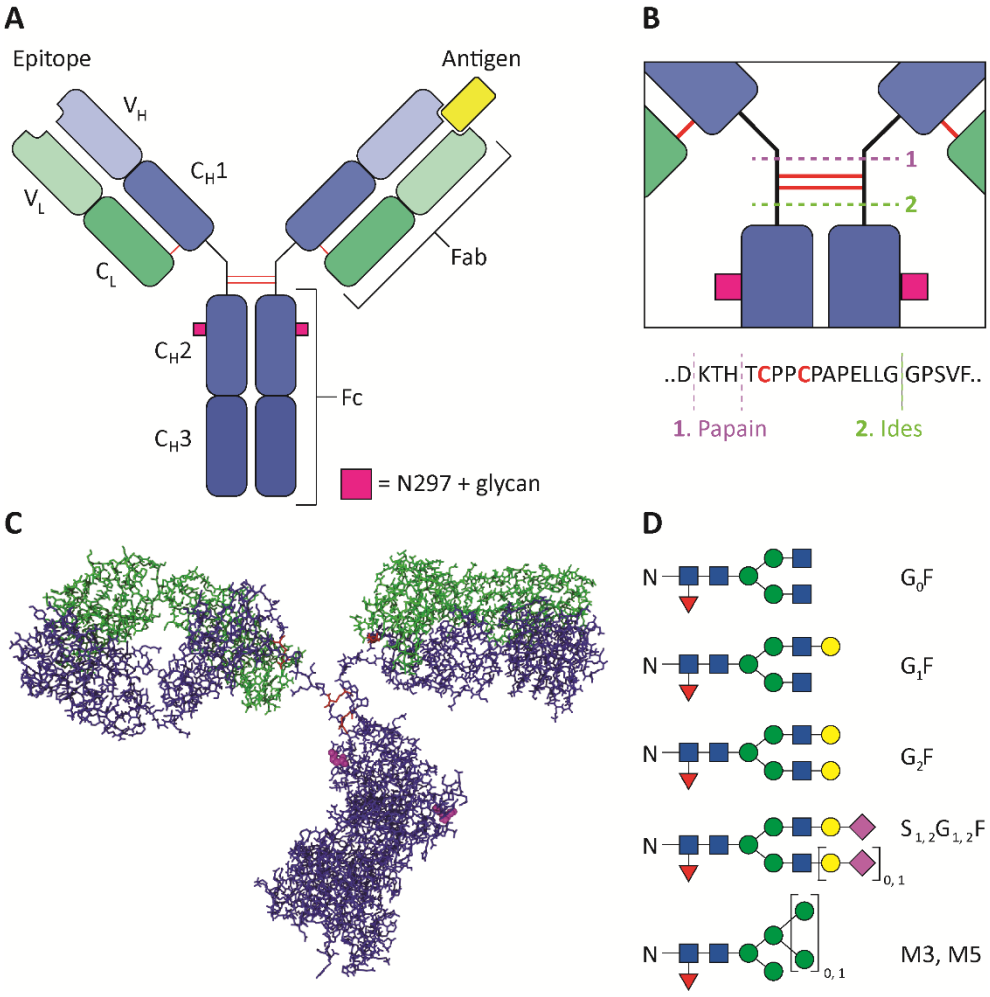



**Figure 1**. (A) Schematic representation of IgG1. Heavy chains are depicted in blue and the light chains in green. The interchain disulfide bonds are depicted in red. (B) Zoom of the hinge-region of IgG1. (C) Crystal structure of an IgG1 (PDB: 1igt[9]). (D) The most abundant[10, 11] glycoforms for IgG1.[i]

### 1.1.1. Antibody digestion and fragmentation

For analytical purposes, digestion of antibodies via enzymatic, chemical or genetic means can be beneficial. Chemical digestion consists mostly of reducing the disulfide bonds by β-mercaptoethanol (BME), dithiothreitol (DTT), or tris(2-carboxyethyl)phosphine (TCEP), resulting

[i] The graphical representation of glycans are according to their nomenclature.[10]

in two 25 kDa fragments (light chains) and two 50 kDa fragments (heavy chains).[12] This is done primarily for analytical purposes, as it can simplify both quantitative and qualitative analysis by SDS-PAGE, HPLC and LC-MS, as well as generate handles for conjugation strategies. Enzymatic digestion can be done by a variety of methods, most of which is done for analysis of antibody purity and integrity.[12] Whereas chemical cleavage relies on the reduction of disulfide bridges, enzymatic cleavage relies on the hydrolysis of an amino acid sequence-specific peptide bond. These include, but are not limited to, the enzymes IdeS, papain, pepsin, and trypsin (Figure 1B).[12, 13] The unique sites of cleavage by these enzymes, called proteases, allows for analysis of different fragments of the antibody, enabling rapid and detailed analysis of the structure and modifications of the antibody.

Antibody fragments can be generated genetically, by expressing only certain monomeric parts of the antibody and, optionally, fusing multiple fragments together. The generation of antibody fragments via genetic modification is mainly done for pharmaceutical applications, and extensive reviews of these antibody fragments and their functions have been reported.[14, 15] A well-known example of antibody fragments is the single chain variable fragment (scFv), where the $V_H$ and $V_L$ are expressed fused together via the N-terminus of one to the C-terminus of the other via a non-specific peptide sequence consisting of 15–20 amino acids (Figure 2B).[16] ScFvs retain the binding exhibited by their parent antibodies, but lack interaction with the Fc receptors. Bare scFvs have many uses, such as preparation of immunotoxins, therapeutic gene delivery, and as anticancer intrabodies.[16] Furthermore, scFvs can also be linked to an antibody to present a second, unique antigen-binding site via protein fusion techniques, creating so-called bispecific antibodies.[15, 17] The advantage of binding multiple different targets are, amongst others, to increase binding affinity,[18] improve internalization of antibody-based drugs,[19, 20] or redirect cytotoxic T-cells for increased immune response near the target cell.[21, 22]

### 1.1.2. Bispecific antibodies

As mentioned above, bispecific antibodies can be made in a variety of ways, such as expressing two scFvs fused in tandem (Figure 2C) or fusing scFvs to the termini of antibodies (Figure 2D).[17] The latter would result in a bispecific antibody with two binding sites for each epitope, *i.e.* a [2]:2 format.[i] Another way to obtain bispecific antibodies is by using asymmetrical antibodies with two distinct antigen-binding sites. An example of this are the knob-in-hole antibodies; by changing only a few key amino acids in the $C_H3$ 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), heterodimers with different epitopes or other mutations be promoted.[17, 23] This method would result in a bispecific antibody with a single

[i] Regarding bi- and trispecific antibody notation: Numbers represent the quantity of a particular binding site or functionality, the number(s) in square brackets are the antibody's binding sites. Numbers after the square brackets are functionalities fused and/or conjugated to the light or heavy chains.

binding site for each epitope, *i.e.* a [1:1] format. Other variants of these heterodimeric antibodies include charge-pairing, where heterodimerization is promoted via negatively and positively charged residues, have been reviewed in length.[15, 17]

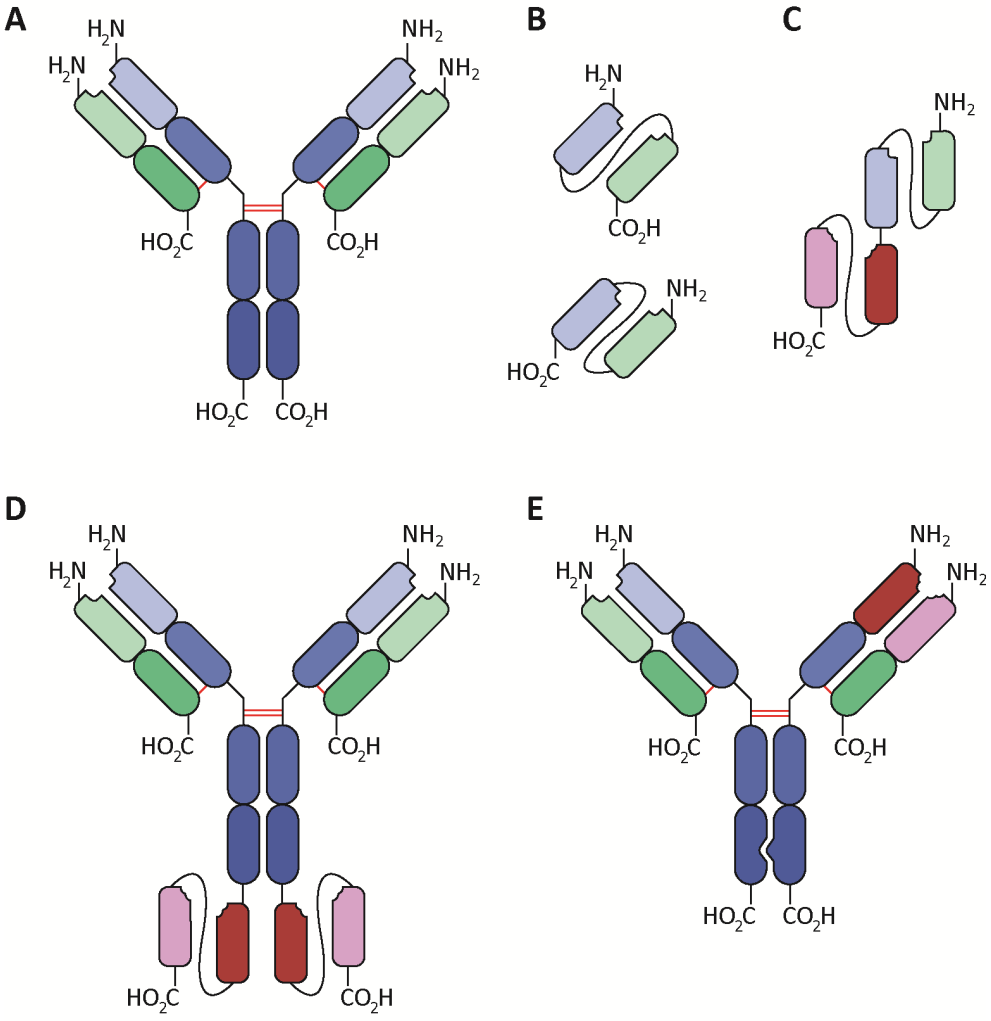



**Figure 2**. Schematic representations of (A) full size antibody with depicted termini, (B) the two permutations of scFv fragments, (C) tandem scFvs with two different scFvs, (D) bispecific antibody with C-terminally fused scFvs as a [2]:2 format, and (E) bispecific knob-in-hole antibody as a [1:1] format.

Higher order functionalities of antibody specificity exist. For example, having a knob-in-hole antibody with identical Fab fragments but two distinct fused scFv fragments would allow for a

trispecific antibody, generating a [2]:1:1 format (Figure 3A).[24] Of note, trispecific antibodies are antibodies that bind to three different targets, whereas trifunctional antibodies are antibodies that have three different functionalities. Functionalities can also include conjugated drugs, radiolabels, cytokines, and fluorophores (Figure 3B).

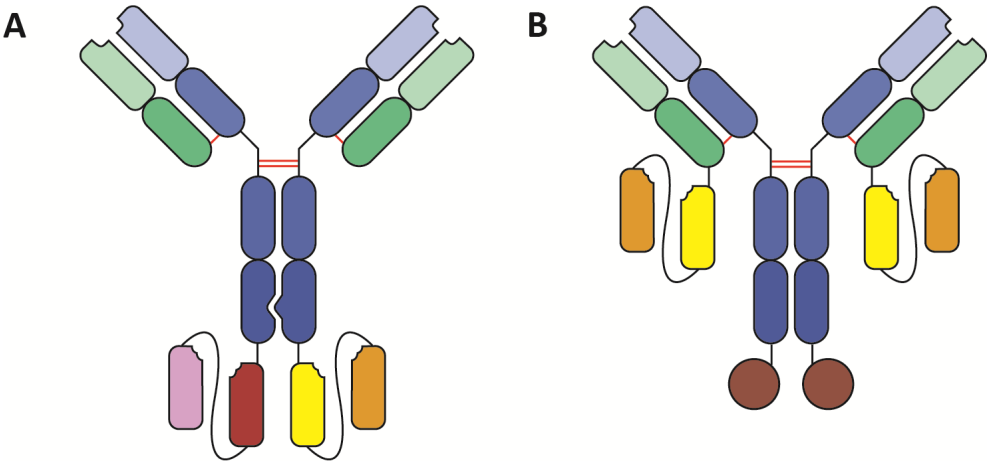



**Figure 3.** (A) Knob-in-hole antibody with two distinct scFv fused, generating a [2]:1:1 antibody format. (B) Trifunctional antibody with two scFvs fused to the light chain and two other functionalities on the heavy chains, generating a [2]:2:2 antibody format.

### 1.1.3. Antibody conjugates

Besides the expression of new functionalities to an antibody via fusion of an additional protein fragments through its termini, (non-natural) functionalities can also be imparted post-recombinantly with a variety of conjugation methods. Via a wide array of chemical and enzymatic methods, functionalities such as fluorescent markers, highly potent cytotoxic agents, radioactive isotopes, and proteins can be introduced in a modular fashion, with potential application in the field of diagnostics,[25] imaging,[26-28] or therapeutics.[28, 29] Notable examples include fluorescent groups or radionuclides on antibodies, allowing cancer cells to be reliably imaged with high sensitivity to provide real-time information regarding cancer screening, staging and treatment.[26-28] Additionally, tumor-specific fluorescent antibodies can greatly aid in surgical procedures with regards to detecting and removing tumors.[25, 26]

Common antibody derivatives with therapeutic applications are antibody-drug conjugates (ADCs)[29, 30] and radiolabeled antibodies.[28] Radiolabeled antibodies are radionuclides with a half-life of a few days conjugated to a tumor-targeting antibody, where the affinity of the antibody results in build-up of radionuclide and therefore localized radiation-treatment. Where radiolabeled antibodies are the localized version of radiation treatment, ADCs are the localized

version of chemotherapy. By coupling a highly potent cytotoxic agent to an antibody, it can be delivered directly to the tumor after binding and internalization. This approach may significantly increases the therapeutic index of the chemotherapy.[i] Release of the drug is important in this matter, which can be achieved via a cleavable linker between the drug and antibody,[31-33] by using bispecific antibodies to increase internalization,[19, 20] and using click-chemistry to release the drug by adding a second chemical probe.[34]

The conjugation of radionuclides or cytotoxic molecules to an antibody of choice is not trivial. Although ADCs can be readily obtained by acylation of the reactive amino group side-chain of lysine residues, random conjugation leads to a highly heterogeneous mixture of conjugates with non-optimal therapeutic index due to decreased binding affinity, accelerated clearance and increased toxicity.[35] Therefore, site-specific conjugation techniques have been widely investigated.[36-39]

### 1.2. Bioconjugate chemistry

Bioconjugate chemistry is the discipline of using chemistry to form covalent bonds between two molecules, of which at least one is a protein, a glycan, a nucleic acid or a lipid.[36] The resulting modified biomolecules can fulfil a plethora of purposes such as targeted drug delivery,[40, 41] cell imaging,[38, 42] improved pharmacokinetics (*e.g.* lower clearance rate, increased thermostability),[43] and diagnostic tools.[44, 45] In this thesis, the focus is entirely on the modification of proteins.

Protein conjugation is unique in the sense that several of the 20 canonical amino acids have specific reactivities,[45] combined with the vast variety of protein structures and functions this leads to endless possibilities for protein conjugates. Employing the innate reactivity of the various amino acids side-chains, in particular lysine and cysteine, is the most straightforward and abundant way to generate protein conjugates.[45] The full extent of these strategies are meticulously described in several review papers,[45-47] therefore only a few examples will be discussed here.

#### 1.2.1. Labeling based on natural amino acids

Lysine residues (**1**) are widely used in protein modification, as the ε-amino function on the side-chains readily reacts with electrophilic reagents such as activated esters (Figure 4A, **2**), yielding stable adducts (**3**).[45] A downside of this innate reactivity is the general lack of selectivity for a specific residue in a given protein, as all or the majority of solvent-accessible residues will undergo acylation, leading to heterogeneous mixtures. Amongst various efforts, one recent

[i] The therapeutic index (TI), or safety window, refers to the ratio between toxic and effective dose. While an indicative TI may be quickly derived based on various animal models, in humans, the TI is defined as the dose where 50% of humans experience adverse side-effects ($TD_{50}$), divided by the dose where 50% of humans experience desired pharmacological effects ($ED_{50}$), resulting in: TI = ($TD_{50}$) / ($ED_{50}$).

report describes the use of sulfonyl acrylate reagents to label the most reactive ε-amino group of one specific lysine in a selective manner.[48] While this method only labels a single lysine residue, it does not allow for targeting of a specific conjugation site. Which can be suboptimal, as different conjugations sites can have varying effects on conjugate functionality and pharmacokinetics.[49]

Cysteine residues (**4**) are, due to their nucleophilic thiol residues and low occurrence in proteins (1-2%),[45] uniquely reactive in proteins and therefore highly employed for bioconjugation. These cysteine residues are either unpaired, resulting in free thiols, or paired to form disulfide bridges vital for the stability of the protein.[50] Generally, thiols can be generated by reducing disulfide bridges, also known as cystines, with reagents such as dithiothreitol (DTT) β-mercaptoethanol (BME), or tris[2-carboxyethyl]phosphine (TCEP).[45] Most commonly, the cysteine thiol moiety is selectively be modified via Michael addition with a maleimide reagent (Figure 4B, **5**), however α-halogenated carbonyl compounds, vinyl sulfones or other electrophilic agents can also be employed.[51] While these modifications are performed easily, maleimide conjugates may suffer from instability issues, while others may be accompanied by side-reactions.[45, 52, 53] Moreover, in case multiple cystines are present in a protein, a heterogeneous mixture of conjugates may result.[54, 55]

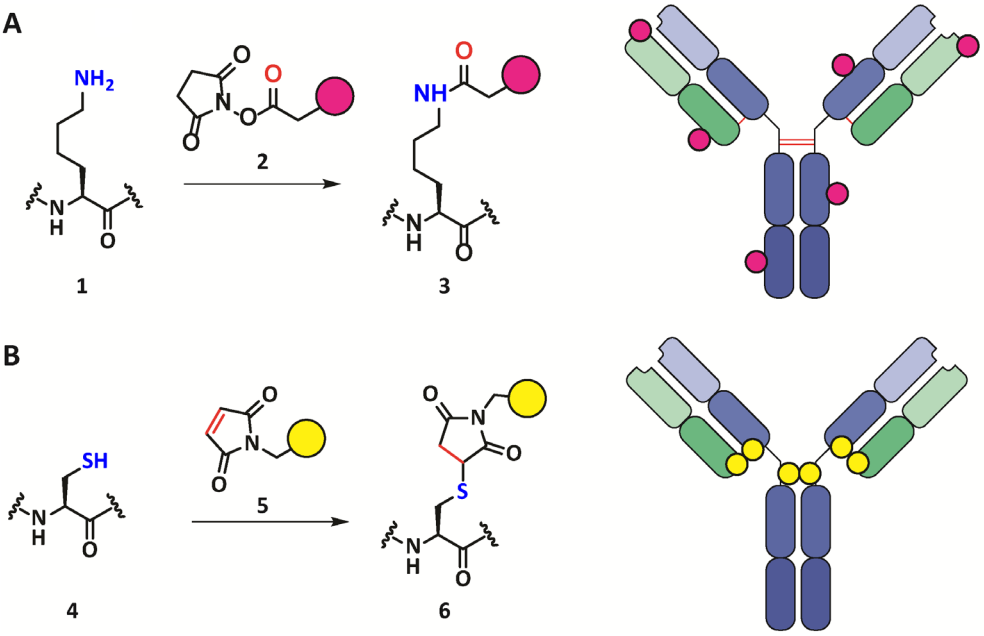
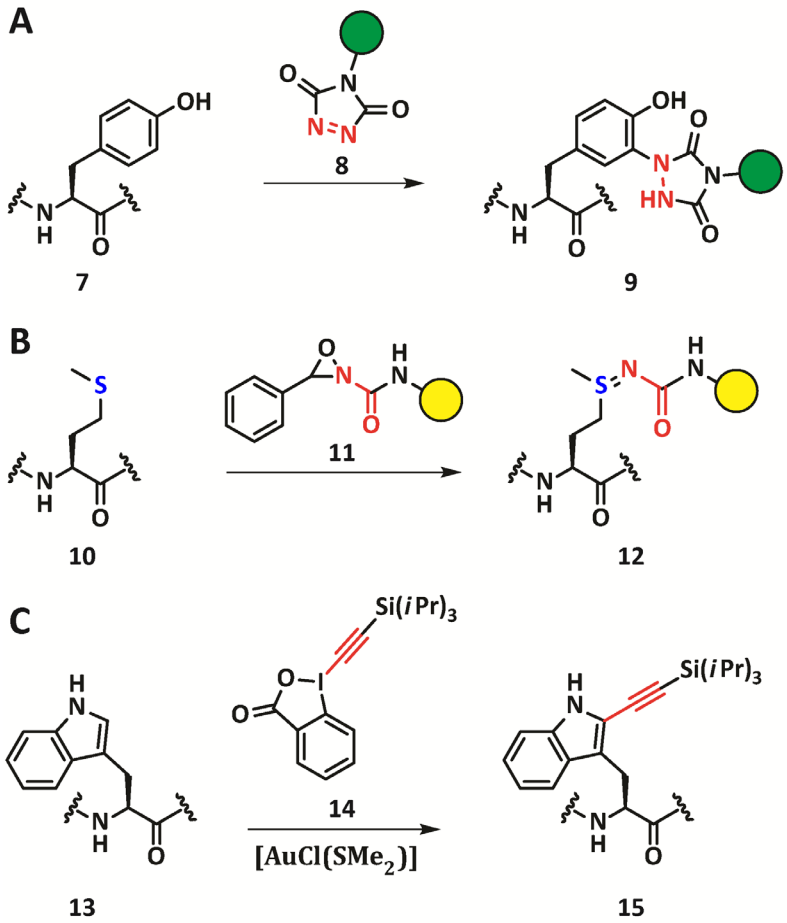



**Figure 4**. (A) Schematic representations of Lysine-NHS conjugation and the resulting random conjugation on antibodies. (B) Schematic representations of Cysteine-maleimide conjugation and the resulting random conjugation on antibodies after disulfide bridge reduction.

Conjugation strategies utilizing less common and reactive amino acids are also available such as PTAD (4-phenyl-3H-1,2,4-triazoline-3,5(4H)-dione)-mediated modification of tyrosine (Scheme 1A),[56] oxaziridine-based methionine conjugation Scheme 1B),[57] or gold-catalyzed ethynylation of tryptophan (Scheme 1C).[58] While these methods provide conjugates with high yield, the use of innate reactivity of an amino acid with multiple occurrences in a protein may not provide site-selectivity. This is clearly demonstrated with the PTAD-modification of tyrosine in human or bovine serum albumin (HSA, BSA),[56] resulting in labeling of 3.9–8.3 tyrosine residues, depending on conditions.




**Scheme 1.** (A) Tyrosine (**7**) conjugation with PTAD (**8**), (B) Methionine (**10**) conjugation with oxaziridine (**11**), and (C) gold-catalyzed tryptophan (**13**) ethynylation.

### 1.2.2. Mutagenesis

Chemically orthogonal reaction sites can be introduced with uniquely reactive groups. In the case of antibodies, this can be achieved by expressing new cysteine groups via site-directed mutagenesis or insertion of an extra cysteine residue.[59, 60] This results in the generation of a protein with a unique single thiol group, which can be selectively labeled with *e.g.* maleimides (Figure 5). While these methods lead to site-selective conjugation, solvent-accessible thiols are susceptible to dimerization and require pre-treatment before conjugation can be attempted.[59, 61] Furthermore, thiol-maleimide conjugates are instable due to *e.g.* retro-Michael side-reactions,[37] which is a major drawback for ADCs as their stability is of paramount importance for a favorable therapeutic index.

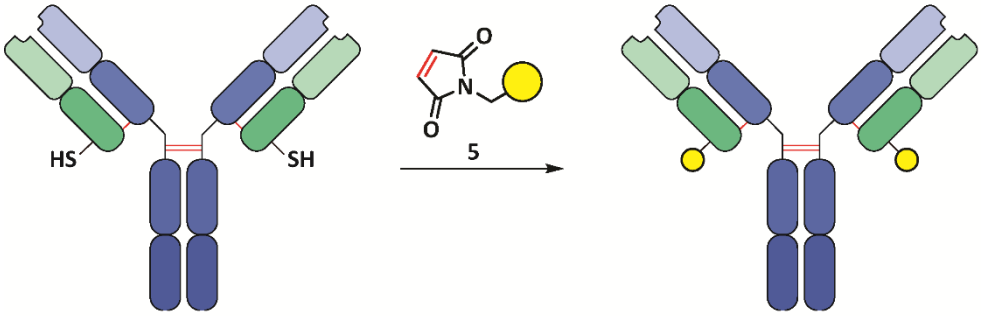


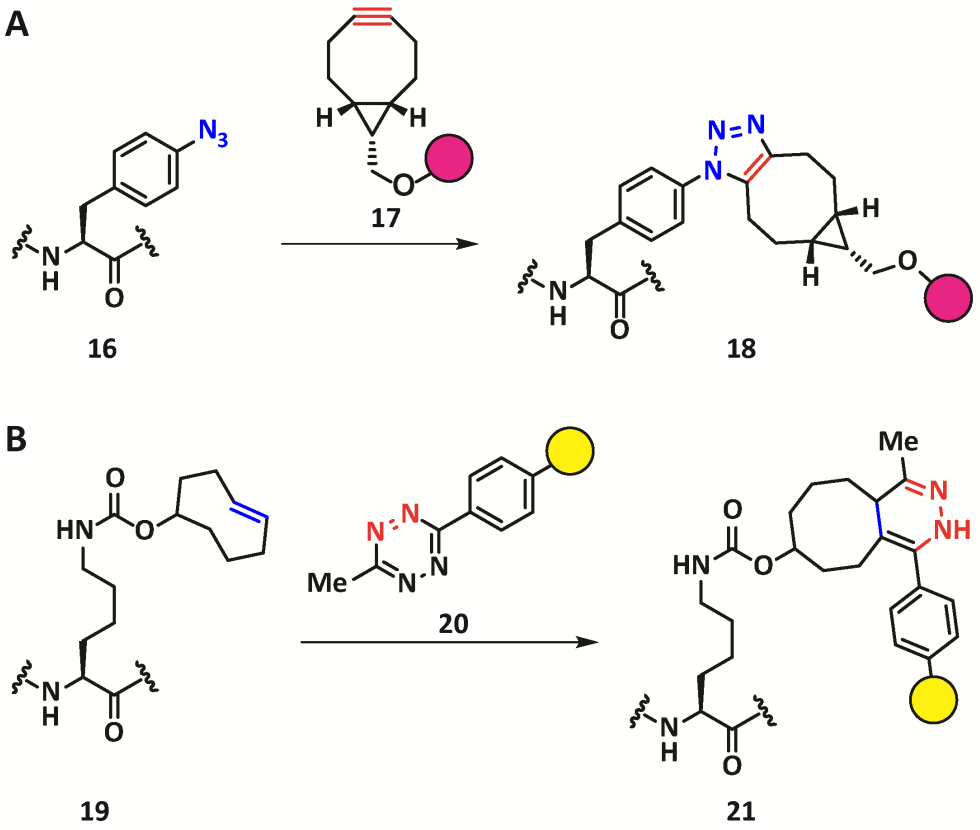

**Figure 5.** Maleimide conjugation of a cysteine-engineered antibody.

To overcome many of the issues associated with conjugation to canonical amino acids, introduction of new, non-canonical amino acids in proteins was developed.[62] By re-allocating the amber stop codon to encode a non-canonical amino acid, the genetic code can be expanded to include new amino acids.[63] With the addition of a non-canonical amino acid, functionalities with fully orthogonal reactivity can be expressed on proteins. This allows for easy and rapid functionalization of antibodies without any side-reactions or pre-treatments.[62]

Noteworthy examples of these bio-orthogonal reactions are the strain-promoted alkyne–azide cycloaddition (SPAAC)[64] and the inverse-electron demand Diels–Alder (IEDDA) reaction with tetrazines,[65] both of which have been reviewed.[38, 66, 67] SPAAC revolves around the (3+2)[i] cycloaddition between azides, such as *p*-azidophenylalanine (*p*-AzF, **16**), and strained alkynes, such as bicyclo[6.1.0]nonyne (BCN, **17**) for rapid and traceless cycloaddition to yield **18** (Scheme 2A).[64] The reaction rate of SPAAC is explained by the fact that the alkyne's SP-hybridized carbon atoms cannot adopt the ideal 180˚ bond angle, resulting in an increase in ring strain.[67] This in

[i] When denoting cycloadditions, round brackets indicate the atoms involved, whereas square brackets indicate the electrons involved. Azide–alkyne cycloadditions are therefore (3+2), [4+2] cycloadditions. Whereas a Diels–Alder is a (4+2), [4+2] cycloaddition. Source: goldbook.iupac.org/terms/view/C01496

turn lowers the barrier of activation for (3+2) cycloadditions, resulting in the spontaneous reaction known as strain-promoted azide–alkyne cycloaddition (SPAAC). Finally, it is noteworthy to mention that BCN can perform inverse-electron demand cycloaddition with azides (IED SPAAC), in particular when an electron-poor azide (*e.g.* 2,6-difluorophenyl azide) is used.[68] The use of electron-poor azides can in fact speed up the reaction (*i.e.* 2,6-difluorophenyl azide increased the reaction rate of SPAAC with BCN by a factor 11), whereas for other commonly used strained alkynes such as DIBAC (aka DBCO) these electron-poor azides reduce the reaction rate (*i.e.* 2,6-difluorophenyl azide decreased the reaction rate of by a factor two).




**Scheme 2**. Schematic representations of (A) azide-bearing phenylalanine and SPAAC with BCN-bearing probes, and (B) TCO-bearing lysine and its IEDDA with MeTz-bearing probe.

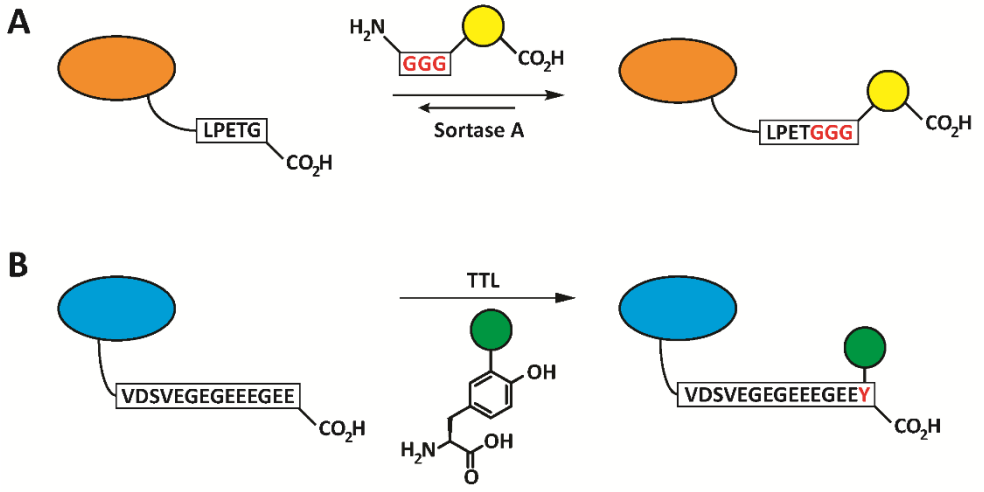
Inverse-electron demand Diels-Alder reactions (IEDDA) are most prominent with 1,2,4,5-tetrazines, of which methyltetrazine (**20**) is a stable and widely used variant.[69] BCN can also perform IEDDA with methyltetrazines at a respectable second-order reaction rate of 80 $M^{-1}$ $s^{-1}$,[70] it is however significantly slower than the *trans*-cyclooctene (TCO) cycloaddition with a rate of

820 $M^{-1}$ $s^{-1}$.[69] While many, faster reacting, *trans*-cyclooctenes exist,[38] regular TCO is a widely used for protein labeling experiments due to its stability and commercial availability. TCO moieties can be expressed on proteins by introducing ε-trans-cyclooct-4-ene lysine (TCO-K, **19**), allowing for labeling with tetrazine-bearing compounds to yield stable conjugates (Scheme 2B, **20**).

### 1.2.3. Enzymatic ligations

As an alternative to the use of non-canonical amino acids, labeling of proteins can be achieved via enzymatic modification of peptide tags.[71] This approach can circumvent issues with the introduction of non-canonical amino acids, such as low protein titers that generally accompany those techniques.[10] By using enzymes, catalysis of reactions can be applied in high specificity under mild conditions on a specific amino acid sequence. This approach is ideal for protein labeling, as this allows for site-selective modification under physiological conditions.

Many examples of enzymatic protein labeling, all of which have been reviewed.[71, 72] One of the most commonly applied enzymes for protein modification employs sortase A, a bacterial $Ca^{2+}$-dependent transpeptidase. Sortase A recognizes the sequence LPXTG, where X can be D, E, A, N, Q, or K (E is used most often).[73] Sortase cleaves the amide bond between T and G residues using the highly reactive thiol in its active center and forms an intermediate thio-ester with the LPXT-bearing protein. Subsequently, the free amino group of an N-oligoglycine-terminated peptide or protein can act as a nucleophile, and yield a new ligated peptide bond (Scheme 3A). Generally, C-terminal LPETG peptide tags are fused to the N-terminus of protein, there are however also procedures describing N-terminal sortase ligation[74] and even sortase ligation in internal protein loops.[75]



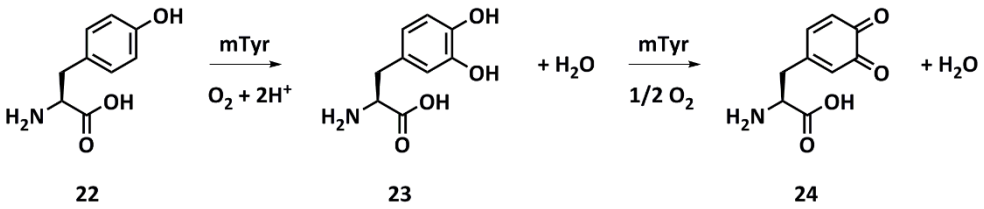

**Scheme 3**. Schematic representations of (A) the sortase reaction, and (B) the tubulin tyrosine ligase.

While sortase A offers an efficient way to enzymatically label some proteins, during ligation the removed C-terminus of an LPXTG-fused protein becomes a substrate for the sortase ligation itself. This reversibility, combined with the fact that water can attack the acyl intermediate to yield the hydrolysis product in an irreversible manner,[76] compromises high ligation yields unless a large excess of glycine-bearing probe is used. This in turn means that protein-protein fusion is unfavorable when using sortase ligation due to high stoichiometric amounts of potentially valuable protein and/or solubility issues due to high protein concentrations. To circumvent this, a step-wise approach may be envisioned based on the introduction of click chemistry handles on proteins for subsequent functionalization, which can lead to efficient production of protein-protein conjugates such as bispecific antibodies.[77-79]

Tubulin tyrosine ligase (TTL) is an enzyme that catalyzes the addition of tyrosine (Scheme 3B), and analogues thereof, to the C-termini of proteins bearing the Tub-tag (VDSVEGEGEEEGEE).[80] By introducing bioorthogonal handles such as azides, subsequent click reactions allow for the conjugation of biomolecules of choice via a two-step ligation strategy similar to sortase A, without the issues of reversible reactions and hydrolysis. Later work demonstrated a much broader substrate scope for TTL, including phenylalanine, L-3,4-dihydroxyphenylalanine (L-DOPA), tryptophan, coumarin, and more.[81] The introduction of L-DOPA is of particular use, as oxidation with sodium periodate ($NaIO_4$) generates quinones, which are highly susceptible to Michael addition or Diels–Alder modification. These quinones are also regularly found in Nature, generally generated by enzymes called tyrosinases.

### 1.3. Tyrosinases

Tyrosinase (polyphenol oxidase, EC 1.14.18.1) is an enzyme that is found in a broad number of species ranging from bacteria, fungi, plants to mammals.[82] While tyrosinases differ significantly with respect to their sequences, size and glycosylation patters,[83] they are similar in their enzymatic activity. The active site consists of six histidine residues coordinating two copper ions, which can perform oxidation by using molecular oxygen. The enzyme can catalyze both the *ortho*-hydroxylation of mono-phenols such as tyrosine (**22**) to di-phenols (**23**), as well as the subsequent oxidation to di-ketones (**24**) (Scheme 4).[82, 84] These di-ketones are called quinones, which due to the *ortho*-hydroxylation are only present in the form of *ortho*-quinones. The formation of quinones via tyrosinases play significant roles in nature, such as the formation of melanins in skin pigmentation to protect against UV radiation via a process called melanogenesis,[85, 86] as well as the browning of food products, especially in mushrooms, bananas, apples, pears, potatoes, avocadoes and peaches.[87, 88]




**Scheme** 4. General mechanism for reactions catalyzed by (mushroom) tyrosinase.[89, 90]

One of the most well-studied tyrosinases is the one found in the common mushroom (*Agaricus bisporus*), or mushroom tyrosinase (mTyr).[91] This tyrosinase is expressed as a heterotetramer comprising of two identical heavy chains of 43 kDa (H subunit) and two identical light chains of 14 kDa (L subunit) (Figure 6).[92] The tetramer is stabilized by calcium ions between the two H subunits, which in the generation of the crystal structure was substituted for Holmium (III) ions (not depicted) to diminish repulsion between the negatively charged residues in the dimer interface (D336, D353, and E351 from one H subunit and the D312 side chain from the other).[92] Each H subunit has an interface with one L subunit, stabilized by two salt bridges: H76 (H)–E139 (L) and E317 (H)–H56 (L).[92] It was found that isolated monomeric H subunit retained its oxidative activity suggesting the H subunit is the tyrosinase part,[93] whereas the function of the L subunits is unknown.

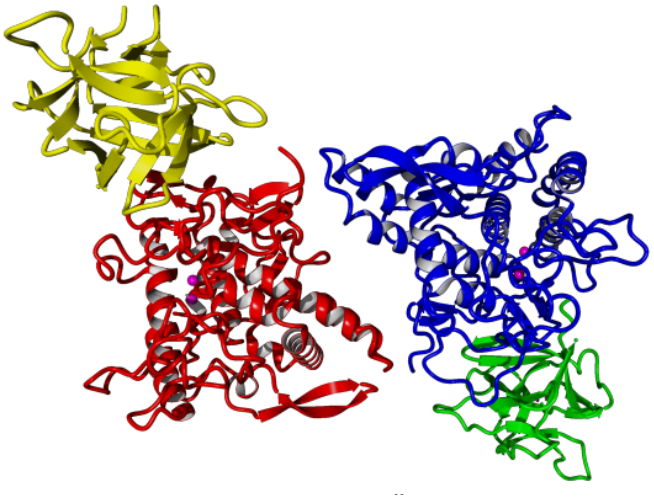


**Figure** 6. Crystal structure of mushroom tyrosinase (PDB: 2y9w).[92] The blue and red subsections are the H subunits, the green and yellow subsections are L subunits, and the purple balls are the copper atoms.

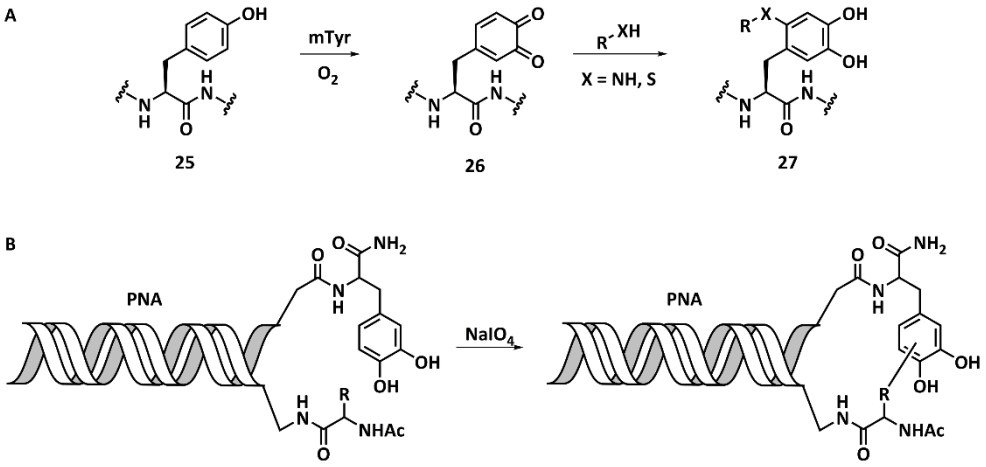
Tyrosinases find many applications in the field of dye production, self-tanning agents, biosensors, tea production and more.[94] The generation of quinones by tyrosinases can also be used for conjugation strategies, as their electron-poor nature allows for rapid Michael additions.[95] Mushroom tyrosinase is generally used in these conjugation procedures, as it is an inexpensive and readily available enzyme.

### 1.3.1. Quinone chemistry[i]

The quinones formed by tyrosinases are highly electrophilic compounds that readily undergo attack by nucleophilic species like a thiol or an amine to afford a stable aromatic conjugate. Within the context of a protein, this means that quinones will react with the sidechains of cysteine, lysine, or histidine residues via Michael addition (Scheme 5A).[96-98]

Utilizing this concept, Liu *et al*. showed that nucleophilic amino acids are able to undergo pseudo-intramolecular cross-coupling when brought in close proximity by two short complementary peptide nucleic acid (PNA) strands.[99] Thus, an in situ generated quinone from 3,4-dihydroxylphenylalanine (DOPA) using sodium periodate (Scheme 5B) underwent Michael addition with specific amino acid residues attached to the complementary PNA strand. Obviously, the hybridized PNA strands ensure close proximity of the intermediate quinone and the nucleophilic amino group on the complementary strand. First, it was found that the α-amino group of alanine was capable of performing this reaction (not depicted), but not when the amine was protected with an acetyl group. Similarly, Liu *et al*. detected products resulting from the cross coupling via the side-chains of lysine, histidine and cysteine (Scheme 5B), with the thiol of cysteine being the most reactive partner. Finally, lack of reactivity of the side-chains of Arg, Glu, Met, Ser, Thr, Trp, Tyr, Phe and Gln residues corroborated the ability of a free amine, imidazole, or thiol group for Michael addition to quinones, but not of alcohol, guanidine, thioether, indole, carboxylic acid, and amide.

[i] Subsections 1.4.1 – 1.4.4 was published as a review; Jorick J. Bruins, Bauke Albada, and Floris L. van Delft, Chem. - Eur. J., **2018**, 24, 4749−4756.

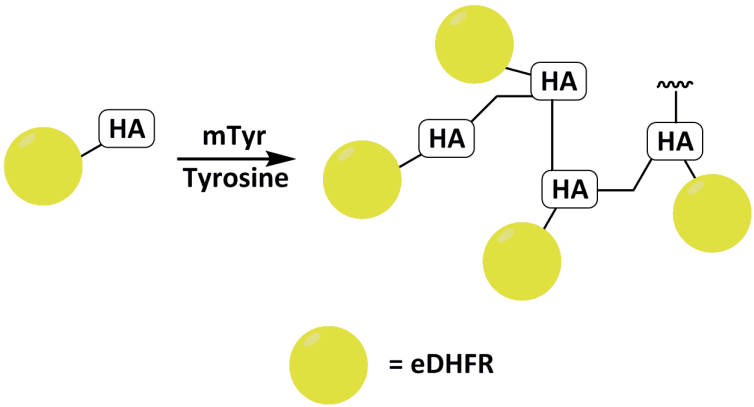



**Scheme 5**. (A) Oxidation of tyrosine to its corresponding quinone, followed by a Michael addition of a nucleophilic side-chain functionality of an amino acid residue R (i.e. Cys, His, or Lys). (B) PNA crosslinking via DOPA-oxidation by $NaIO_4$ and subsequent Michael addition.

### 1.3.2. Polymerization and cross coupling of proteins

The tendency of tyrosine to polymerize upon exposure to oxidizing conditions finds a prominent role in nature in the form of melanin formation, either through copolymerization with cysteine to form pheomelanin,[85] or self-polymerization to eumelanin.[100-102] This phenomenon has been widely studied and applied to e.g. grafting of chitosan,[103] and immobilization of proteins to amino-modified polystyrene beads.[104] Despite the fact that phenolic side-chains of tyrosine residues are themselves incapable of nucleophilic attack on quinone residues,[99] Long *et al*. were able to show that proteins carrying a tyrosine-rich tag (i.e. hemagglutinin-tag, abbreviated as HA-tag) can be used for protein crosslinking upon addition of free tyrosine.[105] Interestingly, when the HA-tag (YPYDVPDYA) was expressed at the C-terminus of Escherichia coli dihydrofolate (eDHFR), oxidation of the tyrosine-rich tag by mushroom tyrosinase (mTyr) led to the formation of quinones, which combined with the addition of free tyrosine to the mixture led to cross-coupled HA-labeled eDHFR (Scheme 6). This was due to polymerization of tyrosine, with incorporation of the tyrosine-rich HA-tag. This effect was intensified in case of introduction of a more tyrosine-rich tag (GYGYGYGYGY). Not surprisingly, in the presence of excess nucleophilic amino acids other than tyrosine, functionalization was observed instead of polymerization, whereas fragmentation of the HA-tag was observed in the absence of any additional amino acid.

The use of tyrosine-bearing tags as employed by Long *et al*. allows for modification on proteins by selective oxidation of tyrosine residues and subsequent functionalization. However, a more selectively addressable coupling strategy is required to prevent non-selective cross coupling between nucleophilic amino acid residue sidechains and the formed quinones.

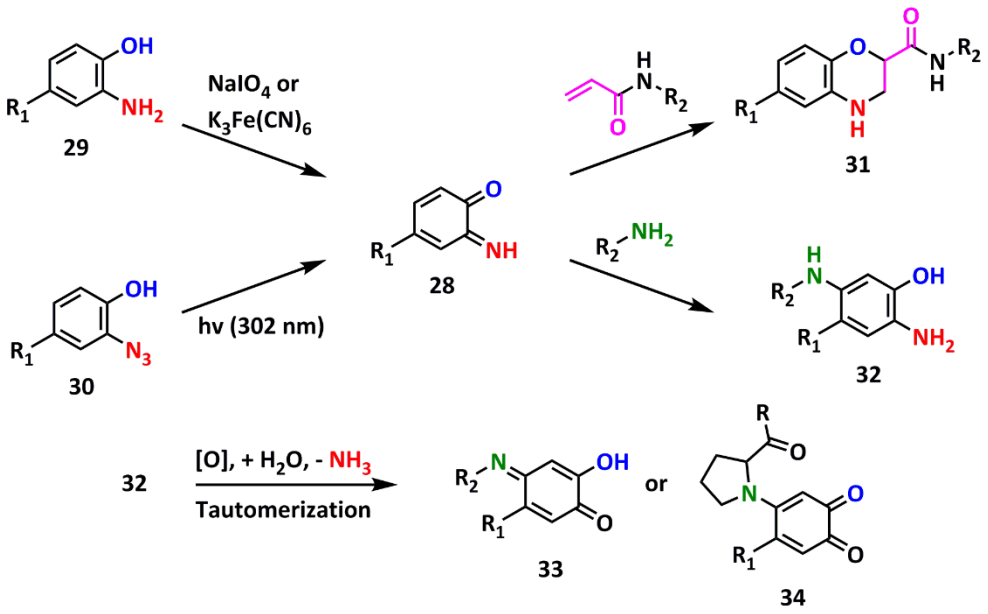



**Scheme 6**. Crosslinking of HA-tagged eDHFR by tyrosine polymerization.

### 1.3.3. Iminoquinones

Similar to regular quinones, *ortho*-iminoquinones (**28**) (from now on referred to as iminoquinones) can be generated from aminophenols (**29**) upon oxidation with $NaIO_4$ or $K_3Fe(CN)_6$,[106, 107] or by photoactivation of azidophenols (**30**) (Scheme 7).[108] Based on their ability to selectively react with nucleophilic nitrogen atoms like the N-terminus of a protein or the amine functionality of anilines,[106, 109] iminoquinone derivatives have found useful applications in bioconjugation chemistry.[110] After Michael addition of an N-terminal amino acid or aniline residue, tautomerization to (**32**) occurs and a second oxidation takes place to yield (**33**) in presence of oxidant (Scheme 7) or (**34**) if N-terminal proline was the utilized as nucleophile.[110] It was observed that proline was most reactive and yielded the highest conversion among all amino acid residues.[109] Francis et al. also observed that $NaIO_4$ was a more potent oxidant than $K_3Fe(CN)_6$, but also gave rise to multiple products whereas $K_3Fe(CN)_6$ only yielded a single product.[107] Furthermore, when photoactivation of azidophenols was performed to obtain iminoquinones, coupling with the N-terminus of proteins was not observed due a much higher reaction rate of aniline addition and a general lack of N-terminal coupling at pH 6.0.[109]

Besides nucleophilic addition, iminoquinones are also able to undergo (4+2) cycloaddition with acrylamides, leading to morpholine-type structures (**31**).[111] Due to the electron-poor character of the acrylamide double bond, cycloaddition in this case proceeds via the regular hetero-Diels–Alder HOMO-LUMO interaction of alkene with the oxygen and nitrogen atom.

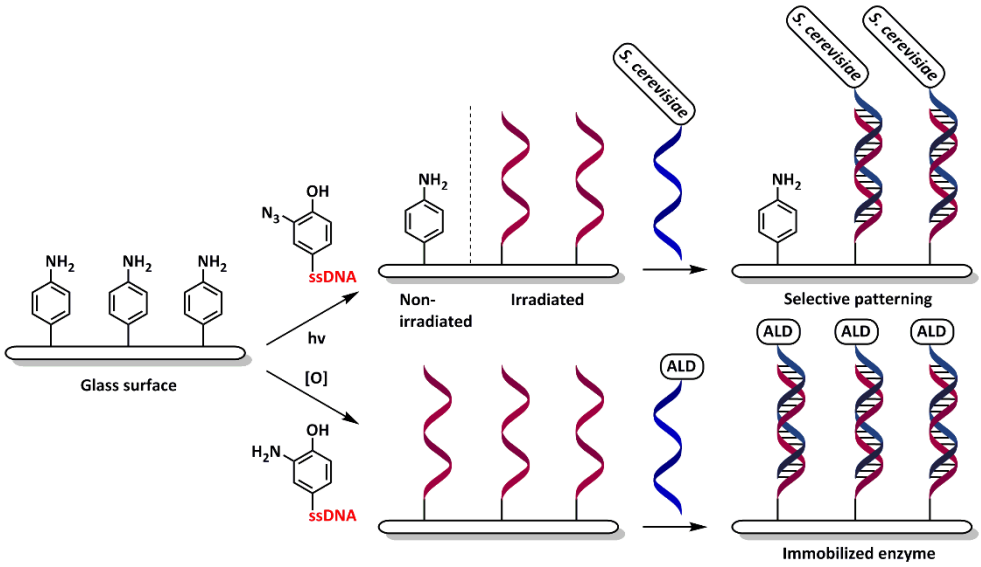



**Scheme 7**. Generation and subsequent reactions of iminoquinones as reported by Francis *et. al*.

Iminoquinone conjugation in biomolecules has been realized by introducing *p*-aminophenylalanine on the external surface of genome-free MS2 capsids using amber stop codon suppression (not depicted).[106] The incorporated aniline moieties could be modified by various *o*-aminophenol compounds after oxidation with $NaIO_4$, including a cyclic penta-amino acid variant of RGD peptide. Comparable results were obtained when the iminoquinone was generated from o-azidophenols upon irradiation with 302 nm light.[108] The applicability of this chemistry was not limited to bioconjugation strategies in solution, but was also applicable on surfaces (Scheme 8, top). For example, glass modification with aniline-bearing trimethoxysilane group coupled followed by selective surface patterning with in situ generated iminoquinone (by light irradiation of *o*-azidophenol) attached to a single stranded DNA. Subsequent binding of Saccharomyces cerevisiae, modified with the complementary DNA strand, allowed visualization of the photolithographic pattern.

Bioconjugation with iminoquinones was also employed to label high-molecular weight polyethyleneglycol [112] chains to the N-terminus of various proteins (not depicted).[109] Using $K_3Fe(CN)_6$, a milder oxidant with less side-reactions than $NaIO_4$,[107] oxidation of *o*-aminophenols allowed for quick and selective modification of N-terminal residues, with proline having the highest degree of conjugation. This was applied to modify aniline-bearing glass surfaces with enzymes.[113] Analogously to the light-induced patterning modification of glass, single stranded DNA was introduced by in situ generated iminoquinone via $K_3Fe(CN)_6$ oxidation. Next, a

complementary strand of DNA was introduced (Scheme 8, bottom), which was linked in an identical fashion to the N-terminal proline residue of fructose-bisphosphate aldolase (ALD). The enzyme was proven to retain its activity after immobilization and could be removed by adding a complementary DNA-strand, regenerating the DNA-bearing glass surface. Finally, fresh enzyme-bearing DNA could be added, and catalytic activity was achieved once more.



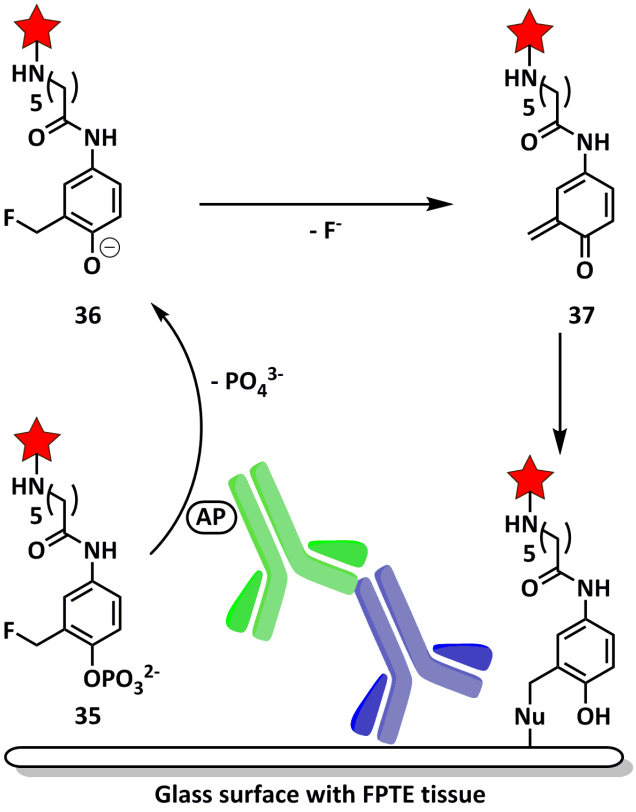

**Scheme 8**. Glass surfaces bearing aniline residues and their subsequent modification for DNA-directed immobilization. ALD = fructose-bisphosphate aldolase.

### 1.3.4. Quinone methides

*Ortho*-quinone methides (from now on referred to as quinone methides) are carbon analogues of regular quinones, with one of the oxygen atoms replaced by a carbon atom (most typically a methylene group). Quinone methide derivatives are widely reported in synthesis and catalysis,[114-118] and, by virtue of their DNA alkylation properties,[119, 120] are known as potent anticancer drugs.

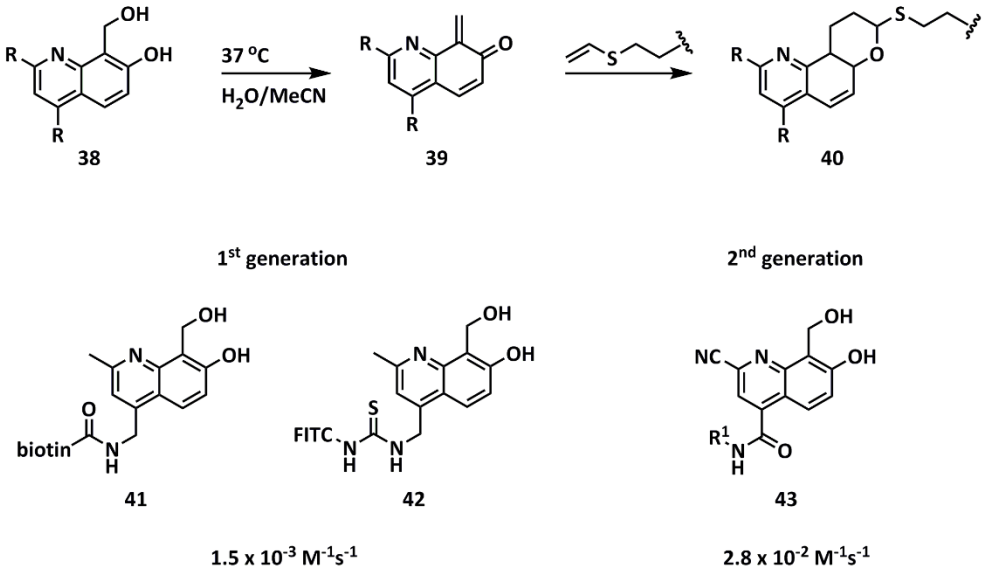
Like quinones, quinone methides have both an electrophilic character and a propensity to undergo (4+2) cycloaddition.[117, 118] In this regard, it is clear that protein modification based on quinone methides requires careful modulation of stability and reactivity. To this end, Rokita et al. explored the effects of substituents on the formation and stability of quinone methide derivatives and their adducts based on nucleophilic addition.[121, 122] Recently, Bieniarz et al. reported covalent labeling of tissue in close proximity of cancer epitopes with in situ generated

quinone methides.[123] To this end, a primary antibody was utilized to recognize and bind to a specific cancer antigen, followed by binding of a secondary antibody conjugated to alkaline phosphatase (Scheme 9). Next, incubation with phosphorylated quinone methide precursor (**35**) led to enzymatic hydrolysis of the phosphate group by alkaline phosphatase to form (**36**), and subsequent 1,4-elimination of the fluoride group yielded the desired quinone methide (**37**). The quinone methide reacted with any proximate immobilized nucleophile, or was quenched by any nucleophile in the reaction media (*e.g.* Tris or water). Despite the high reactivity of the quinone methide, Bieniarz et al. were able to visualize B-cell lymphoma marker BCL6 on tonsil with both biotin and fluorophore markers. This method of proximity-based generation of a quinone methide is similar to a method developed by Li et al.,[124] where conjugation was achieved by using an affinity tag and subsequently generation of quinone methide by activation with UV light.




**Scheme 9**. Selective tissue modification by in situ dephosphorylating and generation of quinone methides.

Another quinone methide bioconjugation method was developed by Lei et al., whom reported that a (4+2) cycloaddition reaction of a vinyl thioether with *ortho*-quinolinone could be employed for cellular organelle imaging (Scheme 10).[125-127] After incubating quinolinone (**38**) at 37 ˚C, quinone methide derivatives (**38**) were formed that were found to undergo cycloaddition with a vinyl thioether. Michael addition of free thiols to the formed quinone methide was also observed, but due to reversibility of this reaction, the (4+2) cycloaddition product (**40**) was obtained as the main product. When biotin or fluorescein (FITC) were attached to the quinone methide precursor (**41** and **42**, respectively), labeling and imaging of vinyl thioether-bearing BSA and vinyl thioether-bearing Taxol inside live cells was achieved.[125] A 2nd generation *ortho*-quinolinone (**43**) was developed with approximately 20-fold higher reaction rate (2.8 x $10^{-2}$ $M^{-1}$ $s^{-1}$ versus 1.5 x $10^{-3}$ $M^{-1}$ $s^{-1}$) and its bioorthogonality to SPAAC was demonstrated.[127] While this method is chemoselective and bioorthogonal to the broadly applied SPAAC conjugation approach, the *ortho*-quinolinone quinone methide requires introduction of the vinyl thioether via a non-selective lysine-NHS conjugation strategy. Furthermore, with a reported rate of 2.8 x $10^{-2}$ $M^{-1}$ $s^{-1}$ for the second generation, the reaction is considerably slower (approximately 105-fold) the iminoquinone conjugation methods.[110] Similar to acrylamides reaction with iminoquinones, cycloaddition takes place via normal electron-demand Diels–Alder cycloaddition on the oxygen and methylene group.




**Scheme 10**. (4+2) cycloaddition with vinyl thioethers and *ortho*-quinolinones, for the 2nd generation precursor: $R_1$ = biotin or fluorescein piperazine.

### 1.4. Strain-Promoted Oxidation-Controlled *ortho*-Quinone cycloaddition (SPOCQ)

We recently showed that quinones undergo fast strain-promoted oxidation-controlled *ortho*-quinone cycloaddition (SPOCQ) with bicyclo[6.1.0]nonynes (BCN).[128, 129] In a model reaction between 4-*tert*-butyl-1,2-quinone (Figure 7A, **44**) and BCN-alcohol (**45**), a reaction rate of 496 ± 70 $M^{-1}$ $s^{-1}$ was determined, leading to the cycloaddition product (**46**). A noteworthy observation is that SPOCQ proceeds via an inverse-electron demand Diels–Alder cycloaddition of the LUMO of the quinone with the HOMO of BCN. As a logical consequence, the strained alkyne DIBAC,[66] also known as DBCO, reacts over 1000x slower (0.19 $M^{-1}$ $s^{-1}$) than BCN, due to its much lower-lying HOMO orbital. This difference in reactivity between strained alkyne and quinone parallels that of the relative reaction rate with 1,2,4,5-tetrazine or electron-poor azide.[68, 130, 131]

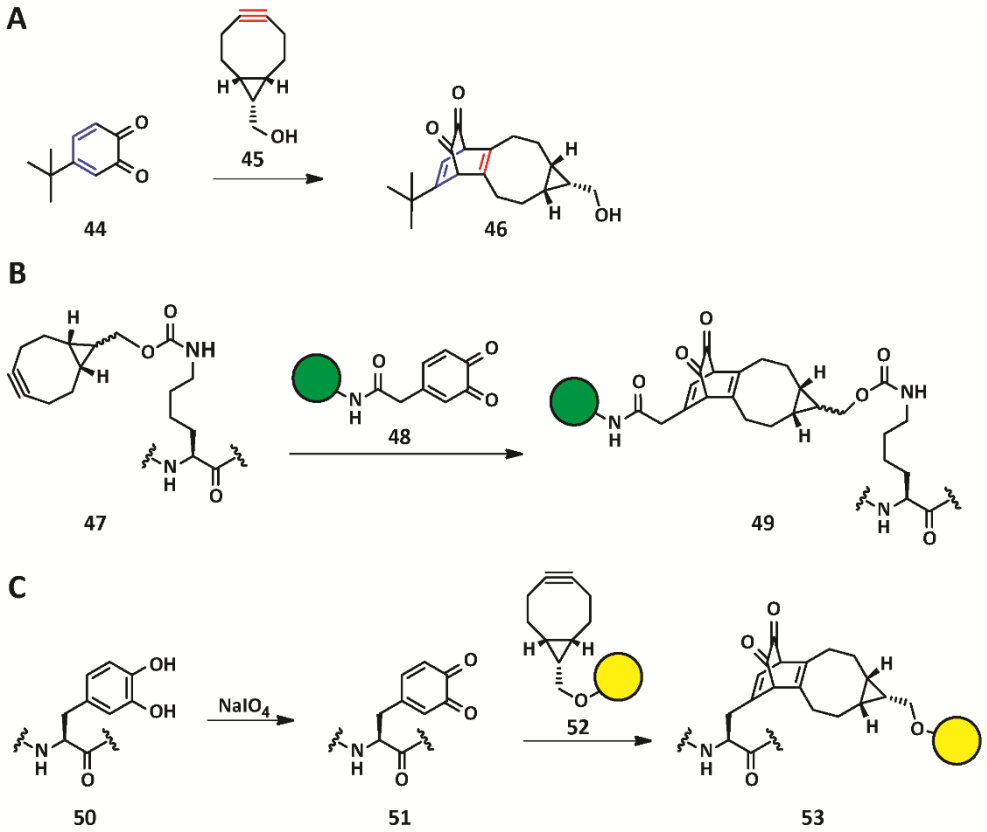



**Figure 7.** SPOCQ between (A) *tert*-butyl quinone and BCN-OH, (B) genetically encoded BCN-lysine and quinone-bearing probe, and (C) oxidation of L-DOPA into a quinone and subsequent SPOCQ with BCN-bearing probe.

SPOCQ was used to conjugate on proteins via the introduction of non-canonical amino acids BCN–lysine (Figure 7B, **47**) and *L*-Dihydroxyphenylalanine (Figure 7C, **50**).[128, 132] With the BCN–bearing proteins, SPOCQ could be achieved be adding quinones (**48**) bearing fluorescent tags within a few minutes.[128] This approach however exposes nucleophilic amino acids such as lysine, cysteine, and histidine to quinones prone to Michael addition, resulting in unwanted side-reactions. The alternative is to introduce L-dopa by virtue of non-canonical amino acid (**50**) and perform the cycloaddition by oxidizing the diol with sodium periodate,[132] the generated quinone (**51**) is then available to perform SPOCQ with BCN-bearing probes (**52**). While this method works and yields clean conjugates, the use of non-canonical amino acids is suboptimal due to aforementioned decrease protein titers.

## 1.5. Outline of this Thesis

The research described in this thesis centers around the chemoenzymatic, site-specific modification of proteins, in particular monoclonal antibodies, by the combination of (a) introduction of an exposed tyrosine residue, (b) oxidation of tyrosine side-chain by mushroom tyrosinase (mTyr) to quinone, allowing for (c) a one-pot conjugation strategy based on strain-promoted cycloaddition of strain ring to the resulting quinone (SPOCQ).

In **chapter 2**, we describe the concept of employing SPOCQ for site-specific labeling of an engineered protein based on C-terminal fusion of a tetra-glycyltyrosine tag ($G_4Y$-tag) based on tyrosinase-mediated quinone generation and reaction with cyclooctyne, and application of the new methodology for the generation of fluorescently labeled antibodies and antibody-drug conjugates (ADCs). In **chapter 3**, we demonstrate that SPOCQ can also be achieved with a strained alkene, *i.e.* cyclopropanated *trans*-cyclooctene (cpTCO), and its orthogonality with SPAAC, thereby allowing the generation of dual-functionalized antibodies by performing two reactions in tandem. In **chapter 4**, it is described how knob-in-hole (KiH) technology can be applied to introduce a single molecule (or protein) of interest on an antibody with either SPOCQ or sortase ligation. In **chapter 5**, we demonstrate a two-step conjugation method for rapid and selective protein-protein conjugation by combination of sortase ligation and SPOCQ, and use the combination of various technology to generate trifunctional antibodies-protein conjugates, bearing scFvs and cytokines. In **chapter 6**, we report on SPOCQ performed on tyrosine residues residing in solvent-exposed loops residing in an internal sequence of engineered antibodies. C**hapter 7** contains an overarching discussion of the work reported in this thesis, give a detailed overview of performing SPOCQ on a technical level, and we examine future possibilities of SPOCQ on proteins. Finally, the **appendix** contains a step-by-step protocol for performing SPOCQ on proteins.

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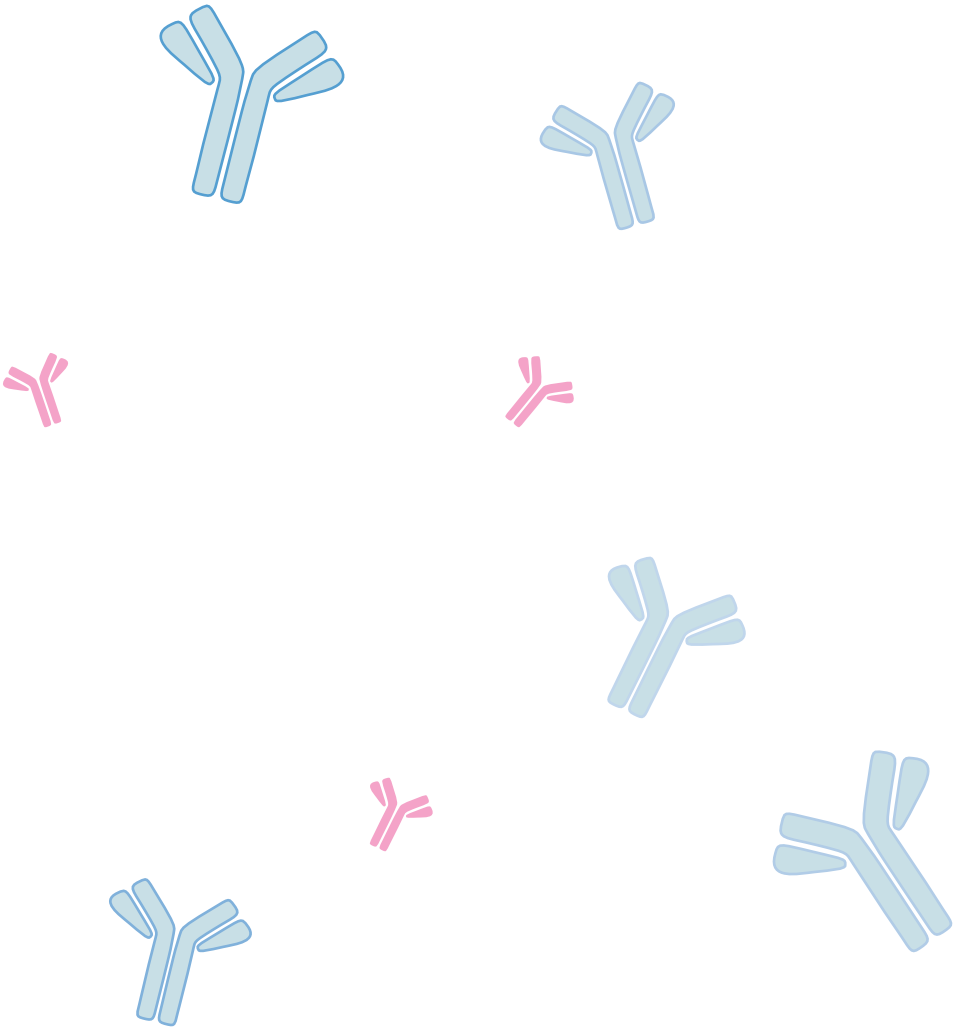
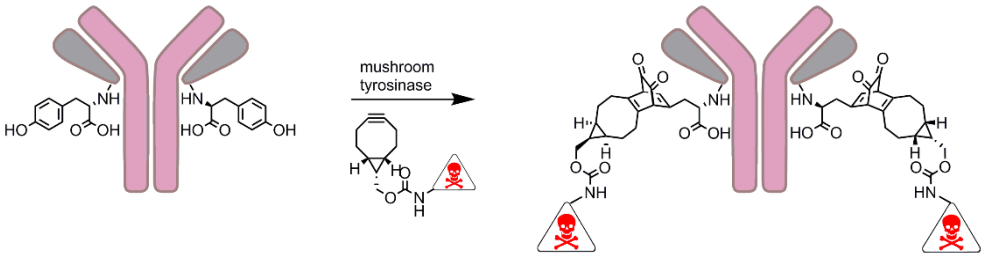
# Chapter 2

# Inducible, Site-specific Protein Labelling by Tyrosine Oxidation–Strain-Promoted (4+2) Cycloaddition



**Abstract:**




*Genetically encoded tyrosine (Y-tag) can be utilized as a latent anchor for inducible and site-selective conjugation. Upon oxidation of tyrosine with mushroom tyrosinase, strain-promoted cycloaddition (SPOCQ) of the resulting 1,2-quinone with various bicyclo[6.1.0]nonyne (BCN) derivatives led to efficient conjugation. The method was applied for fluorophore labelling of laminarinase A and for the site-specific preparation of an antibody-drug conjugate.*

**This work was published as:**

Jorick J. Bruins, Adrie H. Westphal, Bauke Albada, Koen Wagner, Lina Bartels, Hergen Spits, Willem J. H. van Berkel, and Floris L. van Delft
*Bioconjugate Chem.*, **2017**, *28* (4), 1189-1193

## 2.1. Introduction

Functional modification of proteins can be achieved in a wide variety of ways. Early generation approaches, entailing the reaction with amino or thiol groups of amino acid side chains,[1-3] are effective and facile but in most cases lack selectivity due to relatively high natural abundance of lysines and cysteines in proteins.[3] Other methods involving conjugation to tyrosine, histidine, or tryptophan side chains or to the N-terminus of proteins have also been developed. As an alternative to native proteins, full control of regioselectivity can be achieved by introduction of a non-natural amino acid containing a functional handle such as an azide, a ketone or an *ortho*-aminophenol but often at the expense of protein expression yields.[4-6] Site-specific conjugation can also be achieved through enzymatic means, as, for example, with sortase-mediated conjugation, formyl-generating enzyme (FGE), tubulin tyrosine ligase, or activation of dihydrotetrazines by oxidation for tetrazine-TCO ligation via horseradish peroxidase or a photocatalytic agent.[7-10]

Tyrosine residues show promise as selective conjugation sites, as the relative hydrophobicity of tyrosine combined with the tendency of π–π stacking of the aromatic rings results in the limited exposure of tyrosine residues on the periphery of proteins, resulting in generally low accessibility.[11, 12] As a consequence, reactive small molecules may conjugate to tyrosines for less selective conjugation,[13-15] while more exposed tyrosine residues can allow for a wide variety of enzymatic reactions.[12, 16-20] For example, exposed tyrosine residues can be oxidized by mushroom tyrosinase to generate a 1,2-quinone, which can undergo nucleophilic attack by amines or thiols from the side chains of lysine, histidine, or cysteine.[21-23]

We recently showed that a 1,2-quinone undergoes fast strain-promoted oxidation-controlled quinone–alkyne cycloaddition (SPOCQ) with bicyclo[6.1.0]nonyne (BCN).[24-26] Here, we report that SPOCQ finds useful application in protein labelling via *in situ* generation of a quinone by oxidation under the action of mushroom tyrosinase (mTyr). We demonstrate that fast and complete C-terminal labelling of proteins, including an enzyme and a monoclonal antibody, can be readily achieved with fluorescent BCN-lissamine **1**. The potential usefulness of the SPOCQ labelling approach is exemplified by fully controlled, site-specific generation of an antibody-drug conjugate based on anti-influenza AT1002 and BCN bearing the highly potent tubulin binder monomethyl auristatin F (BCN-MMAF) **2**.

## 2.2. Results and discussion

**Laminarinase A.** Given the inaccessibility of native tyrosine residues by mTyr,[11, 12] it was envisioned that an exposed tyrosine required installation in a protein of interest by means of a short spacer. Thus, tetra-glycyltyrosine ($G_4Y$) was genetically fused to the C-terminus of a model protein laminarinase A (LamA), a hyperthermostable endo-β-1,3-glucanase from *Pyrococcus furiosus*, which contained an N-terminal His-tag for purification.[16, 27] Modifications were achieved via site-directed mutagenesis and expression in *Escherichia coli*, resulting in a C-terminal $G_4Y$ fusion (LamA–$G_4Y$, here referred to as Y-tag).

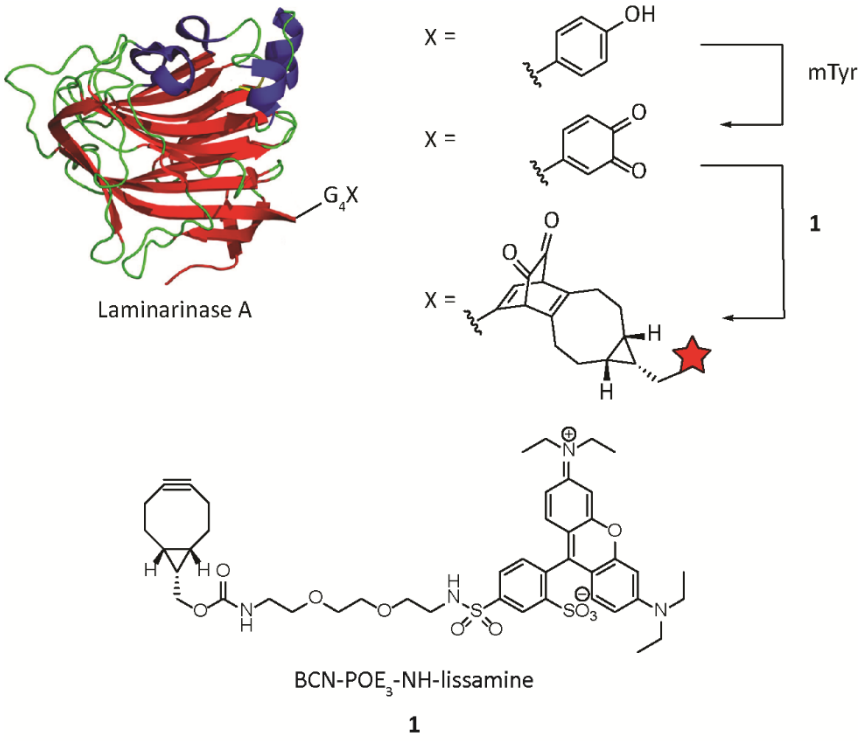



**Figure 1**. SPOCQ labelling of $G_4Y$-tagged laminarinase A by reaction of BCN-lissamine **1** with in situ generated 1,2-quinone.

Purified LamA–$G_4Y$ was subjected to oxidation by catalytic mTyr (7.5 mol %) to generate the intermediate 1,2-quinone, anticipated to undergo *in situ* SPOCQ with BCN-modified lissamine **1**, present in 4-fold excess (Figure 1). Gratifyingly, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis indicated the conversion of LamA–$G_4Y$ into the labelled

product after incubation at 37 °C for 30 min with an apparent high conversion (Figure 2A). Negative controls indicated the specificity of conjugation: no fluorescently labelled LamA was detected in the absence of mTyr (lane C). Incubation with mTyr without **1** led solely to an unidentified band (lane B), most likely originating from aspecific intramolecular nucleophilic attack of an amino acid residue (*e.g.* Lys and His) to the generated quinone, causing it to appear at the expected position.[22, 23] A No fluorescence was detected when SPOCQ was performed with wt-LamA (lane I), which implies that only the newly introduced C-terminal tyrosine is oxidized and undergoes SPOCQ.

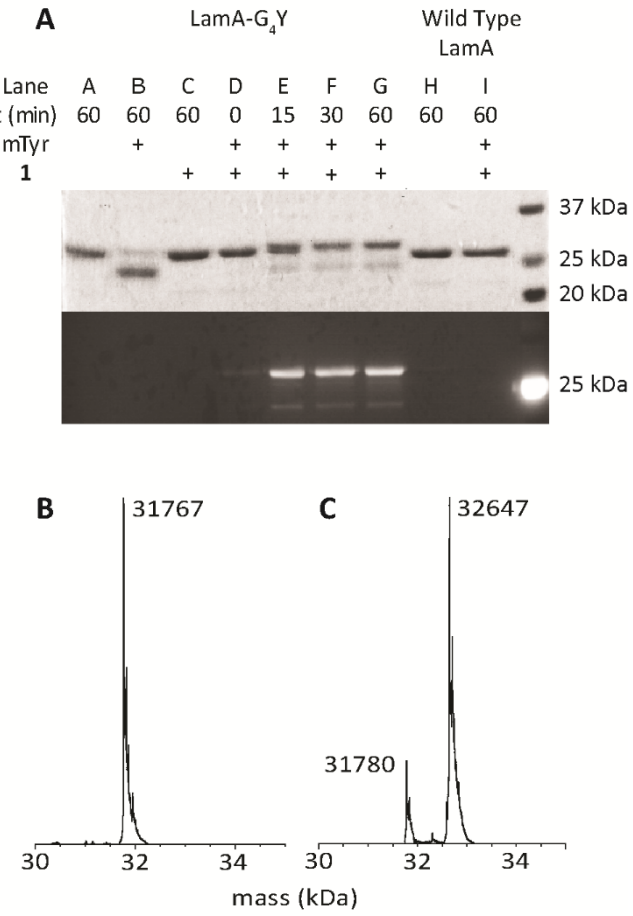
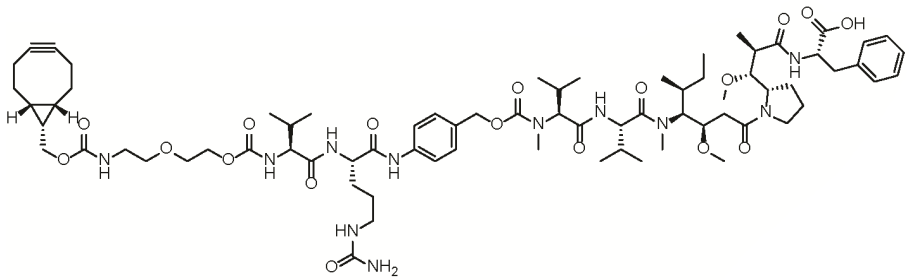



**Figure 2**. (A) SDS-PAGE analysis of SPOCQ on LamA–$G_4Y$ and wt-LamA. (B) MS profile of LamA-$G_4Y$, C) MS profile of LamA-$G_4Y$ after SPOCQ with **1**.

Mass spectrometry (MS) analysis of the SPOCQ reaction show a main product corresponding to the product resulting from oxidation and cycloaddition of **1** (Figure 2B, C), with a deviation of ±1 Da, Figure 2B displays the MS profile of LamA–$G_4$Y prior to oxidation, in agreement with the calculated mass, whereas Figure 2C exhibits LamA–$G_4$Y after oxidation and SPOCQ. The desired product was identified as a peak with molecular weight 32647 Da, indicating an increase of 880 Da (calculated 879 Da) from oxidation of the tyrosine and subsequent conjugation with **1**. The oxidized LamA that underwent aspecific conjugation by a nucleophilic amino acid residue addition was also clearly detected on MS with a mass increase of approximately 13 Da. Further experiments showed that conjugation via SPOCQ could also be performed at 4 and 16 °C and ambient temperature with no observable difference in efficiency (Figure S2).

**Trastuzumab.** Having successfully demonstrated the suitability of SPOCQ for C-terminal protein conjugation, its usefulness for site-specific modification of monoclonal antibodies was investigated next. Trastuzumab with genetically engineered tetra-glycyltyrosine on both light chains (Tras[LC]$G_4$Y) was transiently expressed in CHO-K1 and purified by protein A affinity chromatography. Next, Tras[LC]$G_4$Y was subjected to identical conditions for conjugation with **1** (5 eq.) by SPOCQ at 16 °C as described for LamA. As expected, SDS-PAGE analysis indicated exclusive conversion of Tras[LC]$G_4$Y upon incubation with both **1** and mTyr (SI Figure 1A), while no reaction was detected on either chain for native trastuzumab under identical conditions. Mass spectrometric analysis confirmed successful addition of **1** via SPOCQ (SI Figure 1 B-C). It is worth pointing out that the reaction also proceeded at 4 °C, albeit much slower, but to our surprise, no fluorescent protein could be detected at higher temperature (37 °C), possibly via competing intramolecular reaction of the intermediate quinone with nearby lysine or histidine side chains (Figure S4).



BCN-PEG$_2$-vc-PABC-MMAF

**2**

**Figure 3**. Chemical structure of BCN-MMAF **2**.

**AT1002.** To further assess the applicability of SPOCQ for site-specific modification of monoclonal antibodies, we modified AT1002, a potent anti-influenza antibody, with a C-terminal $G_4Y$ tag (SI S9).[28] To this end, AT1002 with a sortase tag residing on the C-terminus of each light chain was employed to obtain a C-terminally fused $G_4Y$ (AT1002[LC]$G_4Y$). Of relevance, the obtained AT1002[LC]$G_4Y$ possesses a longer C-terminally fused tag (-$G_4$SLPET$G_4Y$) compared to Tras[LC]$G_4Y$, which was anticipated to contribute favorably with regard to accessibility of the tyrosine by mTyr.[29] SPOCQ was attempted on AT1002[LC]$G_4Y$ under identical conditions compared to Tras[LC]$G_4Y$, after which SDS-PAGE analysis demonstrated similar conjugation selectivity with high conversion: only fluorescence on the light chain was detected when reacted with mTyr and **1** (Figure 4B). Interestingly, fluorescence of the labelled AT1002 seems more intense than that of trastuzumab under identical condition, which was taken a confirmation that labelling to the light chain with a short spacer may be encumbered by less steric accessibility. Additionally, a protein band corresponding to the conjugated product was observed by SDS-PAGE analysis upon Coomassie staining, indicating a significantly higher conversion for AT1002[LC]$G_4Y$. MS analysis confirmed SPOCQ on AT1002 (Figure 4C, D). As with trastuzumab, some non-labelled material was still present, which may indicate that also in this case a fraction of the formed quinone reacts with lysine or histidine residues.

Our newly developed conjugation method was envisioned to be suitable as a site-specific approach to access antibody–drug conjugates (ADCs). These are a class of promising chemotherapeutics used for targeted treatment of tumors by combining the high cytotoxicity of a drug such as monomethyl auristatin E (MMAE) or maytansine with an antibody that has high binding affinity to the tumor cell of choice[30] The ability of ADCs to bring highly toxic compounds selectively to the tumor cells allows the treatment of cancers while reducing the effect on healthy tissue as with traditional chemotherapies, which has led to the recent market approval of Adcetris (for the treatment of non-Hodgkin lymphoma and anaplastic large-cell lymphoma) and Kadcyla (for treatment of HER2-positive breast cancer).[31, 32] To this end, AT1002[LC]G4Y was subjected to BCN-monomethyl auristatin F conjugate (BCN-MMAF, 2) after oxidation by mTyr under identical conditions as before, with the exception that DMF was used as co-solvent instead of DMSO. Much to our satisfaction, the desired ADC was seamlessly obtained, as indicated by the expected mass increase of 1459 Da (Figure 4E), which corresponds to oxidation followed by cycloaddition (SPOCQ) with **2**.

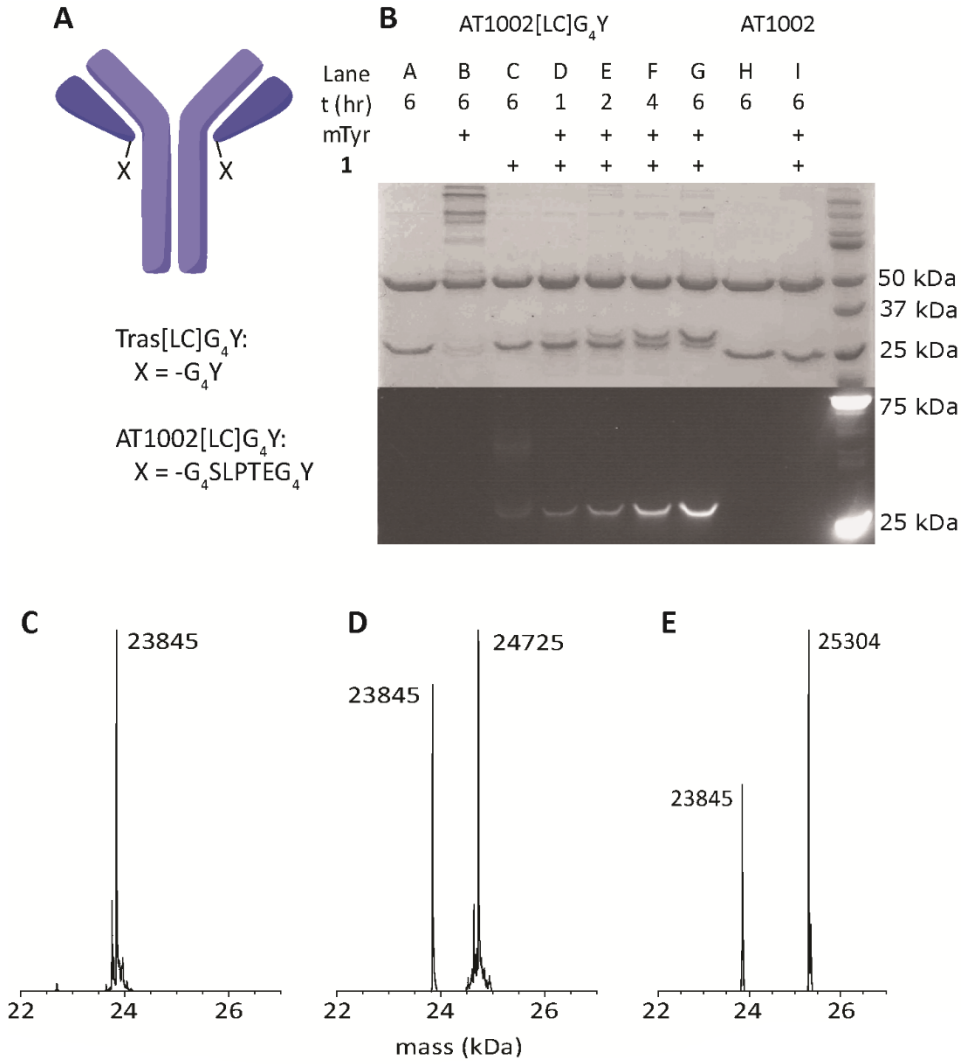



**Figure 4**. (A) Schematic representation of $G_4Y$-tagged antibodies. (B) SDS-PAGE gel analysis of SPOCQ on AT1002[LC]$G_4Y$ and wt-AT1002. (C) MS profile of AT1002[LC]$G_4Y$ (light chain only). (D) MS-spectrum of AT1002[LC]$G_4Y$ after SPOCQ with **1**. (E) MS profile of AT1002[LC]$G_4Y$ after SPOCQ with **2**.

## 2.3. Conclusion

We have successfully developed a site-specific bioconjugation method based on the in situ enzymatic oxidation of tyrosine by mushroom tyrosinase. Various BCN derivatives were successfully conjugated to C-terminal, oxidized tyrosine residues via SPOCQ. This cycloaddition occurs quickly, selectively, and under physiological conditions on a variety of proteins. Extensive optimization of reaction conditions is anticipated to allow fully selective and quantitative conversion. Research along those lines is currently ongoing in our laboratories. We envision that SPOCQ on tyrosine residues can be a valuable tool to create more unique, orthogonal conjugation possibilities for a wide variety of protein modifications, including the preparation of next-generation, site-specifically generated antibody–drug conjugates.

## 2.4. Supporting information

This work can be found at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.7b00046. Further permissions related to the material excerpted should be directed to the ACS

The Supporting Information is available free of charge.

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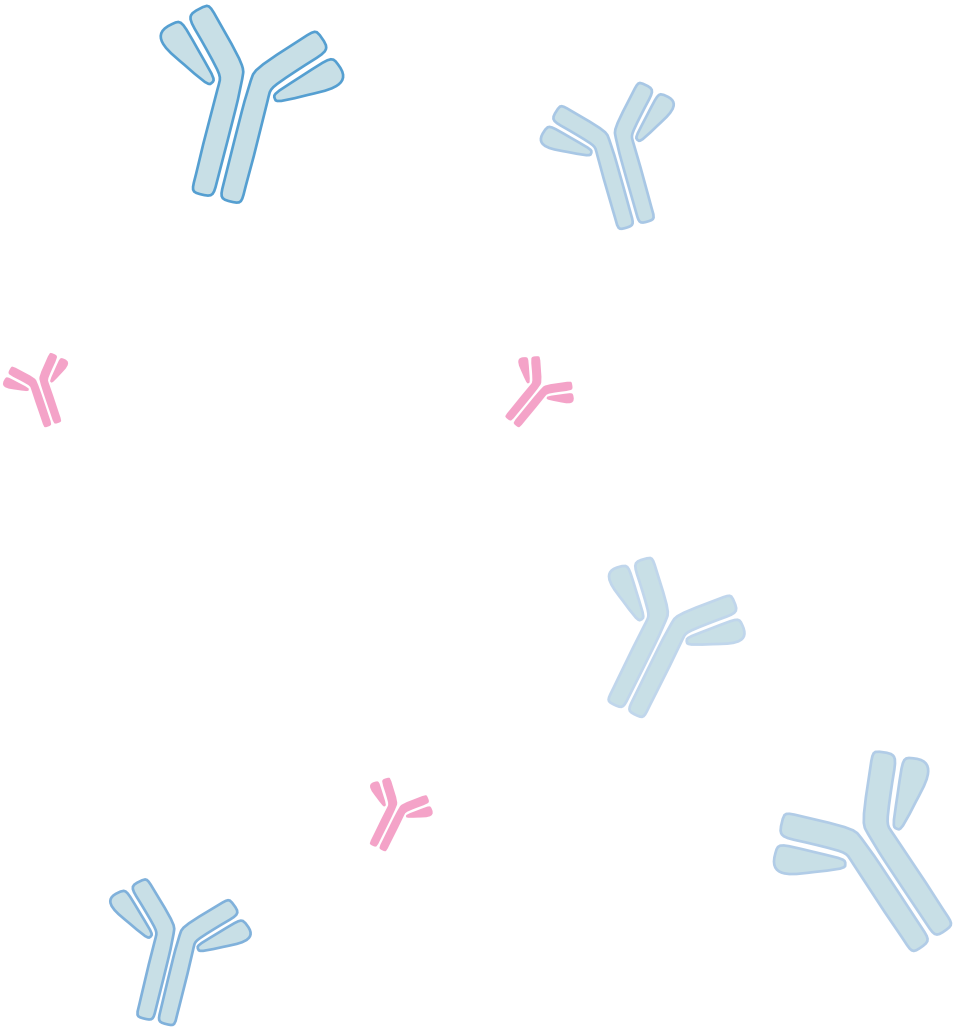
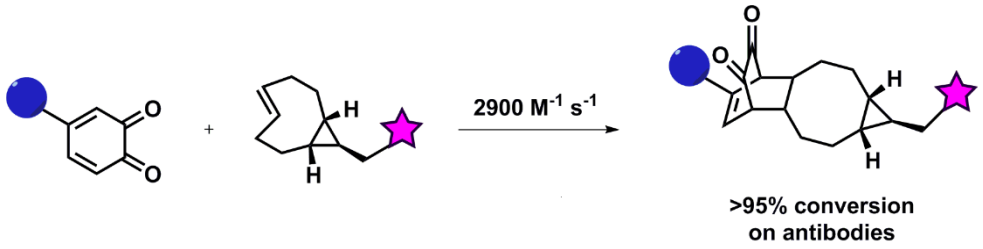
# Chapter 3

# Orthogonal, Dual Protein Labeling by Tandem Cycloaddition of Strained Alkenes and Alkynes to *ortho*-Quinones and Azides



**Abstract:**




*Reaction of cyclopropanated trans-cyclooctene (cpTCO) with in situ generated ortho-quinone is an efficient tool for bioorthogonal protein conjugation. The (4+2)-cycloaddition of cpTCO with ortho-quinone is significantly faster than its cyclooctyne counterpart (BCN). Orthogonal, tandem cpTCO–quinone and BCN–azide cycloadditions afforded a homogeneous, dual labelled antibody–drug conjugate.*

**This work was published as:**

Jorick J. Bruins, Daniel Blanco-Ania, Vincent van der Doef, Floris L. van Delft and Bauke Albada *Chem. Commun*., **2018**, 54, 7338-7341

## 3.1. Introduction

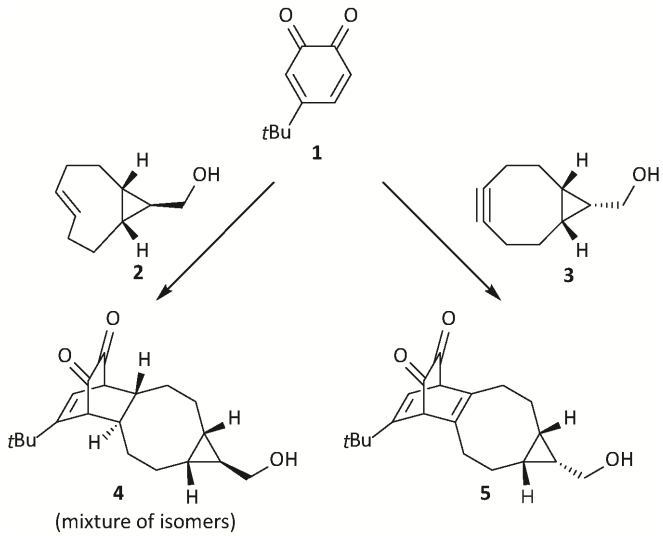
Bioorthogonal chemistry can be defined as the combined arsenal of reactions between two (or more) unique molecular functionalities that together rapidly form a covalent bond, but are inert to the remaining repertoire of chemical groups present in the surroundings.[1-3] As a consequence of this high functional group compatibility, bioorthogonal reactions have inter alia been broadly applied for detection, immobilization and functionalization of biomolecules, such as carbohydrates, nucleic acid and proteins.[4] For example, the Staudinger ligation[5] paved the way for the selective imaging of cell-surface azidosugars, azide–alkyne click chemistry is a well-established tool in the field of DNA/RNA origami,[6] and oxime ligation[7] has proven a powerful technology for protein modification.[8] Arguably the most powerful bioorthogonal reactions are those defined by metal-free, strain-promoted cycloadditions of alkynes or alkenes,[9] of which stand out the strain-promoted azide–alkyne cycloaddition (SPAAC)[10] and the inverse electron-demand Diels–Alder cycloaddition (SPIEDAC)[11] of strained alkenes and tetrazines.

Conveniently, it has been established that with judicious choice of functionalities, a next level of orthogonality can be achieved, involving mutually exclusive, bioorthogonal reactions proceeding in tandem or even concurrently.[12, 13] Besides inherent orthogonality in reactivity, or the application of targeting agents,[14] the use of external stimuli to induce bioorthogonal reactivity with temporal and spatial control has also been described, mostly based on photolysis of tetrazole[12] or cyclopropenone[13] to nitrile imides or cyclooctyne derivatives, respectively. We,[15] and others,[16] recently described that enzymatic oxidation of aromatic rings can also be used for temporally controlled bioorthogonal chemistry. Specifically, we demonstrated that strain-promoted oxidation-controlled *ortho*-quinone cycloaddition (SPOCQ) with cyclooctyne,[17, 18] can be successfully applied for the site-specific conjugation of a cyclooctyne-functionalized fluorophore or a toxic payload to tyrosine-engineered proteins, including the monoclonal antibody trastuzumab,[15] as well as for highly efficient and complete surface modification.[19] Interestingly, with these developments, tyrosine is emerging as a very versatile amino acid for various peptide[20] and protein[21] derivatization strategies.

With the present study, we have expanded the arsenal of SPOCQ chemistry by demonstrating that besides cyclooctynes strained alkenes also display high reactivity for *ortho*-quinones. Specifically, we show that cyclopropanated *trans*-cyclooctene (cpTCO)[22] rapidly reacts with *ortho*-quinones (a.k.a. 1,2-quinones),[23] enabling clean and efficient tyrosinase-mediated protein conjugation. In addition, the inertness of cpTCO for azides provides a next level of dual, orthogonal bioconjugation, which may be executed without additional purification steps in between reactions.

### 3.2. Results and discussion

Before we embarked on the potential application of *trans*-cyclooctenes for SPOCQ modification of proteins, we tested if the strain-promoted click reaction between *trans*-cyclooctenes and *ortho*-quinone would take place. To this end, 4-*tert*-butyl-1,2-quinone **1** was mixed with cpTCO **2** (Scheme 1), and the disappearance of the absorbance that corresponds to the presence of *ortho*-quinone **1** at 395 nm was monitored.[24] Much to our delight, the reaction of quinone **1** with cpTCO **2** not only occurs (Figure 1 and Figure S3–S5), but was actually found to take place faster than that with bicyclo[6.1.0]non-4-yne (BCN) **3**.[25] Specifically, the SPOCQ reaction of quinone **1** with cpTCO **2** proceeded with a rate-constant of 2900±115 $M^{-1}$ $s^{-1}$, a near 3-fold increase versus the (corrected) rate constant of BCN **3** with quinone **1** (1112±8 $M^{-1}$ $s^{-1}$ , N = 3).[15] In the same setup we found a rate constant of 10.4±1.8 $M^{-1}$ $s^{-1}$ for the reaction of regular TCO with quinone **1**. Apparently, the fused cyclopropane ring increases the rate approximately 300-fold, in analogy with enhancement of reaction rate with tetrazine.[22] For comparison, cpTCO–SPOCQ is more than $10^3$ times faster than reaction of quinone **1** with a cyclopropene (<1.7–1.9 $M^{-1}$ $s^{-1}$)[26] and strain-promoted cycloadditions involving azides (0.01–1 $M^{-1}$ $s^{-1}$),[9] however slightly slower than SPIEDAC reaction of regular TCO with tetrazines (2000 to 45000 $M^{-1}$ $s^{-1}$),[16] and much slower than the fastest known cpTCO–tetrazine ligations (reaching above $10^6$ $M^{-1}$ $s^{-1}$).[27-29] Nevertheless, in contrast to azide and tetrazine, an *ortho*-quinone is readily generated from a canonical amino acid (tyrosine), and therefore has the inherent advantage of (inducible) bioconjugation chemistry to native proteins.




**Scheme 1**. Reaction between quinone **1** and either exo-cpTCO **2** or endo-BCN **3** to give alkene SPOCQ product **4** (this work) or alkyne SPOCQ product **5**.[17]

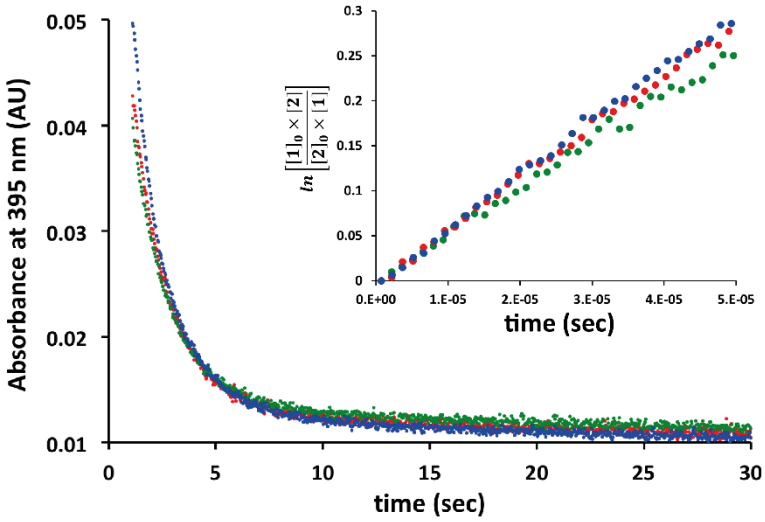



**Figure 1**. Determination of the reaction rate constant of quinone **1** (50 µM) with cpTCO **2** (100 µM) in H2O:MeOH (1:1). Three separate experiments are depicted in the graph.

After this, we explored the suitability of cpTCO-quinone cycloaddition for protein conjugation (Figure 2 and Figure S6–S9). To this end, a cpTCO-functionalized lissamine–PEG conjugate was synthesized and compared head-to-head to its BCN counterpart in labelling of a model antibody (trastuzumab), which contained an exposed $G_4Y$-tag on each heavy chain (abbreviated as Tras[HC]$G_4Y$). Previously, we already showed that without this $G_4Y$-tag, labelling of a smaller protein or an antibody did not proceed.[15] In analogy to our previous work, we studied the alkene–SPOCQ reaction between cpTCO–PEG–lissamine and Tras[HC]$G_4Y$ upon incubation with mushroom tyrosinase (mTyr) to oxidize the exposed tyrosine phenol ring of the $G_4Y$-tag to *ortho*-quinone.[23] Indeed, when Tras[HC]$G_4Y$ was mixed with mTyr in the presence of cpTCO–lissamine, rapid labelling of the antibody was observed that apparently levelled off after approximately 90 minutes (Figure 2, lanes 4–11); no labelling was observed in the absence of mTyr (Figure 2, lane 3). In the absence of a $G_4Y$-tag on the light chain, labelling of this part of the antibody was not observed (*i.e.* no fluorescent bands around 25 kDa were observed), thus demonstrating (1) the bioorthogonality of alkene–SPOCQ, and (2) the need for a $G_4Y$-tag on the protein. Based on HPLC analysis, a yield of approximately 95% may be calculated (Figure S10–S12), with reduced side-product formation in comparison to alkyne-SPOCQ (Figure S9), *i.e.* antibody oligomers formed by cross-linking of the *ortho*-quinone with endogenous protein nucleophiles (Figure 2, lane 2). The reduced formation of protein oligomers for cpTCO versus BCN further corroborates the higher reaction rate with *ortho*-quinones. Since oxidation of tyrosine residues by mTyr is the rate-limiting step in either SPOCQ reaction,[24] the total reaction time does not decrease despite

the higher reaction rate for cpTCO. However, the short lifetime of the generated quinone favors labelling with the fast reacting cpTCO by reducing side product formation.

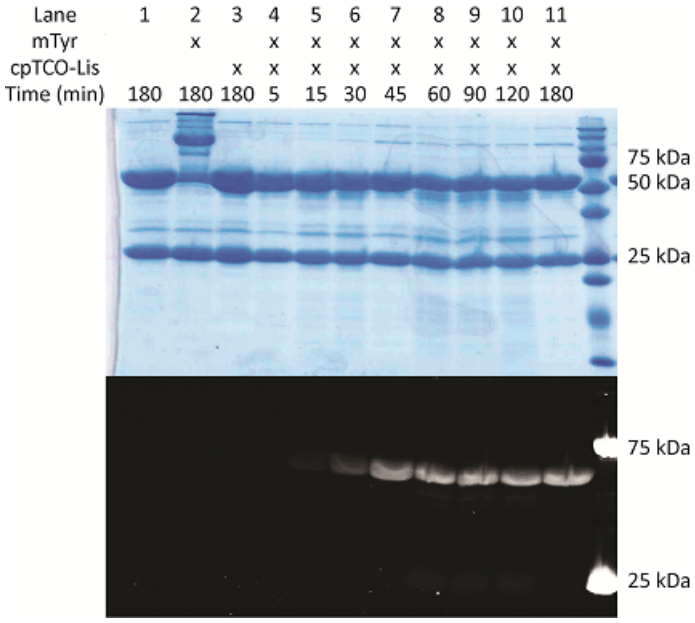



**Figure 2**. Time-resolved SDS-PAGE analysis, based on heavy chain labelling, of cpTCO-PEG-lissamine SPOCQ with Tras[HC]G4Y (lane 1) in the presence of mTyr (lanes 4–11). No reaction is apparent after 3 h in the absence of mTyr (lane 3), while the present of mTyr alone (absence of cpTCO reagent) leads to degradation/oligomer formation (lane 2).

In order to exploit the lack of reactivity of cpTCO for azides, and high reaction rate with *ortho*-quinones, we envisioned that tandem, dual labelling of an antibody should be accessible by orthogonal reactions of BCN and cpTCO with azide and (exposed) tyrosine, respectively. To test this, we explore the possibility of tandem installation of two different functional moieties onto a glycan-remodeled monoclonal antibody[30] by cpTCO–SPOCQ and BCN–SPAAC, executed in either order. We started by the cpTCO–SPOCQ reaction on the Tras[HC]$G_4$Y C-terminus followed by SPAAC modification of azido-glycan with BCN–MMAE (method A), but also checked method B (Figure 3A).

To ensure the same conjugate is obtained via both routes, we used cpTCO–PEG–lissamine and BCN–sulfamide–MMAE (SI) with a cathepsin-cleavable linker (MMAE = monomethyl auristatin E, a potent antimitotic agent that inhibits cell division). In addition, the sulfamide spacer in the BCN unit enhanced solubility and conjugation efficiency.[31]

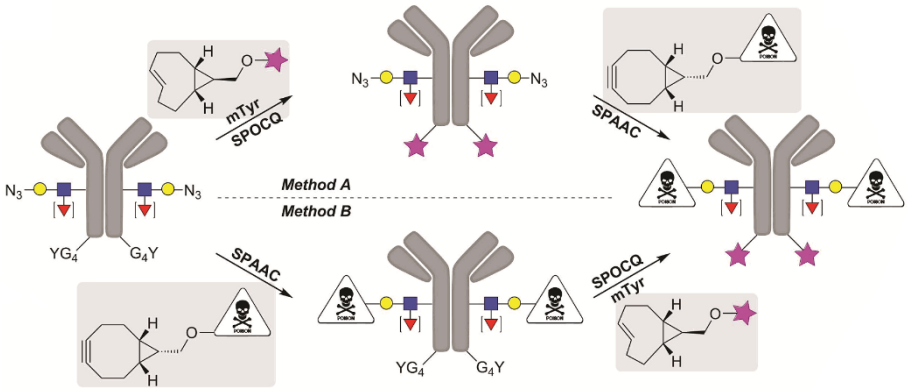



**Figure 3**. Schematic depiction of the two routes for dual labelling of antibodies with lissamine and MMAE. Method A: modification of the $G_4Y$-tag using the cpTCO–SPOCQ reaction with cpTCO–lissamine, followed by SPAAC reaction with BCN–MMAE. Method B: employs first a SPAAC reaction on the azido-glycan followed by SPOCQ on the $G_4Y$-tag of the heavy chain.

Thus, clean and efficient enzymatic glycan remodeling was achieved by endoglycosidase and glycosyltransferase, to install 6-azido-GalNAc onto the core-GlcNAc of transiently expressed Tras[HC]$G_4Y$ (Figure 3A and Figure S13). Next, a SPOCQ reaction between cpTCO–lissamine and the $G_4Y$-tag on the antibody heavy chain was performed in the presence of mTyr (Figure S14). The observed mass of 25768 Da (Figure 3B, yellow peak) corresponds to the SPOCQ product of the azido-glycan antibody and cpTCO–lissamine (expected: 25766 Da), and corroborates the complete lack of reactivity of the cpTCO moiety with the azido-group on the glycan chain. Subsequently, SPAAC modification of the azido-functionalized glycan chain with BCN–MMAE resulted in the clean formation of the dual labelled fluorescent antibody–drug conjugate. HPLC analysis showed almost complete conversion (Figure S15), and MS analysis identified the presence of the target compound with a FW of 26516 Da (expected: 27275 Da) (Figure 3B, green peak in left spectrum). The difference between the observed an expected mass corresponds to loss of the C-terminal part of MMAE due to fragmentation in the mass chamber (Δ762 Da).

Lastly, the tandem labelling steps were performed in reverse order, *i.e.* first SPAAC reaction on the remodeled azido-glycan chain using BCN–MMAE SPAAC (Figure S16), then cpTCO– lissamine SPOCQ reaction on the $G_4Y$-tag upon tyrosinase oxidation (Figure S17). As expected, the same final product is obtained, albeit via a different intermediate product. We were happy to find that in this case, besides the mass of the linker-fragmented final conjugate (at 26514 Da), the mass of the desired dual labelled ADC is also observed at 27279 Da (expected: 27275 Da) (Figure 3, right set of MS spectra). This difference is presumably due to some experimental variability during the MS analysis.

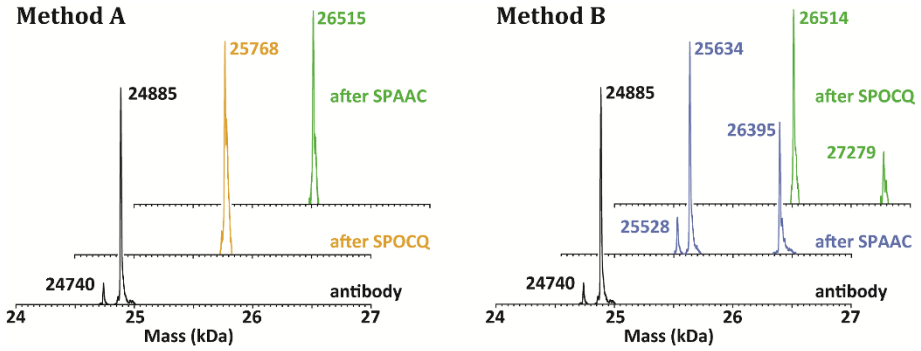



**Figure 4**. The deconvoluted mass spectra of the different steps of the tandem bioconjugation process using the two methods are shown. The main peak in the black trace identifies with GlcNAc-(1,6-Fuc)-6-$N_3$-GalNAc, the minor peak at Δ -145: GlcNAc-6-$N_3$-GalNAc; in-line fragmentation of the MMAF toxin is also visible.

### 3.3. Conclusion

In conclusion, we have uncovered a useful addition to the toolbox of copper-free click chemistry, *i.e.* the rapid reaction of strained alkenes and *ortho*-quinones, and used this reaction to establish a convenient and high-yielding new strategy for the labelling of antibodies. The rate of the SPOCQ reaction between cpTCO and *ortho*-quinone is approximately 2.5 times higher than that for BCN, approximately 300 times higher than that for TCO, and >$10^3$ times higher than that for cyclopropene; the last two strained alkenes would be too slow for efficient SPOCQ labelling of proteins, due to the high reactivity of the quinone, *e.g.* with competing nucleophiles. This high reactivity of cpTCO enables faster and cleaner conjugation, as superior labelling of a $G_4Y$-derivatized antibody with a fluorescent moiety was demonstrated when compared to its BCN counterpart. Ultimately, we developed a convenient protocol for the dual labelling of an antibody with a toxic payload and a dye using the orthogonal character of cpTCO and BCN, which may find application in the assessment of internalization potential of glycan-conjugated antibody–drug conjugates for targeted therapy.[31] Our setup allows a convenient preparation of a glycan-remodeled azido-containing antibody that also contains a peptidic $G_4Y$ tag, and to use this in a two-step dual-labelling strategy to obtain homogeneous ADCs. We note that other methods have been reported for metal-free click conjugation to unnatural amino acids genetically encoded into monoclonal antibodies, for example p-acetylphenylalanine[32] or p-azidomethylphenylalanine,[33] followed by oxime ligation or SPAAC, respectively. However, low antibody titers (1 g $L^{-1}$ maximum), which are significantly lower than expression titers on monoclonal antibodies based on canonical amino acids alone (3-7 g $L^{-1}$ ), limit such applications. Similar arguments apply to genetic encoding of L-DOPA into proteins, which is also suitable for SPOCQ modification.[34] We expect that our current method will find widespread application, not only in the preparation of ADCs but extending to other applications in the field of bioconjugation,

such as PEGylation, immobilization, protein–protein dimer formation, and beyond. Efforts along those lines are currently ongoing in our laboratory.

### 3.4. Supporting information

The Supporting Information is available free of charge on the RSC Publications website at DOI: 10.1039/C8CC02638F

## 3.5. References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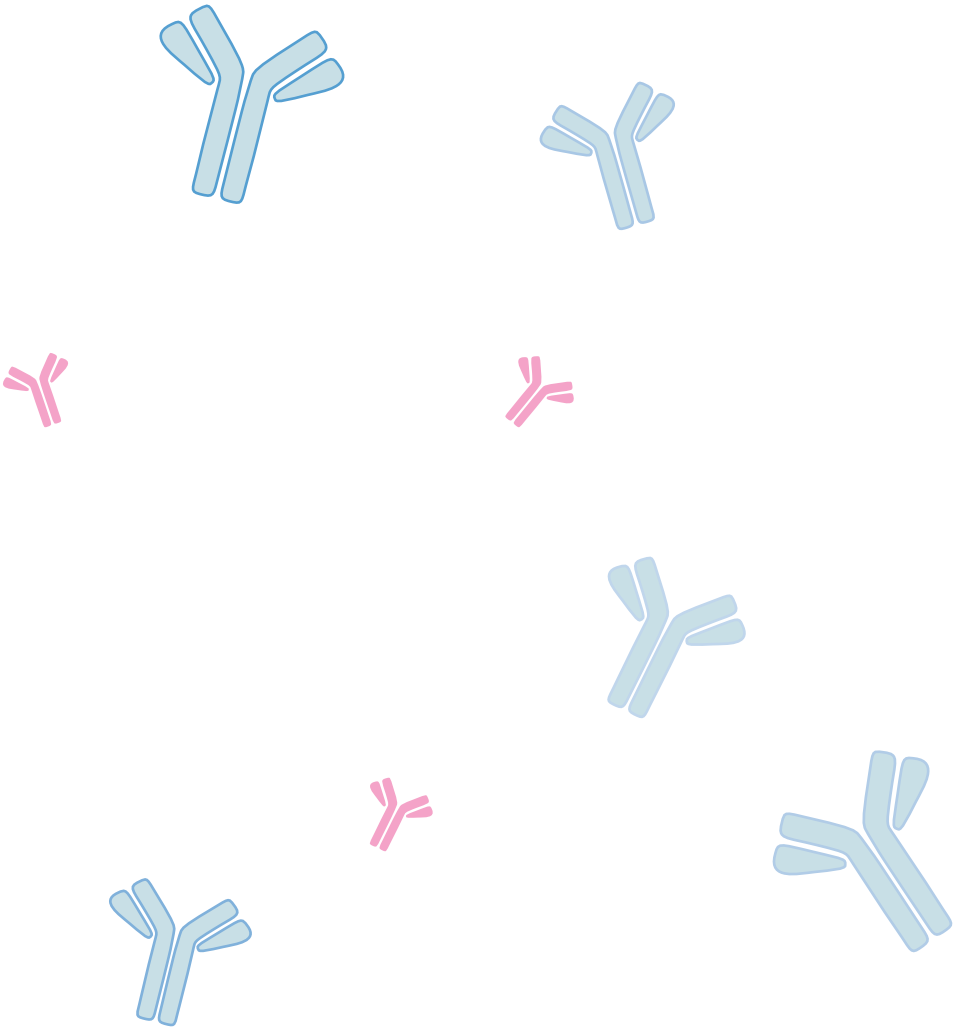
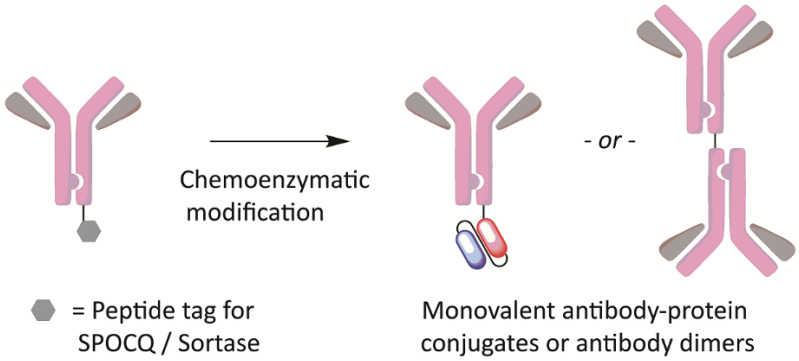
# Chapter 4

# Highly Efficient Mono–functionalization of Knob-in-hole Antibodies with Strain-Promoted Click Chemistry



**Abstract:**




*Knob-in-hole antibodies can be utilized to introduce a single tag for chemoenzymatic functionalization. By either introducing a single C-terminal sortase tag (LPETG) or tyrosine tag ($G_4Y$), 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.*

**This work was published as:**

Jorick J. Bruins, Criss van de Wouw, Koen Wagner, Lina Bartels, Bauke Albada and Floris L. van Delft
*ACS Omega.*, **2019**, 4 (7), 11801-11807

## 4.1. 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 non-canonical amino acids, chemically orthogonal handles can be introduced at predefined sites in a given protein sequence,[8, 9] which allows for chemo-selective oxime ligation[10] 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,[14, 15] phosphopantetheinyl transferase,[16] and transglutaminase.[17] Recently, we demonstrated that selective labelling of monoclonal antibodies can be ensured in a single step based on introduction of a C-terminal tetra-glycyltyrosine tag ($G_4Y$, Figure 1A).[18-20] Specifically, oxidation of the phenol moiety of the tyrosine in the $G_4Y$ 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 labelling in high yields and under mild conditions, with reaction rates exceeding those of the alkyne–azide cycloaddition (SPAAC) 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,[21] or could be further accelerated by employing cyclopropanated *trans*-cyclooctene (cpTCO).[22]

Given the symmetrical nature of an antibody, the site-specific methods described above lead to dual labelling 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 PBD dimer[23] or by radiolabeling with □-emitters such as Thorium-227.[24] Whilst 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, resulting in a significant loss of binding activity and stability.[23] Similarly, various antibody conjugates with a [2]:1 format to enhance the therapeutic window, *e.g.* with IL-2[25] and α-CD3[26], have been reported. The latter conjugates were obtained by fusion of an immunocytokine or T-cell engager to a single chain of an asymmetric antibody format, known as knob-in-hole (KiH) antibodies.[27, 28]

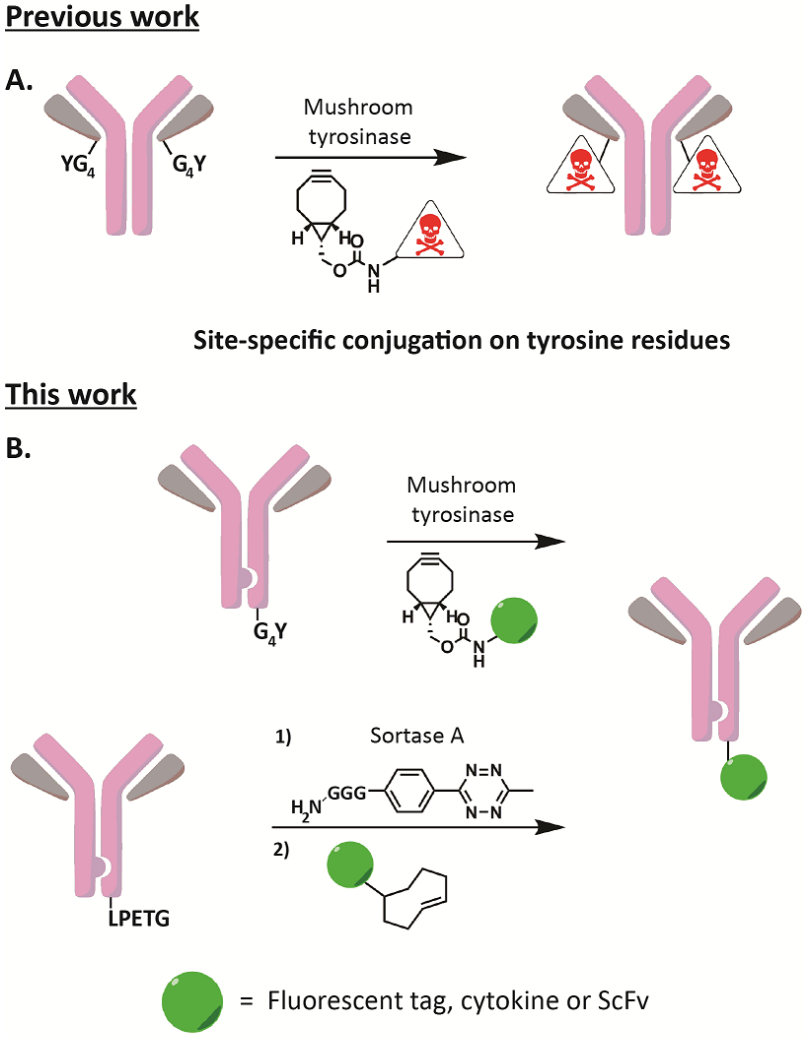



**Figure 1**. (A) Schematic depiction of chemoenzymatic antibody engineering using tyrosinase. (B) The current chemoenzymatic modification of a $G_4Y$ or LPETG tagged hole-LC of the antibody by means of tyrosinase or sortase, respectively.

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.[29, 30] While KiH technology has been applied to create immunocytokines based 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 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.

## 4.2. Results and discussion

**Two-stage antibody conjugation based on tetrazine ligation.** A knob-in-hole variant of trastuzumab was generated by 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.[14, 31] After transient expression and subsequent purification by protein A affinity chromatography, analysis by SDS-PAGE, HPLC and LC-MS confirmed the existence of two distinct heavy chains (Figure 2A).

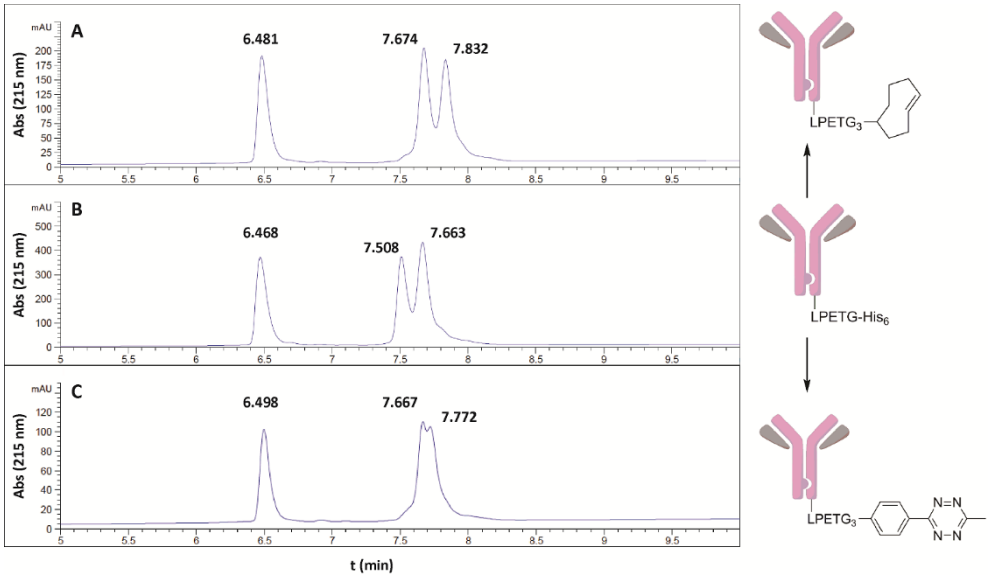



**Figure 2**. HPLC traces of DTT denatured (A) Tras[KiH-HC]ST, (B) Tras[KiH-HC]TCO, and (C) Tras[KiH-HC]MeTz.

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 25565 Da, corresponding to the hole-HC containing the sortase-tag (calc.: 25564 Da). The second peak, corresponding to the knob-HC, was found to have a molecular weight of 24220 Da (calc.: 24221 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 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 min 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**). LC-MS analysis confirmed 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-labelled 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 labelled band could be identified. HPLC analysis displayed an efficiency exceeding 90%, and LC-MS confirmed the formation of the desired hole-HC IEDDA product, whilst 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 □-CD3 T-cell engager. To this end, a MeTz moiety was attached to the C-terminus of either the cytokine interleukin 2 (IL-2)[14] or to the short-chain variable fragment to UCHT1 (□-CD3 scFv)[34] 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 clean labelling 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. HPLC analysis showed full conversion of the TCO-labelled hole-HC, which was confirmed by LC-MS analysis (SI). Similarly, the reaction of Tras[KiH-HC]TCO with MeTz–UCHT1 showed near full conversion to the expected 80 kDa band, with HPLC and LC-MS confirming efficient formation of the expected bifunctional antibody via this method.

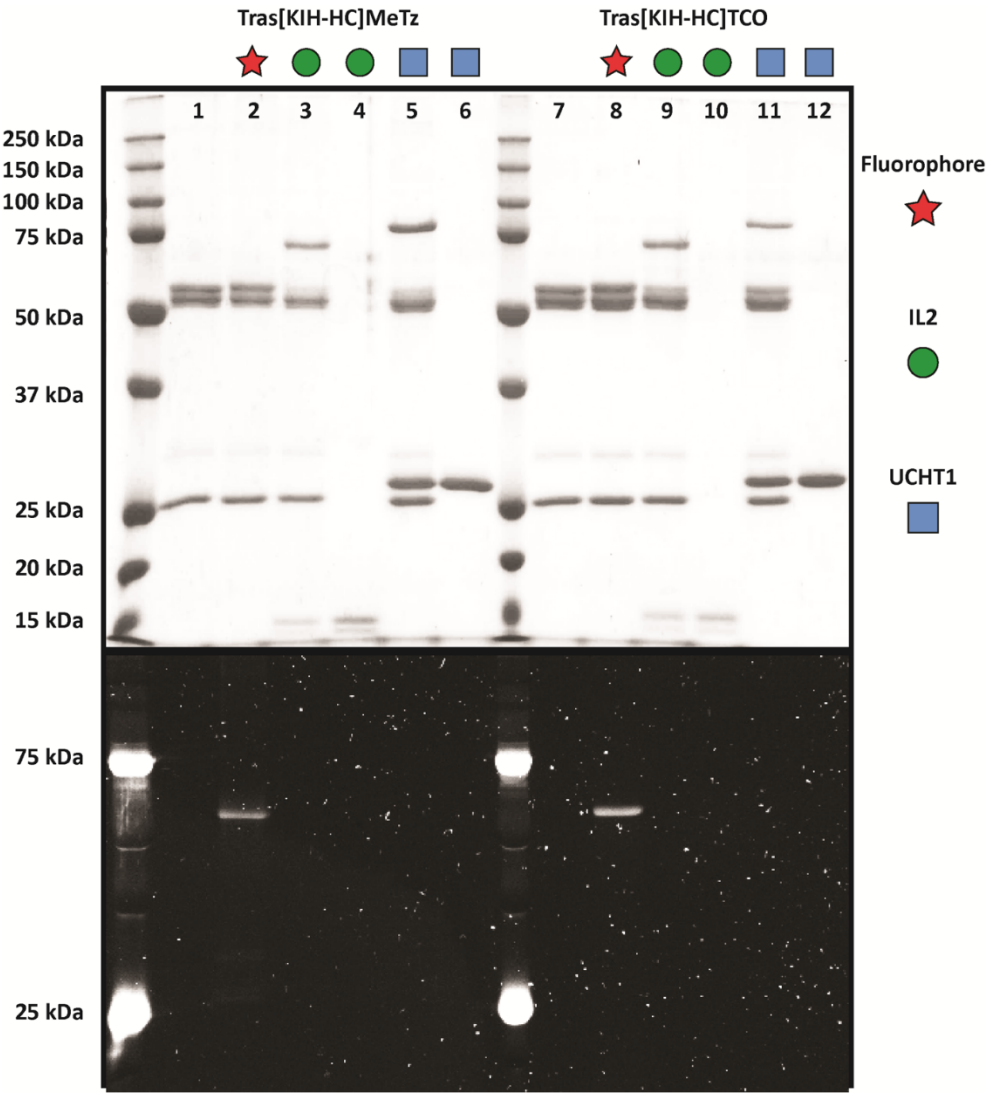



**Figure 3**. SDS-PAGE analysis of IEDDA reaction of Tras[KiH-HC]TCO with MeTz–TAMRA (lane 2), MeTz–IL-2 (lane 3) and MeTz–UCHT1 (lane 5), and Tras[KiH-HC]MeTz with BCN–lissamine (lane 8), TCO–IL-2 (lane 9), and TCO–UCHT1 (lane 11). Lanes M are loaded with protein ladders, lanes 1 and 7 contain the unmodified KiH antibodies.

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), 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 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 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.[35] In this light, having the MeTz on the antibody and the TCO/BCN on the reactive protein partner is clearly the preferred strategy towards 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 capture with TCO-modified agarose beads, leading to clean antibody dimers (SI). Utilization of the modularity of these antibodies allows for 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[36, 37] or are based on chemical approaches yielding highly heterogeneous antibody dimers.[38]

**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 labelling of knob-in-hole antibodies with SPOCQ chemistry was investigated next. SPOCQ (strain-promoted oxidation-controlled *ortho*-quinone cycloaddition) was originally developed based on chemical generation of *ortho*-quinones.[18] Recently, we showed it can be conveniently applied in a one-pot process involving 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).[19]

Earlier we described that SPOCQ on the symmetrical antibody Tras[HC]$G_4$Y converts to 95% of the desired bis-functionalized adducts when small fluorophores were conjugated.[22] However, this level of conversion could not be achieved when protein-protein conjugation was attempted on symmetrical $G_4$Y-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.[39] 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 $G_4Y$ tag (Tras[KiH-HC]$G_4Y$, 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 of Tras[KiH-HC]$G_4Y$ with BCN–lissamine, and subsequent HPLC analysis showed >95% conversion of the $G_4Y$-HC within 20 minutes (Figure 4C), significantly faster than the 90 minutes for the dual labelling of symmetrical Tras[HC]$G_4Y$ as reported earlier.[22]

Analogous to Tras[KiH-HC]ST, the knob-HC was found to have a molecular weight of 24221 Da (calc. Mw: 24221 Da), and 24349 Da for the hole-HC containing the $G_4Y$ moiety (calc. Mw: 24350 Da) (Figure 4D). After SPOCQ, the molecular weight of the hole-HC increased with 879 Da to 25229 Da (calc. Mw: 25229 Da). This corresponds to the addition of 14 Da during the oxidation by mTyr and addition of 865 Da by BCN–lissamine.

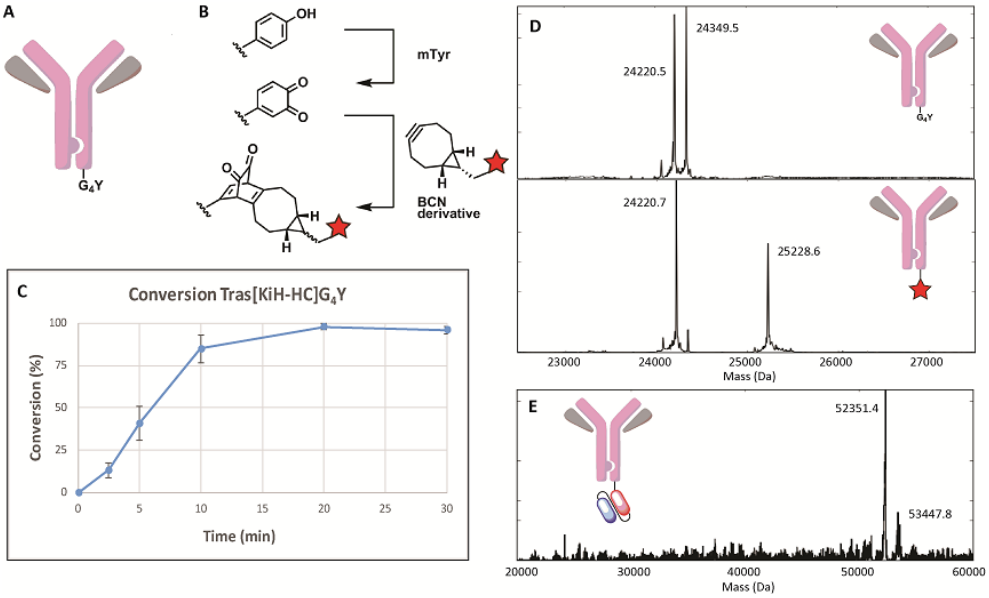



**Figure 4**. (A) Schematic representation of Tras[KiH-HC]$G_4Y$. (B) Scheme of the SPOCQ reaction of an oxidized tyrosine side chain functionality with BCN. (C) Graphical depiction of the time-resolved HPLC analysis of SPOCQ between Tras[KiH-HC]$G_4Y$ and BCN–lissamine. (D) Mass spectra of Tras[KiH-HC]$G_4Y$ (top) and its SPOCQ product with BCN–lissamine (bottom). (E) Mass spectrum of Tras[KiH-HC]UCHT1 as formed by SPOCQ.

Finally, we explored the suitability of Tras[KiH-HC]$G_4Y$ for the generation of protein-protein conjugates. SDS-page (SI) and LC-MS analysis showed the formation of trastuzumab-UCHT1 conjugates by SPOCQ conjugation of Tras[KiH-HC]$G_4Y$ (Figure 4E). Two masses were found by LC-MS: the first being 52351 Da (calc. Mw: 52351 Da), corresponding to the hole-HC plus 14 Da for

oxidation by mTyr and addition of BCN–UCHT1 (Calc. Mw: 27987.0 Da), and the second peak is 53448 Da, corresponding to the same conjugate with incomplete deglycosylation by endo S2, corresponding to a $G_0F$ glycan (calc. Mw: 53446 Da).

### 4.3. Conclusion

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 chemoenzymatic functionalization of one heavy chain by an indirect approach using sortase or by direct labelling with SPOCQ. Subsequently, the method was shown to allow for 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 have increased significantly over the last years, since bispecific antibodies can directly target immune cells to tumours,[40] with many of them in clinical trials.[41] With SPOCQ, near-quantitative conversions are obtained after only 20 minutes of reaction time. We envision this modular approach may open up possibilities for new and potent therapeutic agents. Comparison of our technology with 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.



### 4.4. Supporting information

This work can be found at https://pubs.acs.org/doi/full/10.1021/acsomega.9b01727. Further permissions related to the material excerpted should be directed to the ACS

The Supporting Information is available free of charge.

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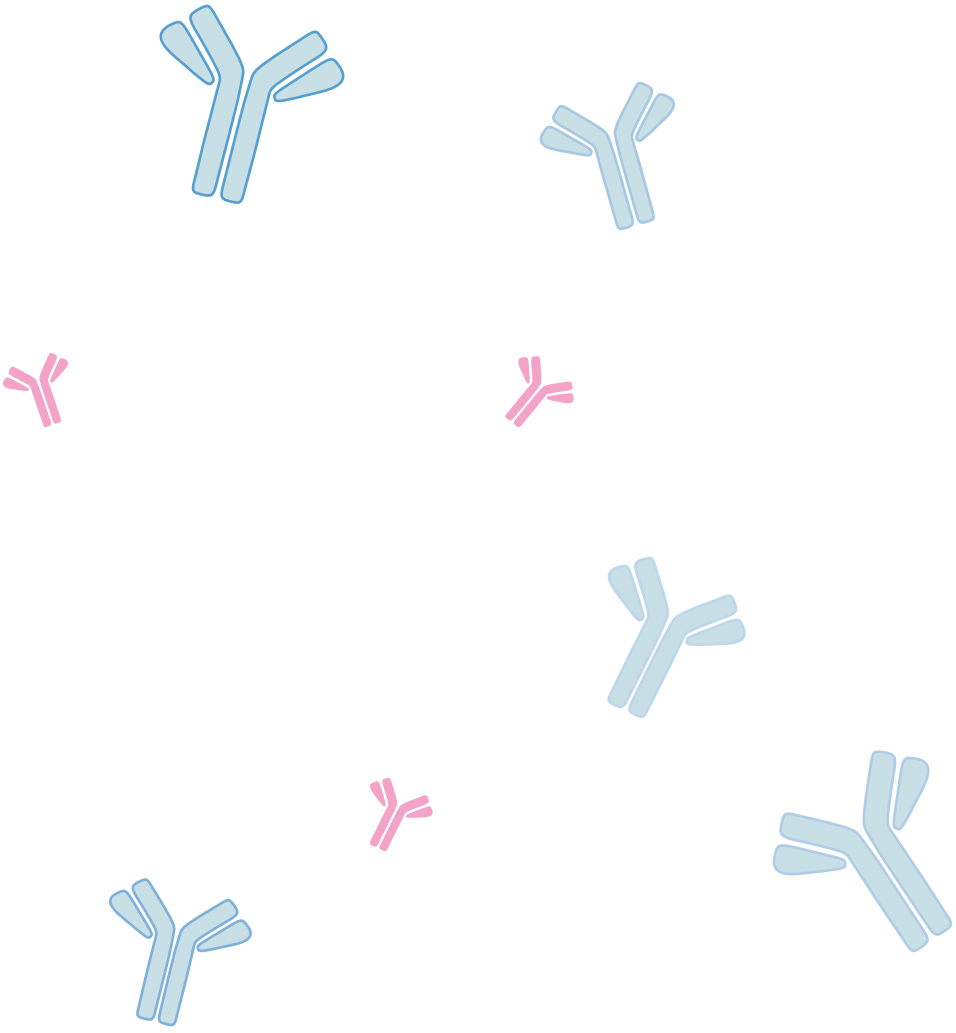
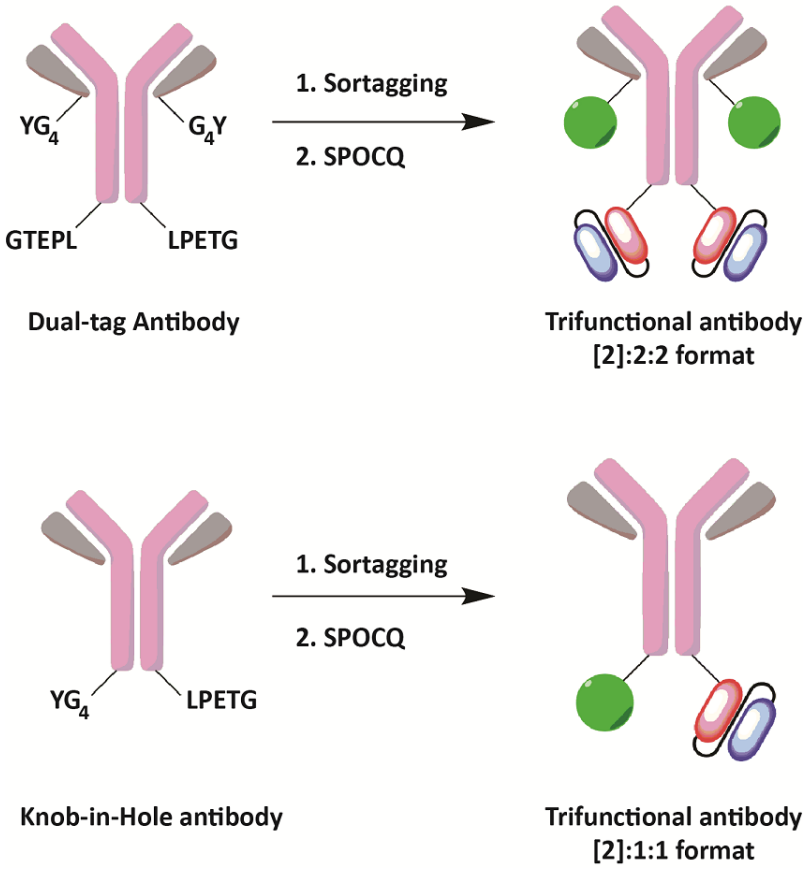
# Chapter 5

# Modular, Chemoenzymatic Preparation of Trifunctional Protein-antibody Conjugates with Tailored Valencies



**Abstract:**




*Tyrosine-tags ($G_4Y$) can be modified with SPOCQ for protein-protein conjugation, and is applied to introduce short-chain variable fragments (scFvs) and cytokines on antibodies in a modular fashion. Combined with sortase A ligation, trifunctional antibodies can be prepared by modifying both the light and heavy chains, generating a [2]:2:2 format, or by modifying asymmetrical knob-in-hole antibodies, generating a [2]:1:1 format.*

## 5.1. Introduction

Bispecific antibodies, *i.e.* antibodies with two structurally distinct CDRs each of which binds monovalently to a different antigen, have the potential to increase binding affinity,[1] improve internalization of antibody-based drugs,[2, 3] or redirecting cytotoxic T-cells for increased immune response near the target cell.[4, 5] Bifunctional antibodies, by comparison, are antibodies that retain the original bivalent binding fashion of a traditional antibody, however feature an additional protein module for additional functionality, for example an IL-2 fragment for T-cell activation.[6] A significant amount of research regarding bispecific and bifunctional antibodies has been performed in recent years, with architectures ranging from hetero-dimerized Fab-domains to C-terminally fused scFv groups.[7, 8] Recently, we reported the generation of monovalent antibody-scFv and antibody-cytokine conjugates from knob-in-hole antibodies combined with SPOCQ or sortase ligation (chapter 4).[9] Alongside this work, divalent antibody-scFv and antibody-cytokine conjugates were achieved based on sortase ligation.[10, 11]

Trifunctional antibodies can be considered the next step for enhanced tumor targeting and/or payload delivery.[8] Amongst trifunctional constructs are trispecific antibodies capable of binding to three different antigens, which have shown promising activity to combat HIV by binding multiple HIV glycoproteins.[12] They can be generated via a plethora of methods such as the fusion of a single single-chain variable fragment (scFv) to a bispecific antibody (OrthoTsAb),[13] or by the fusion of two unique scFv units to the light chains (LC) or heavy chains (HC) of a monospecific antibody.[14] Similarly, trifunctionals in the form of bispecific ADCs can employ improved internalization of the antibody for enhanced lysosomal delivery of the toxic payload.[2, 3, 15] Furthermore, trifunctionals in the form of bispecific immunocytokines[16] and bispecific antibodies carrying radiolabels have been reported.[17] However, the fusion of a scFv units or a cytokine to a monoclonal antibody based on recombinant technology is a strategy that is effective but rather time-consuming.

The combination of preparing a trifunctional containing both UCHT1 and IL–2 would be of particular interest. UCHT1 is an antibody targeting CD3, a T-cell co-receptor which bind to T-cells for subsequent activation.[18] By introducing the scFv variant of UCHT1 on an antibody targeting acute myeloid leukemia (AML) cells, binding of both AML and T-cells was achieved, and suppressed tumor growth in mice inoculated with AML-cells.[11] This effect might be further enhanced by exposing the recruited T-cells to IL-2,[19] a cytokine that can activate T-cells, allowing for localized immune responses.[6] By combining UCHT1 and IL-2 on tumor-targeting antibodies, we envisioned to create a system capable of efficiently recruiting and activating the immune system. Furthermore, by creating a modular approach of introducing UCHT1 and IL–2 on an antibody, the effect of varying the conjugation site can be explored, and the combination of other scFvs, cytokines, and other pharmaceutically relevant molecules can be investigated rapidly and efficiently.

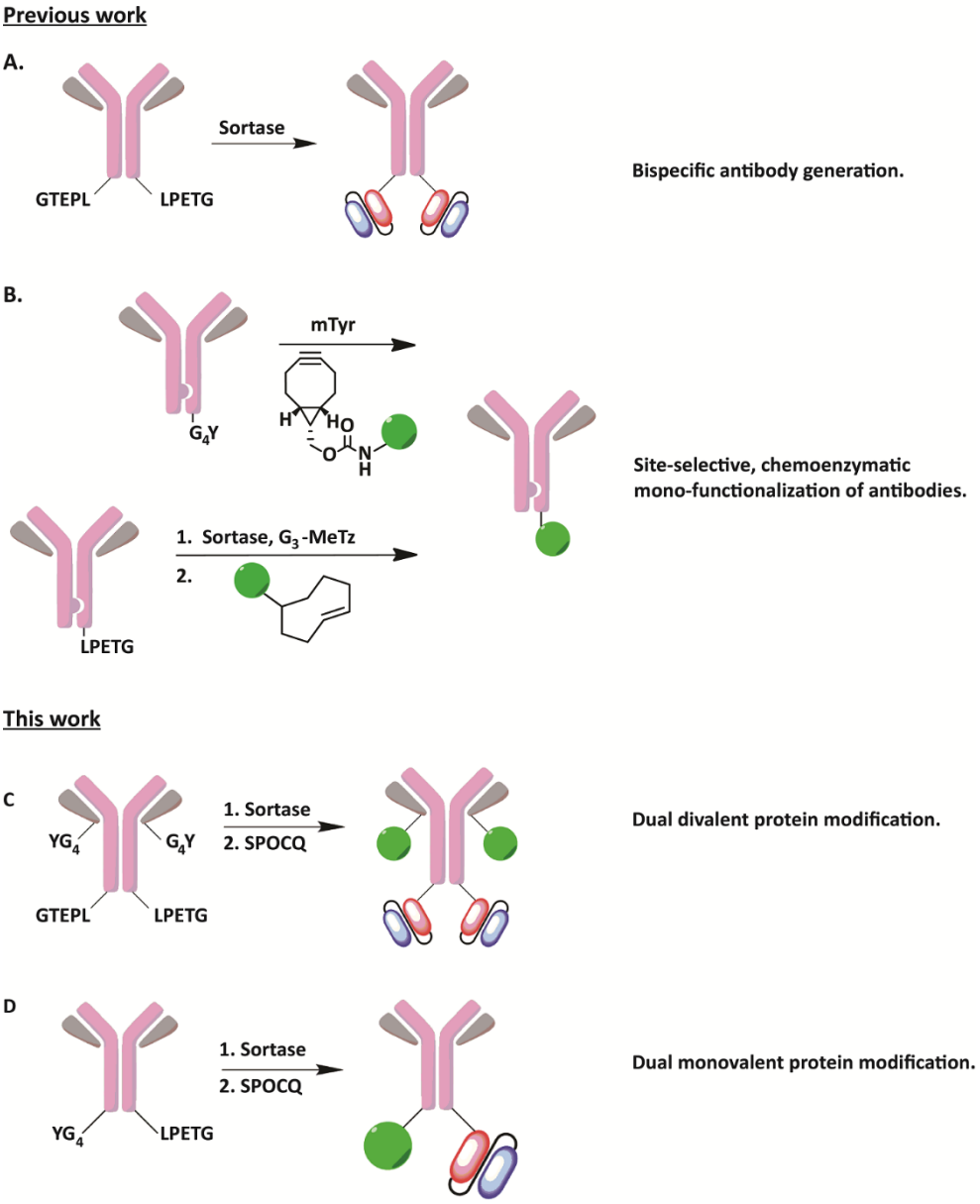



**Figure 1.** (A) Generation of [2]:2 bispecific antibodies via sortase ligation, (B) generation of [2]:1 bifunctional antibodies via SPOCQ or sortase, (C) generation of [2]:2:2, and (D) [2]:1:1 trifunctional antibodies via sortase and SPOCQ.

The potential to create trifunctional antibodies by chemoenzymatic modification based on the combination of SPOCQ and sortase ligation allows for the modular preparation of trifunctional antibodies without the need of antibody reengineering. Here, we describe a two-step conjugation method for efficient post-recombinant antibody functionalization based on the combination of strain-promoted chemistries. After selection of the correct probe(s) for this two-step conjugation, we optimized the procedure for conjugation with scFvs, cytokines, and oligonucleotides. Finally, the method was combined with sortase A conjugation strategies to create trifunctional antibodies with various valencies.

## 5.2. Selection of a bifunctional linker

In chapter 4 we discussed the possibility of performing SPOCQ with BCN–UCHT1 on antibodies. It was found that knob-in-hole antibodies can undergo mono-functionalization in a clean and rapid fashion,[9] however symmetrical AT1002[HC]$G_4$Y does not perform SPOCQ to full completion (Figure 2). One potential explanation is that after conjugation of BCN–UCHT1 to one of the heavy chains, the second $G_4$Y site becomes inaccessible for mTyr to oxidize, preventing a second cycloaddition from occurring.

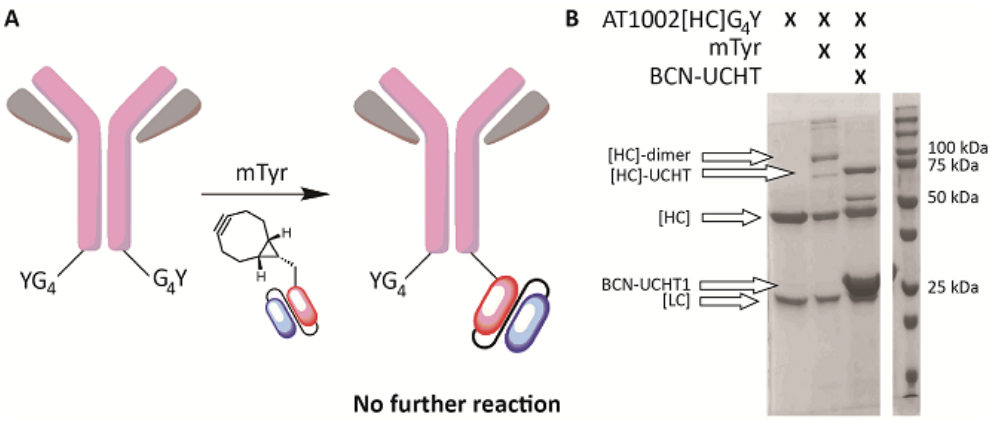
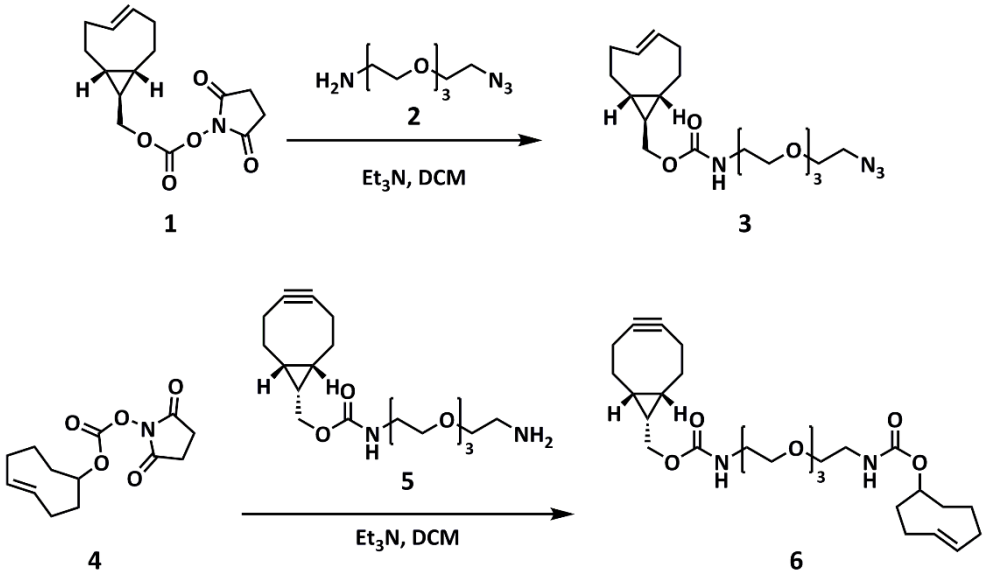



**Figure 2.** (A) Schematic representation of SPOCQ between BCN–UCHT1 and Tras[HC]$G_4$Y, and (B) SDS-PAGE gel of SPOCQ between BCN–UCHT1 and Tras[HC]$G_4$Y.

To ensure selective and efficient protein–protein conjugation, we opted to apply SPOCQ to introduce an elongated linker with a terminal chemical handle for subsequent protein–protein functionalization. To this end, a bifunctional linker was envisioned with an azide on one end and the strained alkene cpTCO (cyclopropanated *trans*-cyclooctene) on the other end, based on the assumption that azide and cpTCO are mutually inert,[20] by cpTCO-NHS (**1**)[20] and a PEG-bearing azide (**2**). However, synthesis of the cpTCO–azide (**3**) consistently gave no product, and $^1$H-NMR showed the disappearance of the alkene peaks at 5.1 and 5.8 ppm after conjugation of cpTCO-

NHS with an azide-bearing PEG-moiety (SI S5.1). Subsequent testing revealed that the alkene peaks of cpTCO disappear within hours when an organic azide was present in the solution (SI S5.2).




**Scheme 1**. Synthesis of bifunctional linkers cpTCO-PEG$_3$-azide (**3**) and BCN-PEG$_3$-TCO (**6**).

As an alternative, we considered to replace cpTCO with its less reactive TCO, which undergoes (4+2) cycloaddition with quinones, but TCO would be too slow for efficient conjugation (*i.e.* 100-fold lower reaction rate than BCN).[20] Therefore, we reasoned that a chemoselective reaction would be feasible for a bifunctional linker with BCN on one end (for SPOCQ, *i.e.* cycloaddition with quinone) and TCO on the other end (for ligation with tetrazine-functionalized protein). To confirm this assumption, Tras[LC]G$_4$Y was treated with mTyr in the presence of BCN-bearing fluorophore lissamine (BCN–lissamine), or TCO-bearing fluorophore AF568 (TCO–AF568). In contrast to our expectation, both BCN–lissamine (Figure 3B) and TCO–AF568 (Figure 3C) gave clean and full conversions to the fluorophore-bearing product, which for TCO had not been demonstrated before. These findings were later confirmed by SDS-PAGE, where a fluorescent band was formed after SPOCQ with TCO–AF568 (Figure 6), and by LC-MS, where the mass increase corresponding to oxidation and subsequent cycloaddition were observed (Figure S7, S13). However, in line with the significant reaction rate difference between BCN and TCO, when SPOCQ was performed by subjecting Tras[LC]G$_4$Y to an excess of a 1:1 mixture of BCN–lissamine and TCO–AF568, only the BCN-SPOCQ product was detected (Figure 3D).

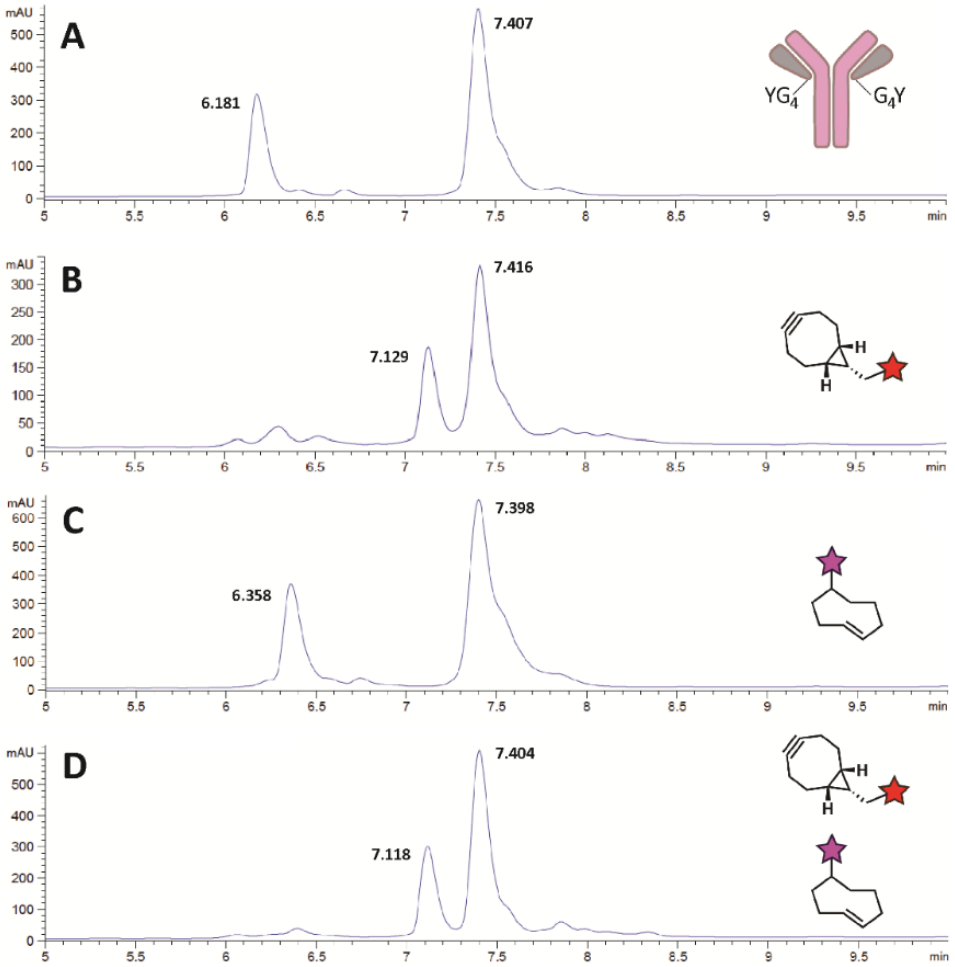



**Figure 3**. HPLC chromatograms of (A) Tras[LC]$G_4Y$ and SPOCQ with (B) BCN-lissamine or (C) TCO-AF568, and (D) competitive SPOCQ between BCN-lissamine and TCO-AF568. It is noted that there seem to be multiple small peaks present in figure 3B; these are degradation products of the excess BCN–lissamine due to exposure to the relatively harsh HPLC conditions (0.1% formic acid at 80 ˚C).

The ability of BCN-SPOCQ to outcompete TCO-SPOCQ paves the way for the selective introduction of TCO-moieties at $G_4Y$ sites by using bifunctional linkers that contain both BCN and TCO. To this end, BCN-NHS (**4**) was subjected to $H_2N$-$PEG_3$-TCO (**5**) to yield BCN–$PEG_3$–TCO (**6**). Subsequently, **6** was compared to commercially available BCN–$PEG_3$–BCN (**7**) in a 2-step conjugation process (Figure 4B). First, SPOCQ with **6** or **7** was performed, after which excess linker was removed by Amicon® centrifugal filters (50 kDa MWCO). Then, methyltetrazine bearing the fluorophore TAMRA (MeTz–TAMRA, Figure S4) was added for inverse-electron

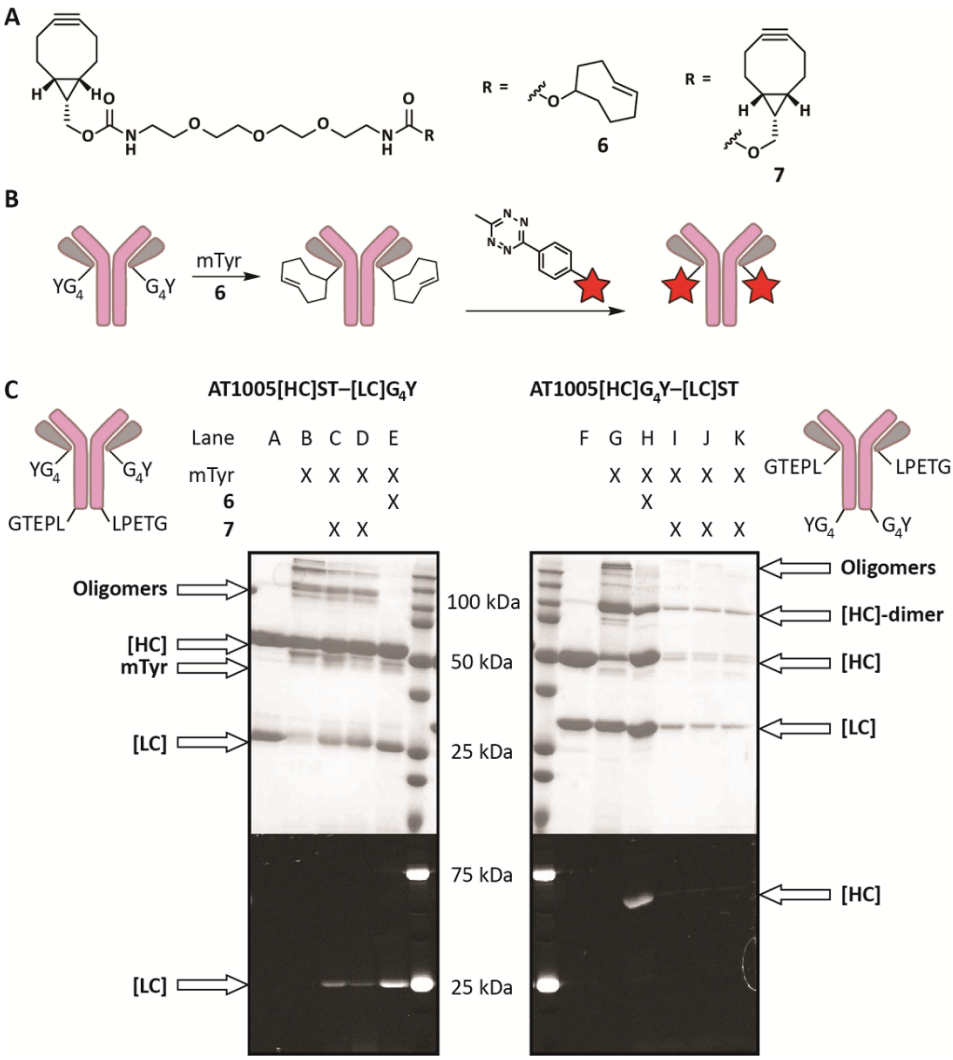



**Figure 4.** (A) Structures of BCN-PEG$_3$-TCO (**6**) and BCN-PEG$_3$-BCN (**7**), and schematic representation of MeTz–TAMRA. (B) Reaction scheme of the two-step conjugation to introduce MeTz-bearing handles, only TCO-bearing antibodies are displayed. (C) SDS-PAGE analysis of AT1005[HC]ST–[LC]G$_4$Y before oxidation (lane A), after mTyr oxidation (lane B), SPOCQ with 2.5 or 5 eq. of bis-BCN **7** (lanes C, D), or SPOCQ with 5 eq. of BCN-TCO **6** (lane E), and AT1005[HC]G$_4$Y–[LC]ST before oxidation (lane F), after mTyr oxidation (lane G), SPOCQ with 5 eq. of **6** (lane H), or SPOCQ with 86, 172 or 344 eq. of **7** (lanes I, J, K).

demand Diels–Alder (IEDDA) with the TCO- or BCN-bearing antibodies (Figure 4B). The two-step conjugation method was tested on AT1005 with either a G$_4$Y on the light chain and sortase on the heavy chain, or vice versa.[21] The sortase tag (LPETG) is not expected to perform any reaction

under SPOCQ conditions, but does result in a difference in molecular weight and subsequent shift of the protein band on SDS-PAGE gel (Figure 4C). Interestingly, two-step conjugation with 2.5 or 5 equivalents of BCN–BCN linker **7** on AT1005[LC]$G_4$Y gives rise to a fluorescently labelled light chain, but also generates high-molecular weight side products (Figure 5, lane C and D). In contrast, two-step conjugation with 5 eq. of BCN–TCO linker **6** results in efficient labelling of the light chain without any visible high-molecular weight side products (Figure 4C, lane E). Furthermore, the lack of any reaction on the heavy chain of the antibody confirms the orthogonality of SPOCQ with the LPETG moiety of the sortase tag.

When the two-step conjugation method was attempted on the antibody heavy chain as in AT1005[HC]$G_4$Y however, neither **6** nor **7** gave full conversion. Even with 10 eq. of **6**, the heavy chains of AT1005[HC]$G_4$Y seem to dimerize significantly (Figure 4C, lane H). One possible explanation is that the first generation of quinone and conjugation with BCN proceeds according to expectation, however after the quinone is subsequently formed on the other heavy chain, a pseudo-intramolecular reaction takes place with TCO-moiety on the firstly introduced linker. The suggestion of such heavy chain dimerization is corroborated by the fact that this effect is enhanced with bis-BCN **7**, where >300 eq. is not enough to prevent full dimerization (Figure 4C, lane K).

From these results we conclude that BCN-$PEG_3$-TCO **6** gives rise to significantly cleaner conjugates than bis-BCN **7**. However, the proposed two-step conjugation method does not yield satisfactory results with tyrosines on the C-terminus of antibody heavy chains. One obvious approach to circumvent heavy chain dimerization would be by avoiding a chemical moiety that undergoes cycloaddition with quinone altogether. However, another likely option we followed is to investigate a similar approach on the antibody light chains.

### 5.3. Accelerating SPOCQ by increasing linker length

While the light chain C-termini are structurally significantly more distant than the heavy chain C-termini, thereby naturally avoiding issues related to dimerization, SPOCQ on the light chain of antibodies is significantly slower than the heavy chain: while various trastuzumab $G_4$Y heavy chain mutant undergo complete SPOCQ in 0.25–1.5 h,[9, 20] Tras[LC]$G_4$Y required overnight incubation to obtain high yields.[22] Therefore, we reasoned that increasing the availability of the tyrosine residue for oxidation, by elongating the peptide linker, would increase the reaction rate. Whilst there are many peptide linkers with varying functions and properties,[23] $G_4$S-peptide linkers were investigated due to their inert nature, flexibility and stability.[24] To this end, trastuzumab was expressed with either 0, 1 or 2 additional $G_4$S spacers inserted before $G_4$Y, resulting in Tras[LC]$G_4$Y, Tras[LC]$G_4S G_4$Y, Tras[LC]$G_4S G_4S G_4$Y (Figure 5).

When time-resolved SPOCQ was performed on all three trastuzumab variants with TCO–AF568 as reactant, significant differences in reaction rates were observed (Figure 5). Tras[LC]$G_4Y$ shows the slowest reaction rate, with about 90% conversion after eight hours. Tras[LC]$G_4SG_4Y$ shows over fourfold enhanced reaction rate, with the reaction nearing completion after two hours and showing full completion after three hours. Finally, SPOCQ on Tras[LC]$G_4SG_4SG_4Y$ is nearly complete after one hour, and full completion is detected after two hours.

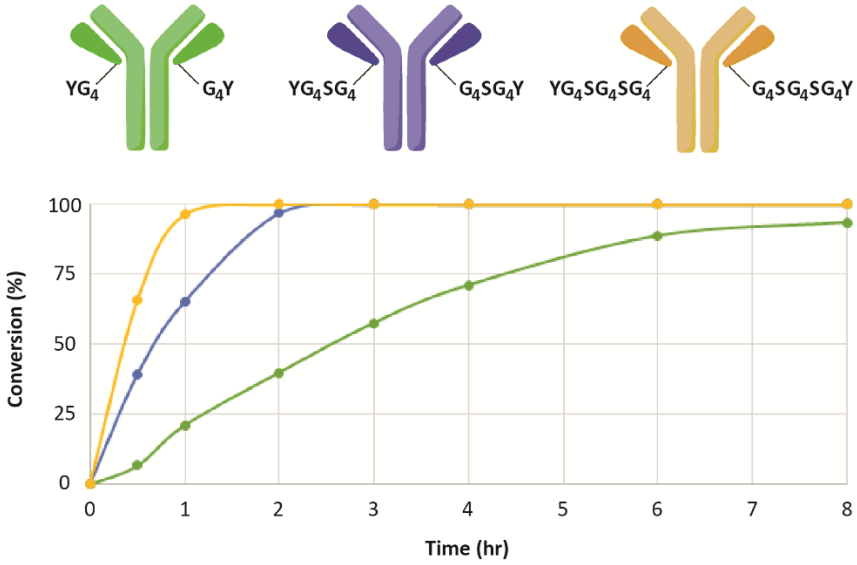



**Figure 5.** Structures of Tras[LC]$G_4Y$, Tras[LC]$G_4SG_4Y$, and Tras[LC]$G_4SG_4SG_4Y$ and time-resolved SPOCQ with TCO-AF568.

The significant increase of reactivity by insertion of even a single $G_4S$-spacer is most likely due to the LC-HC disulfide bridge, which for the light chain is located at the very C-terminus (C214). In Tras[LC]$G_4Y$ the tyrosine residue is only four amino acids removed from the disulfide bridge, whereas in Tras[LC]$G_4SG_4Y$ the residue is nine amino acids removed. As anticipated, having the tyrosine residue more exposed allows it to enter the active site of mTyr more easily, resulting in faster oxidation and subsequent cycloaddition. By increasing the linker to $G_4SG_4SG_4SG_4Y$ one would expect even faster conjugation. While this might be true, increasing the length of flexible linkers such as the $(G_4S)_n$ linker can lead to significant drops in expression yields and/or loss of biological activity, including reduced antibody-antigen or scFv-antigen binding.[23, 25] Therefore, the significant increase in reaction rate that comes with one or two $G_4S$-spacers should be sufficient for this approach.

Finally, a time-resolved SPOCQ on Tras[LC]$G_4SG_4SG_4Y$ with BCN–lis was performed. This reaction yielded identical results compared to the reaction with TCO-AF568. The lack of difference in the

conversion over time for two compounds that have significantly different reaction rates for the cycloaddition indicate that the rate-limiting step of SPOCQ is the oxidation by mTyr. This is in line with all our findings so far, where the reaction rate of SPOCQ on proteins is based on the accessibility of the tyrosine residue.

### 5.4. Protein-protein conjugation with BCN-PEG$_3$-TCO

To ability of the two-step conjugation method to introduce proteins on trastuzumab was investigated next. Using BCN-PEG$_3$-TCO **6**, TCO moieties were introduced on Tras[LC]G$_4$SG$_4$Y and Tras[LC]G$_4$SG$_4$SG$_4$Y and the products were subsequently purified with protein A spin columns. Next, the obtained TCO-bearing antibodies were subjected to conjugation with one of the following constructs: the fluorophores azido–lissamine (N$_3$–lis) or MeTz–TAMRA, the scFv MeTz–UCHT1, cytokine MeTz–IL-2, or the oligodeoxynucleotide (MeTz–ODN1826).[26] The products of these reactions were analyzed by SDS-PAGE (Figure 6).

SDS-PAGE analysis of the TCO-bearing antibodies show a clean product after protein A purification (Figure 6, lanes 1 and 7), while no reaction was observed when incubated overnight with N$_3$–lis (Figure 6, lanes 2 and 8). This further confirms the selectivity of BCN over TCO for the initial conjugation with **6**, leaving only free TCO on the antibody. Since reaction of TCO with azides is highly sluggish (0.0064 $M^{-1}$ $s^{-1}$),[27] it is too slow for efficient labelling with azido-lissamine under relatively high dilution conditions. However, fluorescence is clearly visible when incubated with MeTz–TAMRA (Figure 6, lanes 3 and 9), confirming that TCO is present on the light chains. These findings were confirmed by LC-MS (SI S5.5).

Analogously, conjugation of MeTz–UCHT1, MeTz–IL-2 and MeTz–ODN1826 with TCO-bearing antibodies were analyzed. The mass of MeTz-UCHT1 (27740 Da) is similar to the light chains, and SDS-PAGE analysis does not depict the conjugation properly (Figure 6, lanes 4 and 10). LC-MS analysis, however, shows the expected mass increase of the Tras[LC]G$_4$SG$_4$Y light chain from 24679 Da to 52392 Da (SI Figure S10), corresponding to the addition of MeTz-UCHT1.[28] The same observations are made for Tras[LC]G$_4$SG$_4$SG$_4$Y conjugation, with the mass of the TCO-bearing light chain shifting from 24994 Da to 52705 Da (SI Figure S16). Finally, MeTz–IL-2 and MeTz–ODN1826 were conjugated to the TCO-bearing antibodies, both showed high-yielding conversion on SDS-PAGE (Figure 6, lanes 5 and 11, and lanes 6 and 12, respectively).

### 5.5. Combining SPOCQ and sortase ligation for the generation of trifunctional antibodies

Having successfully developed the two-step SPOCQ ligation, we next considered the generation of trifunctional antibodies by combination of SPOCQ with sortase A. To this end, antibody AT1002 was modified to contain a sortase tag (ST) on both heavy chains and a G$_4$SG$_4$SG$_4$Y-tag on

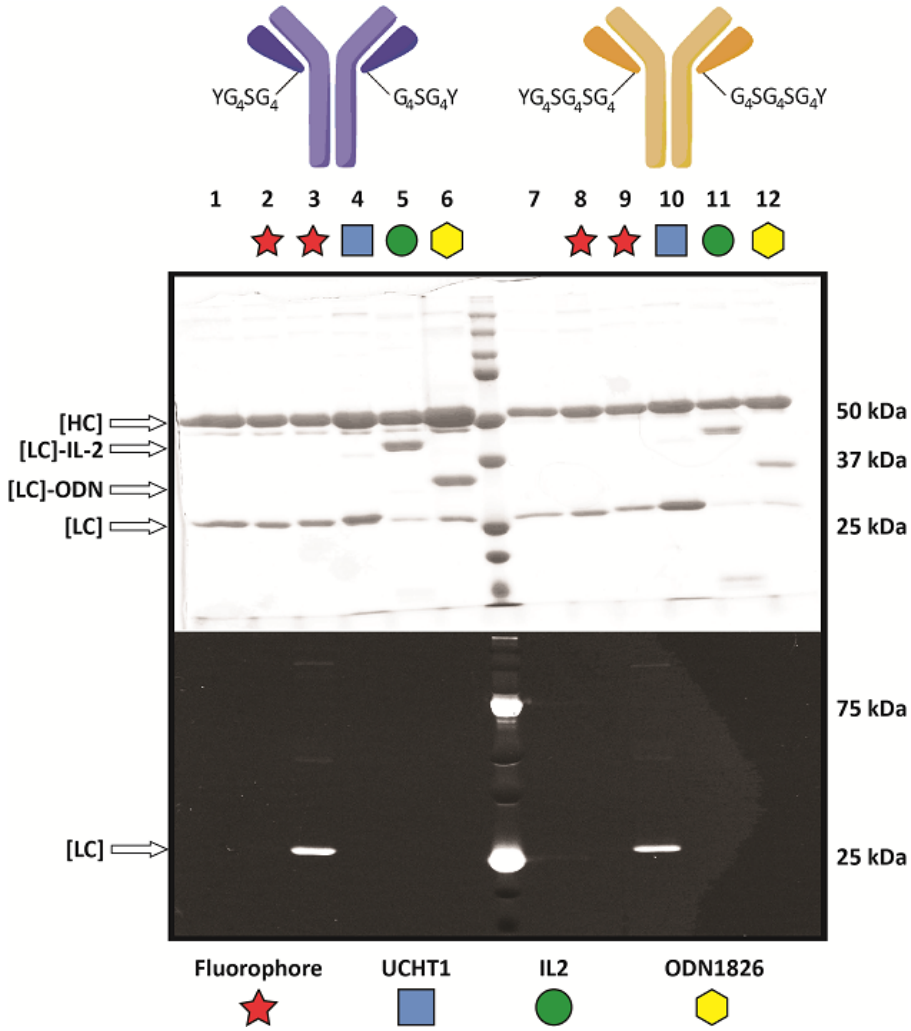



**Figure 6.** SDS-PAGE analysis of SPOCQ product between **6** and Tras[LC]$G_4SG_4Y$ (lane 1), and **6** and Tras[LC]$G_4SG_4SG_4Y$ (lane 7), and subsequent reactions with $N_3$-lis (lanes 2 and 8), MeTz–TAMRA (lanes 3 and 9), MeTz–UCHT1 (lane 4 and 10), MeTz–IL-2 (lanes 5 and 11), and MeTz-ODN1826 (lanes 6 and 12).

each light chain (AT1002[HC]ST–[LC]$G_4SG_4SG_4Y$).[21] In theory, trifunctional antibodies would be accessible by either order of event, *i.e.* sortase A ligation before SPOCQ or vice versa. However, both UCHT1 and IL-2 were modified with TCO or MeTz functionalities by means of sortase ligation, thus generating constructs of a format similar to: protein–LPET–GGG–click probe. Thus, it was reasoned that SPOCQ modification of antibody *prior* to sortase ligation of a third functionality, was likely to cause havoc, due to sortase ligation being a reversible reaction,[29] and

addition of sortase to any antibody-protein conjugate featuring the LPET-GGG linker would lead to sortase-mediated hydrolysis of peptide bond and deconjugation of the earlier attached functional groups. The alternative order of events, *i.e.* sortagging prior to SPOCQ, was therefore explored instead.

First, sortase A was used to introduce a methyltetrazine-tag on both heavy chains as reported.[10] The methyltetrazine-tagged antibody was incubated with TCO–UCHT1 to produce two UCHT1-modified heavy chains. SDS-PAGE analysis indicated a clear conversion for the heavy chain by showing a protein band a 75 kDa product (Figure 7C, lane 1-3), which corresponds to the combined mass of the heavy chain and the UCHT1. Attention was then focused on conjugation of another protein, IL-2, to the antibody light chain based on SPOCQ chemistry. To this end, BCN-TCO bifunctional reagent **6** was incubated in the presence of tyrosinase to introduce a TCO-moiety on the $G_4Y$-terminated light chain, followed by Protein A purification and subsequent incubation with MeTz–IL-2 for subsequent tetrazine ligation with the TCO-moiety (Figure 7A). Gratifyingly, the modified light chain showed up as a band at 45 kDa (Figure 7C, Lane 3-5), which corresponds to the combined mass of the light chain and the IL-2 cytokine.

Having successfully achieved the conversion of an antibody to the trifunctional construct with full loading of heavy and light chains mAb-to-protein ratio [2]:2:2, we investigated if it would be possible to obtain a trifunctional mAb-protein conjugate in [2]:1:1 ratio by execution of two mono-conjugation reactions in sequence. To this end, a AT1002 mutant was designed and transiently expressed based on knob-in-hole technology with two differentiated heavy chain C-termini: one bearing a LPETG sortase tag and one bearing a $G_4SG_4Y$-tag. The generated antibody, AT1002[KiH-HC]ST/$G_4Y$, was subjected to the same sequence of events as described above, i.e. the LPETG-fused heavy chains was subjected to sortase-mediated introduction of methyltetrazine, followed by IEDDA with TCO–UCHT,[9] whereas the C-terminal tyrosine on the other heavy chain was modified based on the reaction sequence by SPOCQ-mediated introduction of TCO followed by IEDDA with MeTz–IL-2 (Figure 7B). Whilst performing the reactions, samples were taken and analyzed with SDS-PAGE (Figure 7D). The starting antibody (lane 1) and the sortase-ligated product (lane 2) do not significantly shift on SDS-PAGE, however a 75 kDa side-product becomes apparent, as well as a band at 27 kDa (sortase A). However, after conjugation with TCO–UCHT1 (28 kDa) and subsequent protein A purification (lane 3), a clear shift to a 75 kDa band was observed, in analogy with the expected mass increase. Furthermore, the 75 kDa band of the modified heavy chain has roughly the same intensity as the 50 kDa band corresponding to the $G_4Y$-bearing unmodified heavy chain, indicating that the generation of a mono-functionalized antibody is efficiently performed. When subsequent SPOCQ with **6** is

performed, the 50 kDa band shows a slight upward shift (lane 4). Subsequent IEDDA with MeTz–IL-2 shifts the remaining 50 kDa band to 70 kDa, corresponding to the newly formed HC–IL-2 conjugate (lane 5).

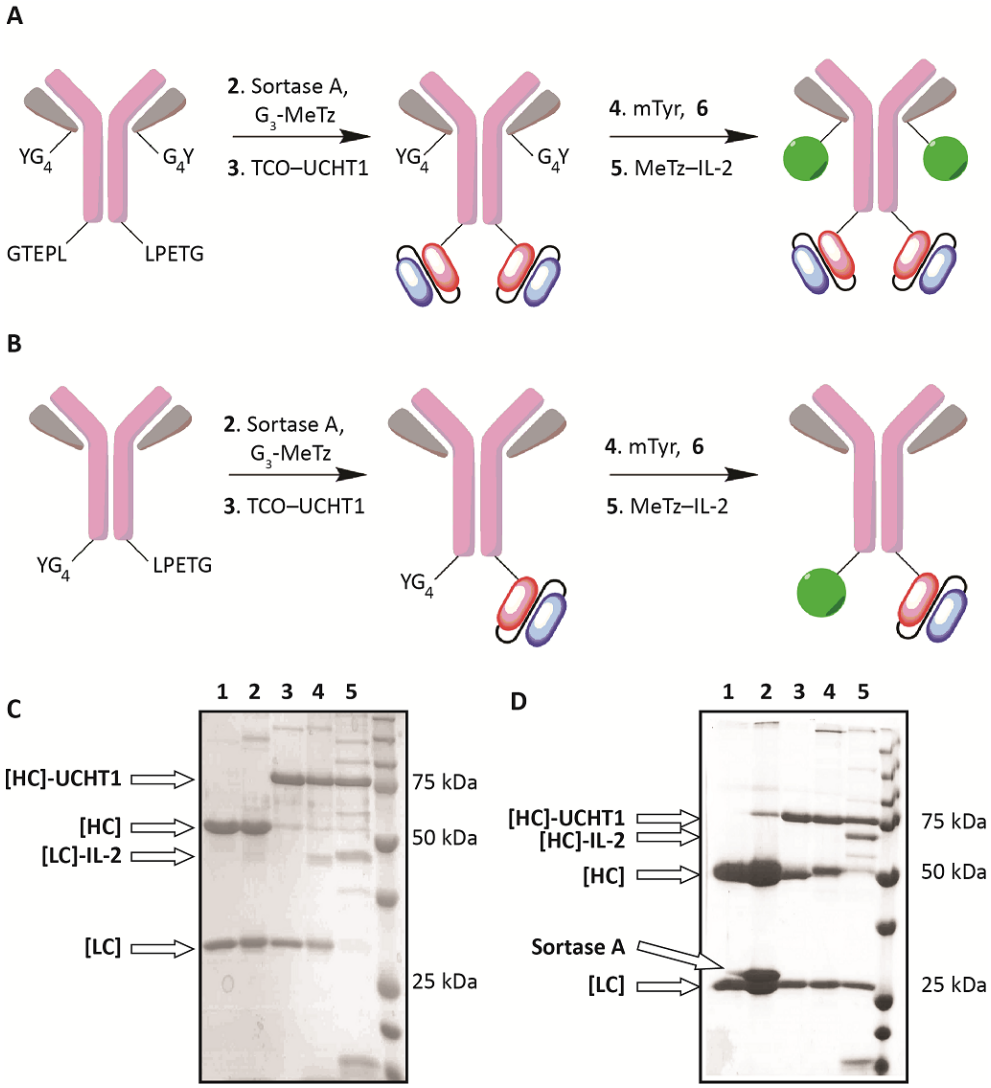



**Figure 7**. Schematic overview and corresponding SDS-PAGE gels of the dual modification of AT1002[HC]ST–[LC]$G_4SG_4SG_4Y$ (A, C) and AT1002[KiH-HC]$G_4Y$/ST (B, D). For both SDS-PAGE gels; unmodified antibody (lane 1), antibody after sortase ligation (lane 2) and subsequent TCO–UCHT1 treatment (lane 3), and SPOCQ with BCN-$PEG_3$-TCO **6** (lane 4) with subsequent MeTz–IL-2 treatment (lane 5).

## 5.6. Conclusions

We have developed various novel methods to antibody-protein conjugates based on step-wise SPOCQ and tetrazine ligation (for monofunctionalization of antibody) or by further combination with sortase-based protein conjugation (for difunctionalization). Specifically, monofunctionalization was achieved based on a two-stage approach starting with introduction of a TCO moiety based on chemoselective SPOCQ with a BCN-$PEG_3$-TCO linker, after which IEDDA can be employed for attachment of a MeTz-bearing protein, oligonucleotide, or small molecule. To introduce these moieties faster and more efficiently, it was found that the length of the peptide spacer between the antibody and the $G_4Y$-tag plays a crucial role in the efficiency of the reaction, in particular with regard to oxidation of the terminal tyrosine residue by mTyr, with longer spacers ($G_4S$ and $G_4SG_4S$) significantly speeding up the efficiency of the reaction.

This two-step conjugation method was subsequently further extended to the generation of trifunctional antibodies with high efficiency. In this case, a first protein is introduced based on sortase ligation followed by IEDDA, and a second protein via SPOCQ followed by IEDDA. In addition, we have shown that the valency of the resulting trifunctional antibodies can be carefully tailored towards either dual divalent functionalization on both light and heavy chains, or dual monovalent functionalization on or two structurally distinct heavy chains. Other formats or protein-to-antibody ratios have become not been investigated here, but are obviously also at hand.

While functional tests with the bi- and trifunctional antibodies presented herein are ongoing (but not ready for presenting), we envision the generation of trifunctional antibodies in a modular fashion will allow for rapid production of bispecific ADCs or immunocytokines, as well as many other interesting multifunctional antibody constructs. This will enable the rapid screening of varying bispecifics based on *e.g.* antigen targets, linker types and lengths, and different payloads to improve cell targeting and subsequent treatment.

## 5.7. Supporting information

The Supporting Information is available free of charge.

## 5.8. References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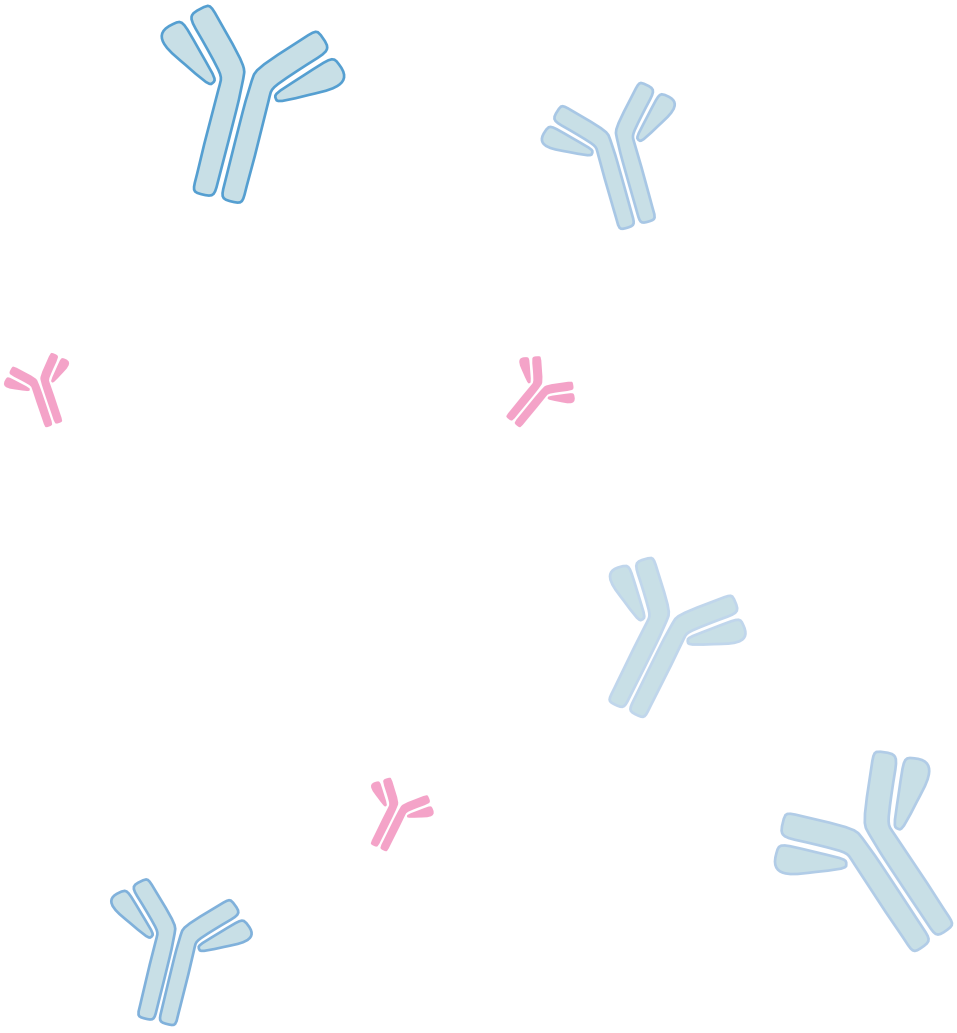
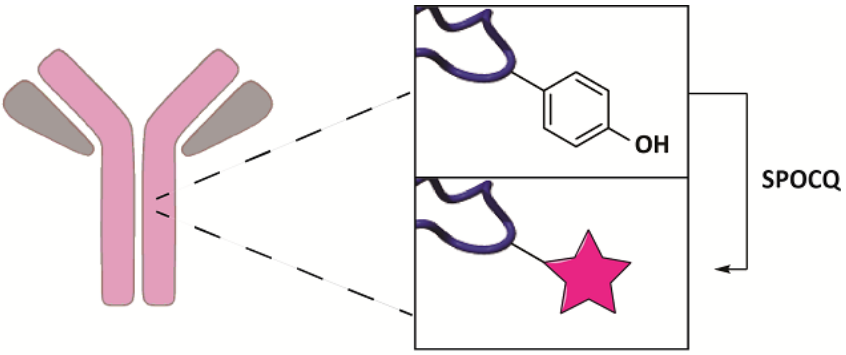
# Chapter 6

# Engineering of Tyrosine-Containing Peptide Loops Enables Non-terminal Protein Labeling



**Abstract:**




**Labeling of tyrosine-containing loops with SPOCQ**

*While termini of antibodies are easily modified, their pharmacokinetic profile is generally inferior to conjugates on internal amino acid residues. Therefore, an internal exposed tyrosine residue was introduced via solvent-accessible loops on the CDR and the Fc-region. Labeling on the CDR-tyrosine went quantitatively, and labeling could be inhibited by co-inhibition with the corresponding anti-idiotypic antibody. Labeling on the Fc-tyrosine was also achieved, though the antibody proved instable overtime.*

## 6.1. Introduction

The pharmacokinetic profile of an antibody conjugate depends not only on the homogeneity of the conjugation method,[1] but also on the selected site of conjugation.[2] First of all, highly solvent-accessible conjugation sites may be susceptible to increased deconjugation through various mechanisms, such as reverse Michael reaction of thiol-maleimide conjugate,[3] or valine-citrulline-*p*-aminobenzylcarbamate (vc-PABC) linker cleavage by endogenous esterases.[4] Generally, more readily accessible linker-drugs on a protein display compromised pharmacokinetics, which can be improved by optimizing conjugation site and the type of linker.[5] The enhanced clearance of ADCs obtained by C-terminal conjugation suggests that our previously reported SPOCQ method, although highly stable, is based on labeling of exposed tyrosine tags engineered at either terminus, and might therefore generate antibody conjugates with non-optimal in vivo efficacy.

While functionalization of non-terminal tyrosine residues has been demonstrated, these methods rely either on non-specific oxidation conditions (Fremy's salt[6]) or involve the use of non-natural amino acids bearing a catechol moiety in the form of L-DOPA.[7] While the latter approach allows for mild oxidation conditions (*e.g.* sodium periodate), it also requires the rather complicated and low-yielding genetic encoding of L-DOPA during protein expression. We[8] and others[9] have reported earlier that selective tyrosine oxidation and subsequent conjugation under the action of mushroom tyrosinase (mTyr) is feasible in case the tyrosine residue resides at (or close to) the protein's N- or C-terminus, leaving all other tyrosine residues in the protein sequence unmodified. Based on the premise of potentially improved pharmacokinetics, we explored the conjugation of non-terminal tyrosine residues based on selective oxidation and conjugation. We here report two strategies for non-terminal SPOCQ, either based on the use of an endogenous, naturally exposed tyrosine or based on the engineering of specific tyrosine-containing peptide loop structures.

## 6.2. Cystine knobs

Disulfide bridges in CDR regions are rare, but in some cases are naturally encoded in the third heavy chain loop of the CDR (CDR-H3): approximately 6% of human CDR-H3 sequences contain two additional cysteine residues separated by 0-12 amino acid residues,[10] with the vast majority (91%) separated by 3 or 4 amino acids, forming a short loop, also known as a cystine knob.[10] A recent study of an antibody containing a cystine knob showed that mutagenesis of the two cysteine residues severely hampered binding efficiency.[11] By mutating one or two cysteines to alanines, antigen binding would be decreased $10^2$-$10^4$ fold, demonstrating the importance of the rigid cystine loop. Another antibody known to feature a cystine knob is AT1413. This patient-derived antibody specifically recognizes a sialylated epitope on CD43,[12] a transmembrane cell surface protein expressed on all cell lines of acute myeloid lymphoma (AML).[13, 14]

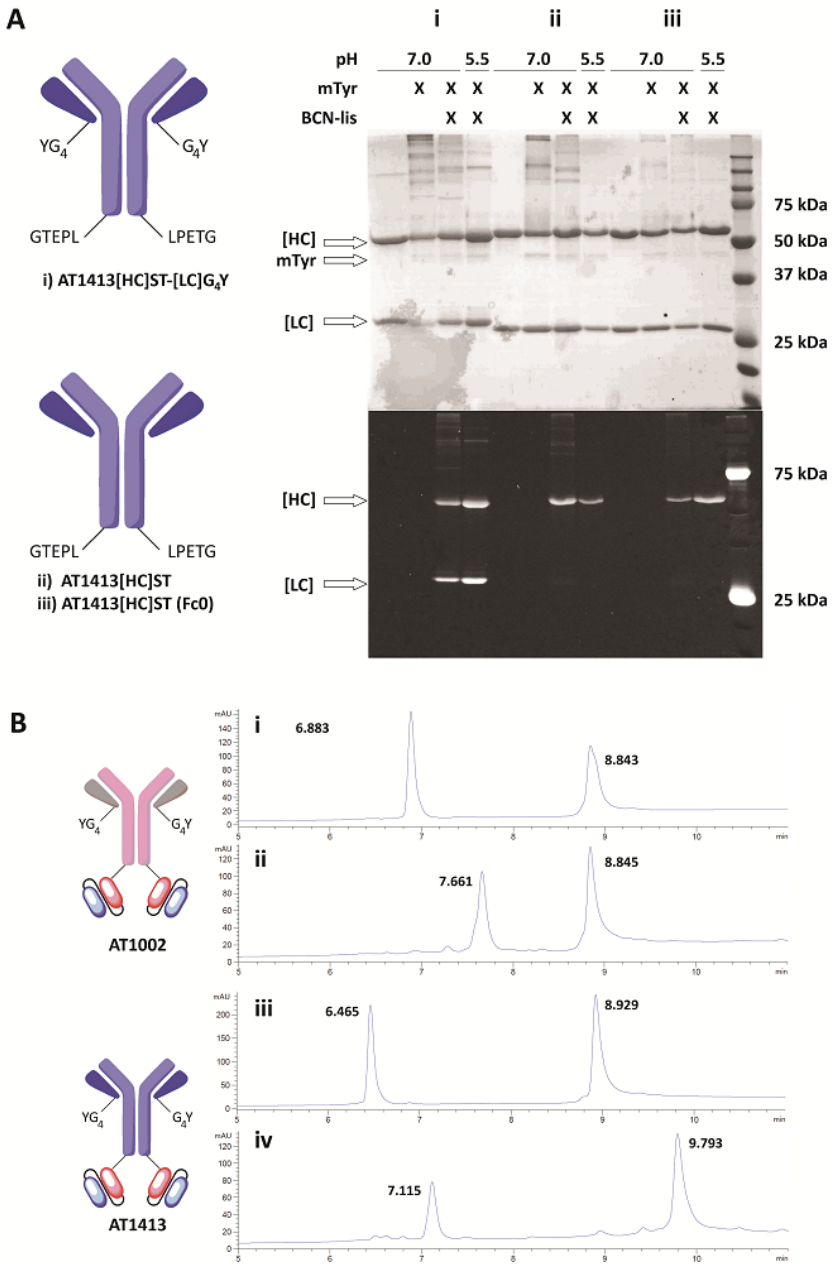



**Figure 1**. (A) Schematic representation of two different AT1413 mutants and SDS-PAGE gel of resulting SPOCQ experiments, (B) HPLC chromatograms of AT1002[HC]UCHT1-[LC]$G_4$Y and AT1413[HC]UCHT1-[LC]$G_4$Y before (i, iii) and after (ii, iv) SPOCQ.

In a previous chapter, we reported that engineering of a C-terminal $G_4Y$ tag on an antibody light or heavy chain enables selective tyrosine oxidation followed by in situ conjugation based on SPOCQ with a BCN-modified fluorophore. SPOCQ with BCN-lissamine was attempted on an AT1413 mutant bearing the C-terminal $G_4Y$ tag on its light chain, as well as a heavy chain sortase tag (*i.e.* AT1413[HC]ST-[LC]$G_4Y$). SDS-PAGE revealed conjugation on the light chain went in identical fashion to earlier findings; however, fluorescence was also detected on the antibody heavy chain with similar efficiency (Figure 1A). This was also the case for the AT1413 mutant AT1413[HC]ST (Fc0), in which Fc0 represents two single point mutations (G236R and L328R) to abolish interaction with other, non T-cell, immune effector cells.[15] Variation of pH (pH 7.0 and pH 5.5) had little influence on conjugation efficiency, with SPOCQ performed at pH 5.5 yielding slightly cleaner conjugates as reported before.[16] Similarly, prior sortagging at the heavy chain C-terminus (with scFv UCHT1), led to identical conjugation on both chains (Figure 1B). In order to exclude any influence of the sortase recognition sequence of the antibody or fusion of UCHT1 to the antibody, SPOCQ was also performed on the similar conjugate based on control antibody AT1002[HC]UCHT1-[LC]$G_4Y$, which showed exclusive labeling on the light chain, corroborated by SDS-PAGE and HPLC analysis (Figure 1). Since we had not earlier observed any labeling of native antibody heavy chain in the past, it was reasoned that the addition reactivity had to be attributed to the presence of the CDR cysteine loop in AT1413.

Earlier, we found that AT1413 is no longer able to recognized to CD43 when tyrosine was mutated (unpublished results), strongly indicating an essential role for the tyrosine residue in target binding. This in turn would suggest that the tyrosine residue, presented in a cysteine loop "...GCG**Y**SSCF..." in the CDR-H3 (HC-Y106), might be the tyrosine oxidized by mushroom tyrosinase. However, this could not be concluded based on SDS-PAGE and HPLC experiments only. While a logical next step could involve expression of an AT1413 mutant at position Y106 or tryptic digest and MS analysis of the peptide fragment before and after SPOCQ labeling, we considered an entirely new approach to confirm the labeling of Y106. We reasoned that the addition to AT1413 of a stoichiometric quantity of an anti-idiotypic antibody, which binds to the CDR by definition, would sterically block to accessibility for mushroom tyrosinase and thereby effectively preventing oxidation of Y106. Indeed, this hypothesis was confirmed when SPOCQ was attempted in the presence of various stoichiometries of an anti-idiotypic antibody (AIA) for AT1413:[17] addition of AT1413-AIA to AT1413[HC]ST under SPOCQ labeling conditions fully blocked oxidation and subsequent cycloaddition (Figure S1). To the best of our knowledge, no such transient 'protection' of native amino acid functionality with monoclonal antibodies during a biochemical processing step has been reported to date and warrants further investigation. At the same time, while these findings clearly provide a clear proof-of-concept for the feasibility of SPOCQ for labeling of non-terminal tyrosine residues, it is also apparent that conjugation on tyrosine in the CDR of an antibody would impede the essential binding of the antibody to its target antigen and was therefore not further explored here.

### 6.3. Loop antibodies

As a second option for non-terminal labeling of antibodies, we envisioned the incorporation of small tyrosine-containing peptide sequences into solvent-exposed loops in an antibody constant domains. These 'loop antibodies' would ideally display better pharmacokinetics than terminally labeled antibodies, in particular in combination with hydrophobic payloads, based on a potential partial covering of the payload by the antibody protein fragments. Using the two-step conjugation method described in chapter 5, these loop antibodies could be used to generate new bispecific antibody structures, which previously required the introduction of entire proteins in antibody loops.[18]

Our design of an antibody with an exposed tyrosine-bearing loop was based upon work performed by Grünewald *et al*.,[19] on a total of 183 trastuzumab mutants containing either of two peptide insertions in a total of 110 distinct positions on the antibody. These peptide insertions (S6 = GDSLSWLLRLLN; YbbR = DSLEFIASKLA) contain a serine residue available for site-specific labeling with enzymes Sfp and AcpS phosphopantetheinyl transferases (PPTases).[20] By using these tags and fusing them inside the solvent-exposed loops in trastuzumab, over 60 different ADCs with a drug-to-antibody ratio (DAR) >1.8 were produced and evaluated.[21] It is noteworthy that the S6-tag has two serine residues, but only a single modification was detected. After mutating the serine residue at position 3 to an alanine (S6-S3A) the conjugate formation was completely abolished, confirming the specificity of the PPTase for that particular residue.[19]



Based on the finding by Grünewald *et*. *al*., a tyrosine-based insertion was envisioned. We selected insertion of a tyrosine loop after residue HC-P294,[i] based on the excellent DAR reported for conjugation of the drug monomethylauristatin F on tags following that position, as well as the high area-under-the-curve (AUC) with regards to pharmacokinetics, indicating long-term stability and low clearance of the conjugates. Thus, two distinct tyrosine-containing peptide loops were inserted after P294, both of which were based on the earlier reported S6 tag.[20] The first tag, Y1, had the S3Y mutation (Figure 2), with the argument that the tyrosine would be located on the same site where conjugation with PPTase occurs.[19] The second tag, Y2, was designed as a W6Y mutation (Figure 2), selected based to the comparable hydrophobicity and size for tyrosine and tryptophan. More importantly, a phage-displayed peptide library optimizing the tag for PPTases indicated that tyrosine was a common mutation of the W6.[20] Tras-Y1 and Tras-Y2 antibodies were transiently expressed in CHO-K1 and purified, after which SPOCQ was attempted on both antibodies.

[i] Grünewald *et*. *al*. defined the residue as P291 by virtue of Eu numbering, however the correct numbering for trastuzumab is P294 (drugbank accession number DB00072).

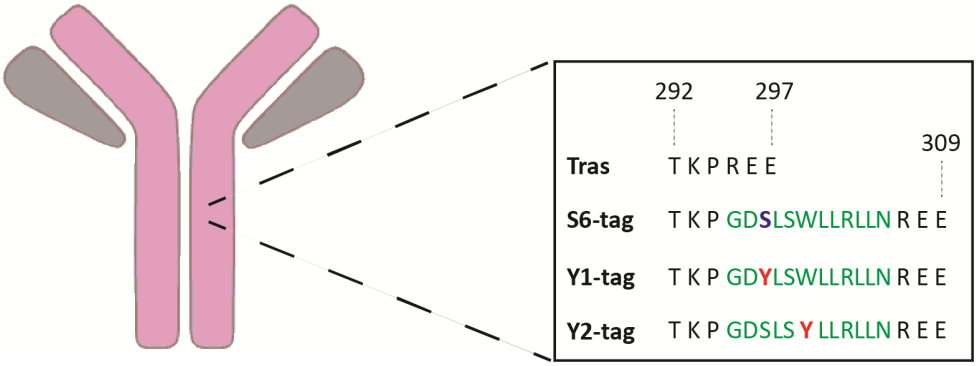



**Figure 2**. Comparison between inserted tags in trastuzumab.

Having the various mutants in hand, SPOCQ was attempted on both Tras-Y1 and Tras-Y2 antibodies in PBS (50 mM phosphate, 150 mM NaCl) at pH 5.5 or pH 7.1, and at 4 °C or 37 °C. The higher temperature and pH would generally increase the reaction rate of the oxidation by mushroom tyrosinase,[8, 22] although more side reactions might occur due to an increase in non-selective Michael additions of nucleophilic amino acid side-chains.[23] The antibodies were modified at 3 mg/mL with 6 eq. of BCN-lissamine with overnight incubation, after which SDS-PAGE and HPLC analyses were performed.

A few interesting observations can be made, the first of which is that Tras-Y1 seems to outperform Tras-Y2 based on a higher fluorescence intensity, which is most clearly visible at pH 7.1 (Figure 3A and 3B, lanes I and L). Furthermore, SPOCQ at pH 5.5 seems to lead to lower yield than at pH 7.1, which is in contrast with SPOCQ on termini where a shift in pH generally only results in different rates of oxidation by mushroom tyrosinase. In those cases, a lower pH resulted in decreased non-specific Michael addition by nucleophilic amino acids, and therefore a better conjugation yield.[23] A case can be made for the oxidation not going to completion due to the inaccessibility of the tyrosine by mushroom tyrosinase, but then one would expect a difference in conversion rate between 4 °C and 37 °C as well, which is not the case (Figure 3A and 3B, lane C vs lane F). A control experiment with mushroom tyrosinase in the absence of BCN-lissamine for Tras-Y1 at pH 7.1 (Figure 3A, lanes H and K), indicated that the antibody seems to fragment into lower molecular weight fragments. In contrast, a similar control experiments with terminal tyrosine-bearing antibodies, generally induced cross coupling between light and heavy chains, resulting in higher molecular weight conjugates.

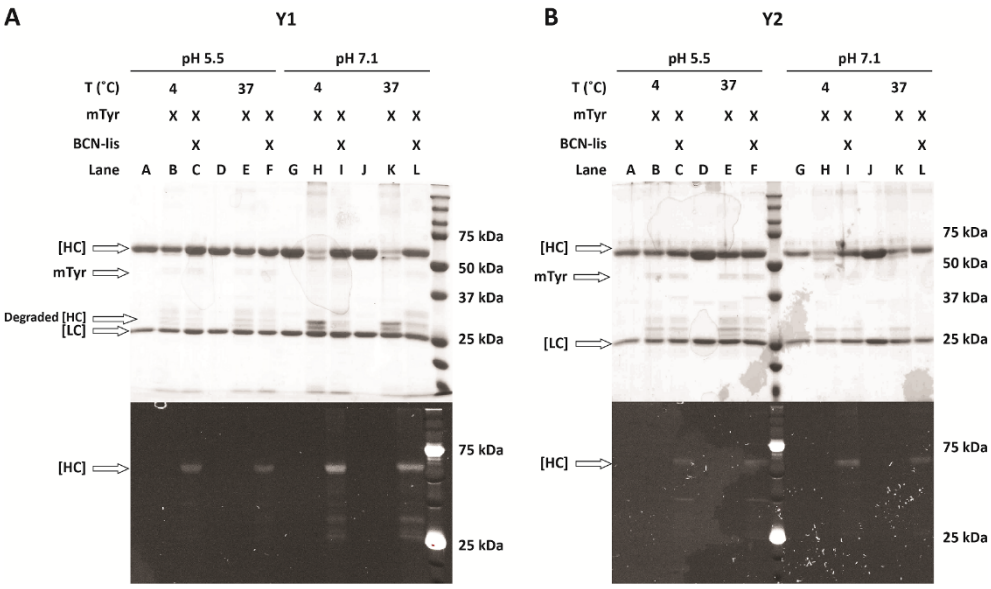



**Figure 3**. SDS-PAGE analyses of SPOCQ attempted on (A) Tras-Y1 and (B) Tras-Y2 with varying pH (5.5 or 7.1) and temperature (4 °C or 37 °C). Each condition was performed with only antibody, antibody with mTyr, and antibody with both mTyr and BCN-lis.

Additionally, HPLC spectra were recorded of all the formed products (SI S6.4). A lack of consistency was observed however; while each attempt at SPOCQ yielded a visible peak with a fluorescent signal, the yield was no higher than 10-20% in each case. Furthermore, there did not seem to be an increase in conjugation yield between pH 5.5 and pH 7.1, and all HPLC spectra displayed a significant amount of unidentified peaks.

Optimization of SPOCQ on Tras-Y1 and Tras-Y2 was attempted by varying the concentration of antibody in the reaction, as earlier work indicated that performing SPOCQ at higher concentrations suppresses side reactions.[8] Thus, overnight SPOCQ was performed on both Tras-Y1 and Tras-Y2 in PBS pH 5.5 at 4 ˚C with various antibody concentrations, after which SDS-PAGE (Figure 4) and HPLC (SI S6.5) analyses were performed. Both SDS-PAGE and HPLC indicate a significant amount of unidentified bands and peaks for Tras-Y1, which might indicate degradation of the product (Figure 4, lane A – E). Even the sample containing only the antibody seems significantly different compared to the previous gel/chromatogram, which was performed six days before this experiment. While Tras-Y2 does not seem to degrade as much as Tras-Y1, there is a new band at 37 kDa that also displays fluorescence (Figure 4, Lane H – J). Furthermore, none of the conditions shows any improvement in reaction yield based on HPLC analysis.

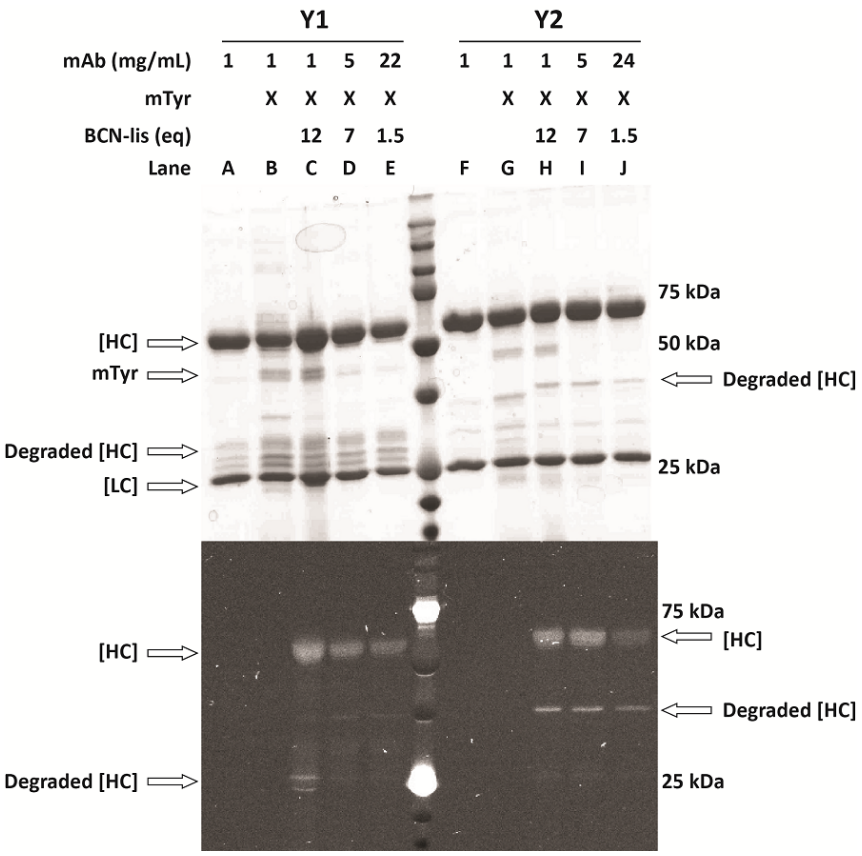



**Figure 4**. SDS-PAGE analysis of optimization of SPOCQ on Tras-Y1 and Tras-Y2. Both antibodies were run without reagents (Lane A, F), with only mTyr (Lane B, G) and with varying protein and BCN-lis concentrations (Lanes C-E and H-J).

A final attempt to perform SPOCQ on Tras-Y1 and Tras-Y2 was based on performing the reaction in phosphate buffer instead of PBS, as sodium chloride is known to inhibit mTyr.[22] An overnight reaction at 4 °C with 22 mg/mL was performed and analyzed with HPLC (SI S6.6). No significant improvement on yields were detected, however even more changes were detected to the heavy chain signals of both antibodies. The signals were shifted significantly and the intensity became lower than that of the light chain, this while the antibody was only stored for 28 days at 4 ˚C.

An overview of the HPLCs of the antibodies without the presence of mTyr or BCN-bearing compounds was made (Figure 5), and it depicts clearly the rapid change of the antibodies. It is unknown what is happening exactly, but Grünewald *et*. *al*. did report on the antibody losing

stability after installing peptide tags in the $C_H2$ region.[19] By further altering the peptide tag and installing it in a region prone to instabilities, the antibody degrades within weeks.

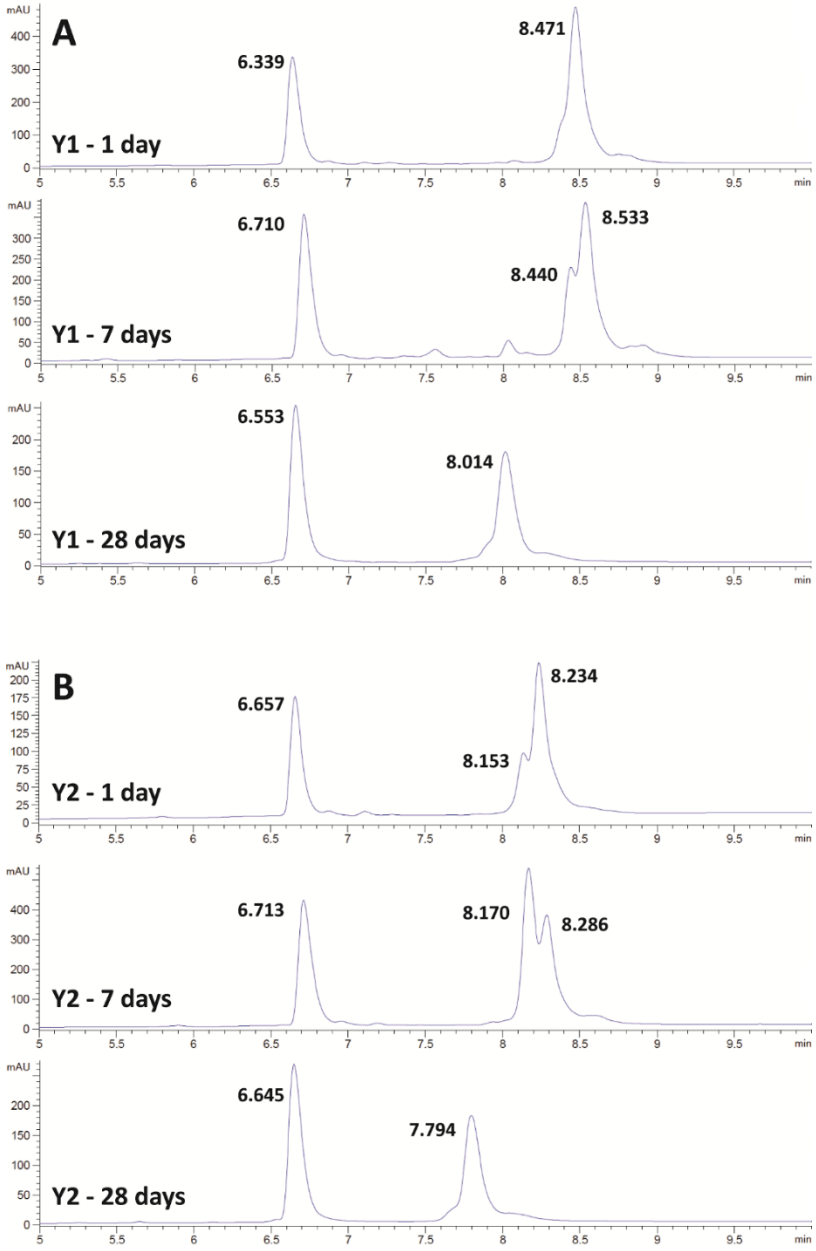



**Figure 5**. HPLC chromatograms overtime of (A) Tras-Y1 and (B) Tras-Y2.

## 6.4. Conclusion and Outlook

The method of installing a tyrosine residue via an internal peptide tag in a solvent-exposed area is clearly viable in various ways. First of all, we found that an exposed tyrosine in the antibody CDR is labeled with high efficiency and homogeneity based on the standard SPOCQ conditions. Unfortunately, the method has limited applicability for the generation of functional antibody conjugates due to the disruption of antigen binding. As an alternative, we explored the insertion of specific tyrosine-containing peptide loops in the antibody $C_H2$ fragment. Indeed, SPOCQ was detected on both Tras-Y1 and Tras-Y2 antibodies, but the instability of both the Tras-Y1 and Tras-Y2 antibodies and the low yield during the conjugation clearly requires further optimization.

First, the location of the tyrosine insert can be altered. Grünewald *et*. *al*. reported residues A121, S122 and T123 all showing high DARs for both S6-tags and YbbR-tags, as well as generally better stability than conjugates formed in the $C_H2$ region.[19] The trade-off for higher stability might be lack of oxidation by mTyr, as the stable, less exposed tags might not be accessible for the large enzyme (130 kDa). If this becomes an issue, using monomeric mushroom tyrosinase (43 kDa) might be a viable alternative.[24] Second, optimization of the installed tyrosine-bearing peptide can lead to better conjugation yields. The Y1 and Y2-tags were based on the S6 tag,[20] which was optimized for PPTases. As seen on the AT1413 antibodies, the tyrosine simply needs to be exposed for oxidation by mTyr. Therefore, the loop could be shortened to minimize protein structure perturbation. Inserting the exact same cysteine loop as present in AT1413 at the positions identified by Grünewald *et*. *al*. might also lead to more stable and higher yielding conjugates. Finally, it might even be possible to modify a single exposed amino acid residue to a tyrosine, to this end single-point mutation of the amino acids where S6-tag insertion led to high DARs to a tyrosine residue might be sufficient, or alternatively careful analysis of the antibody crystal structures may reveal optimal naturally exposed sites for mutation to tyrosine and oxidation by mushroom tyrosinase. To perform all this however, rather than expressing antibodies in high quantities, a large quantity of small-scale expressions to optimize conditions might be preferable. A possible option to facilitate this issue is using an automated Protein Expression and Purification Platform (PEPP).[25]

## 6.5. Supporting information

The Supporting Information is available free of charge.

## 6.6. References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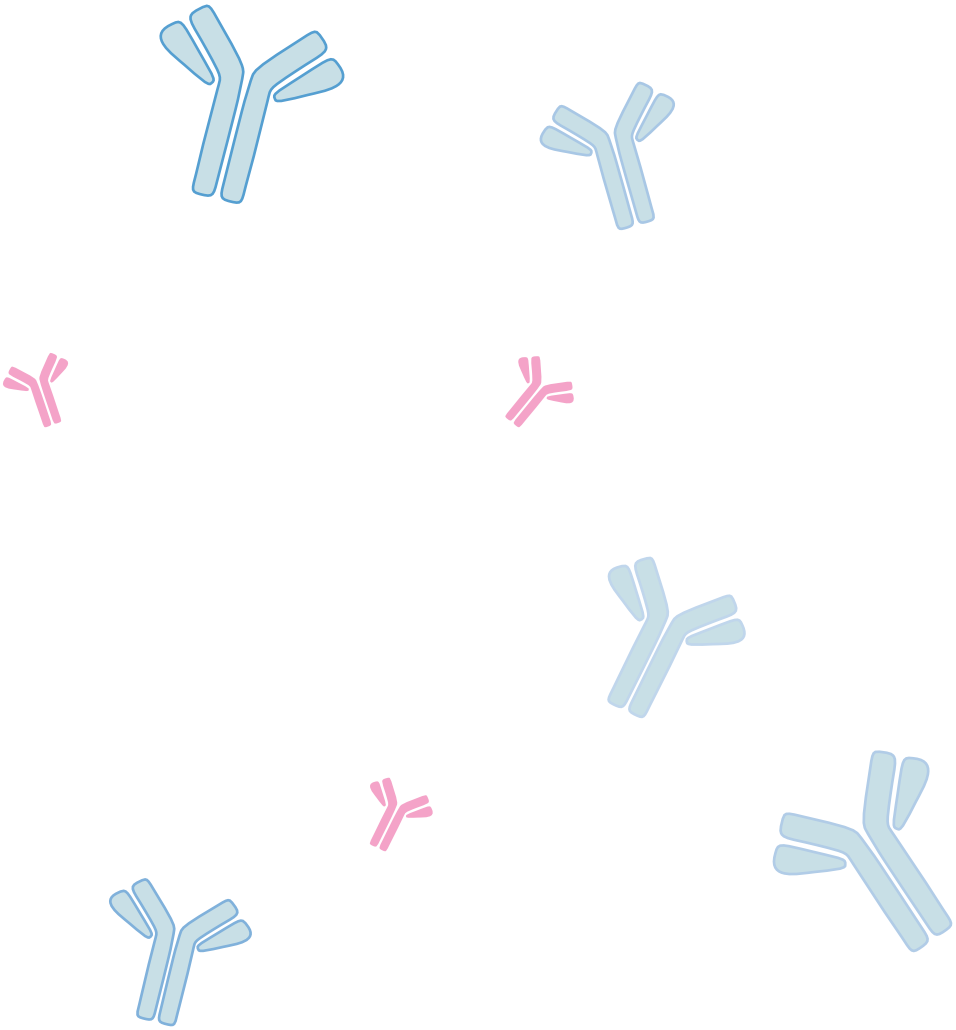
# Chapter 7

# Summary and General Discussion



## 7.1. Short summary

Antibodies can be modified with pharmaceutical compounds for targeted therapies, and have been widely studied for drug delivery (ADCs),[1] localized radiation,[2] and immunotherapies.[3] A key factor in generating these compounds has been the ability to modify proteins in a site-selective manner, as uncontrolled conjugation presents with unfavourable pharmacokinetics.[4] Ideally, the method of conjugation relies on natural amino acids under mild conditions, and yields a single product.

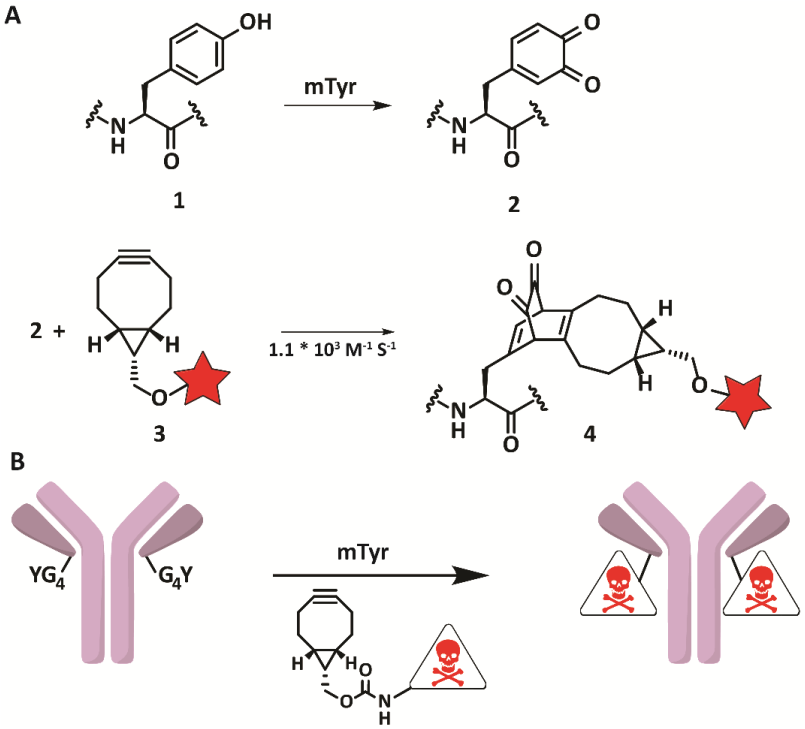
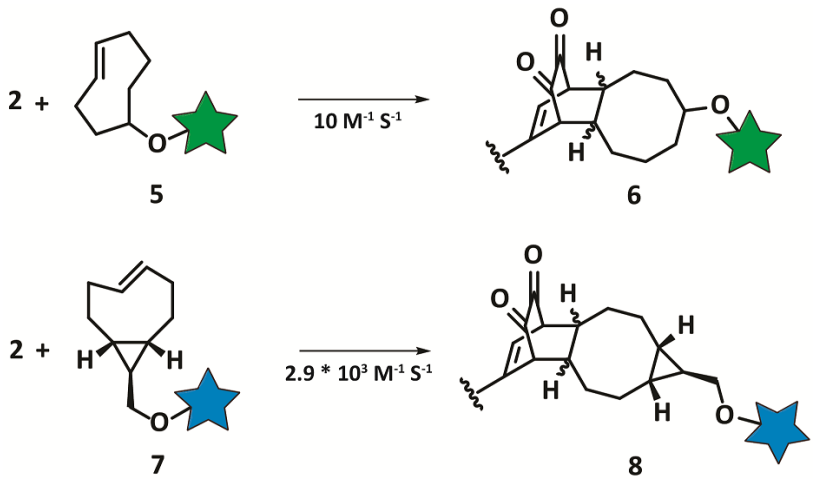



**Figure 1**. (A) A schematic representation of SPOCQ; oxidation of a tyrosine residue by mTyr and subsequent cycloaddition with a BCN-bearing probe. (B) SPOCQ on $G_4Y$-bearing antibody light chains for the generation of ADCs.

This thesis describes a new process for labeling of proteins in an inducible fashion, based on the selective enzymatic oxidation of tyrosine side-chains (**1**) by mushroom tyrosinase (mTyr).[5] As most proteins, including monoclonal antibodies, do not have tyrosine residues accessible for oxidation by the bulky mushroom tyrosinase (130 kDa), selective oxidation is ensured by engineering of an exposed tyrosine tag ($G_4Y$) on any of the protein termini. After oxidation by mTyr, a highly electrophilic quinone group (**2**) is generated that can readily undergo an inverse-electron demand Diels-Alder reaction (IEDDA), such as strain-promoted oxidation-controlled

*ortho*-quinone cycloaddition (SPOCQ). Thus, by performing the tyrosine oxidation in the presence of the strained alkyne bicyclo[6.1.0]nonyne (BCN, **3**),[6] fast cycloaddition takes place to the in situ generated quinone (Figure 1A), leading to a stable conjugate (**4**) in high yield.[7] This protocol was first proven on the enzyme laminarinase A (LamA), and subsequently on various monoclonal antibodies for the generation of antibody-drug conjugates (Figure 1B, **chapter 2**).




**Scheme 1**. SPOCQ with cycloalkenes TCO (**5**) and cpTCO (**7**).

The initial protocol was optimized to increase reaction rate and purity of the products. Cycloaddition chemistry of the intermediate quinone was then extended to SPOCQ with *trans*-cyclooctene (TCO, **5**) and cyclopropanated *trans*-cyclooctene (cpTCO, **7**), and using the cpTCO we were able to perform bioconjugation in an orthogonal fashion to strain-promoted alkyne–azide cycloaddition (SPAAC) for tandem dual labeling of trastuzumab (Figure 2A, **chapter 3**). By application of knob-in-hole technology, trastuzumab variants were generated with two different heavy chain termini, thereby introducing only a single tag for SPOCQ labeling or sortase ligation, and we demonstrate the use of either strategy for rapid and highly efficient mono-functionalization (Figure 2B, **chapter 4**). We then developed a two-step labeling method for highly efficient protein-protein coupling using SPOCQ and IEDDA. Combining this two-step labeling strategy with sortase ligation, trifunctional antibodies were created by engineering a $G_4Y$-tag on light chains and sortase tag (LPETG) on heavy chains. This allowed for the introduction of small molecules, cytokines, scFv fragments or oligonucleotides in high-yielding and selective fashion. We also demonstrate that a knob-in-hole antibody with two distinct heavy chains, one with $G_4Y$-tag and one with sortase tag, allows for two consecutive modifications (Figure 2C, **chapter 5**). Finally, we demonstrate that SPOCQ on internal tyrosine residues is also feasible, by either expressing a tyrosine in a small cystine knob on the CDR or in a solvent-exposed loop in de $C_H2$ region (Figure 2D, **chapter 6**).

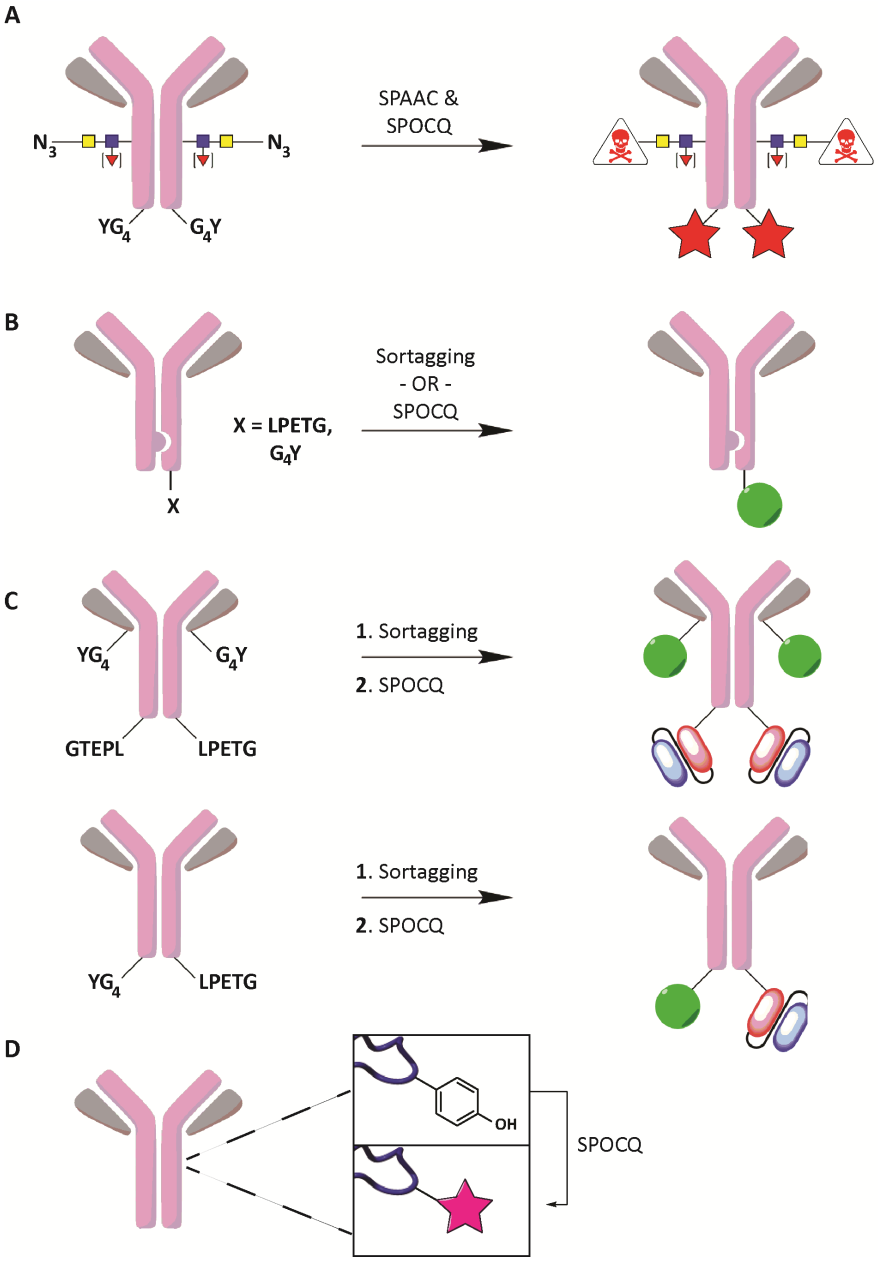



**Figure 2**. (A) Dual modification of antibodies with glycan remodelling and SPOCQ. (B) Generation of [2]:1 bifunctional antibodies via knob-in-hole antibodies with sortase or SPOCQ. (C) Generation of [2]:2:2 and [2]:1:1 trifunctional antibodies via sortase and SPOCQ. (D) Tyrosine-containing peptide for non-terminal protein labeling via SPOCQ.

### 7.2. SPOCQ on proteins – Critical process parameters

As described in this thesis, reaction conditions for SPOCQ have been optimized to enable >95% labeling of antibodies in a reliable fashion. While specific conditions were already included in the supporting information of chapters 3 and 4, a detailed explanation of each process parameter and its impact on SPOCQ has not been provided yet. As concluded in chapter 5, the rate-limiting step of SPOCQ is the enzymatic oxidation of tyrosine, which in this thesis was performed by mushroom tyrosinase (mTyr). Thus, for faster conjugations, the rate-limiting oxidation rate of tyrosine must be accelerated. At the same time, the relative rate of intermolecular cycloaddition with strained alkynes or alkenes versus non-specific side-reactions is critical for product purity: the *in situ* generated quinone has only a limited lifetime before undergoing Michael addition by nucleophilic amino acids, hence cycloaddition by either BCN, TCO or cpTCO must be rapid. Here, the impact of various process parameters on enzymatic oxidation and cycloaddition chemistry are discussed in detail.

**pH**: Over the course of this research, it was found that the pH of the reaction is of paramount importance to the reaction rate and conversion rate of SPOCQ (chapter 3, Figure S10 and S11). At lower pH, reaction rate of Michael addition by nucleophilic amino acids is mitigated due to protonation of amine and thiol residues. Unfortunately, oxidation rate by tyrosinase is also directly correlated to pH, with mushroom tyrosinase having barely any activity below pH 5.0. Generally, a pH of 5.5 is optimal for selective antibody modification, yielding clean conjugates without hampering the rate of tyrosine oxidation.



**Buffer type**: While buffer seems to have little influence on SPOCQ, it is noted that tris(hydroxymethyl)aminomethane (present in Tris buffer) is also a nucleophilic agent that is able to perform Michael addition to quinones and therefore incompatible with SPOCQ. Generally, any non-nucleophilic buffer capable of maintaining the desired pH of the reaction is suitable. Low chloride concentration is preferred, because mushroom tyrosinase is inhibited during storage over time by chloride ions.

**Temperature**: Increase in temperature increases the rate of oxidation by mushroom tyrosinase. However, it also leads to increased non-selective Michael additions. Generally, performing the reaction at 4 °C is optimal (chapter 3, Figure S10).

**Protein concentration & tag stoichiometry**: In case of C-terminally labeled antibodies, 5 mg/mL antibody and 3-5 eq. of BCN, TCO or cpTCO-bearing label results in optimal conversion. However, for N-terminal labeling of antibodies on the light chain, it was found that a significantly higher protein concentration is necessary to prevent non-selective Michael additions. This is most likely due to the tag being near the N-terminus of the heavy chain, as we found the main side-product to be 75 kDa in weight (chapter 3, Figure S10). Generally, a reaction consisting of 5 mg/mL mAb,

1.0 mg/mL mTyr and 5 eq. of BCN-bearing tag is sufficient, but increasing antibody and BCN-tag concentration may be attempted depending on the protein of interest.

**Co-solvents**: The majority of the BCN- and cpTCO-probes applied in this thesis are insoluble in water and therefore require a co-solvent such as DMSO or DMF to maintain a homogenous solution. MeOH is an option, but is more volatile and therefore concentrations of the probe stocks may be more variable. Of note, mTyr activity is reduced at increased DMSO concentration, with about 75% activity at 10% (v/v) DMSO and 40% activity at 20% (v/v) DMSO.[8]

**Tyrosinases and other oxidizing reagents**: In this thesis, only mushroom tyrosinase (mTyr) was used for the oxidation of tyrosine due to its commercial availability. Smaller tyrosinases such as monomeric mTyr[9] or tyrosinase from *Burkholderia thailandensis* (BtTyr), a faster tyrosinase capable of catalysis at lower pH,[10] can be considered. Furthermore, in the unique situation where there is only a single tyrosine present on the protein, Fremy's salt can also be considered.[11]

**Label size and type**: As shown in chapters 2–5, BCN, TCO, and cpTCO are all capable of performing SPOCQ, yielding conjugates with >95% conversion. In chapter 5, we also showed that direct conjugation of larger molecules such as proteins via SPOCQ is unfavorable. Generally, BCN is the optimal reagent for SPOCQ, although bigger probes (>5 kDa) should ideally be conjugated via two-step conjugation (chapter 5).

**Tyrosine tag size and location**: Both C-terminal and N-terminal tyrosine tags are eligible for conjugation by SPOCQ. When expressing an N-terminal $G_4$Y-tag, an additional amino acid (*i.e.* methionine) must be engineered to the very N-terminus to prevent intermolecular Michael addition when the quinone generated, otherwise leading into the formation of dopachrome compounds.[12] In many cases, expression of a $G_4$Y-tag (C-terminus) or MY$G_4$-tag (N-terminus) is enough for rapid and selective oxidation by mushroom tyrosinase. To improve reaction rate, one or two $G_4$S-spacers can be inserted to increase accessibility of the tyrosine residue (chapter 5). Finally, expressing solvent-exposed tyrosine residues in non-terminal positions has been achieved, but application of this method would require careful engineering depending on the protein of interest.

## Future Perspectives

We have shown the capability of SPOCQ for protein labeling, and shown its orthogonality to other, established protein conjugation methods. The unique qualities of SPOCQ are the rapid reaction rate under mild conditions, whilst employing an enzyme that can be extracted from the common mushroom.[13] Many improvements may still be made to the procedure, such as the use of bead-immobilized tyrosinases for easy workup.[14] Faster oxidation of the tyrosine residues can be achieved by more active enzymes such as tyrosinase from *Burkholderia thailandensis* (BtTYR),[10] potentially capable of full conversion within minutes.

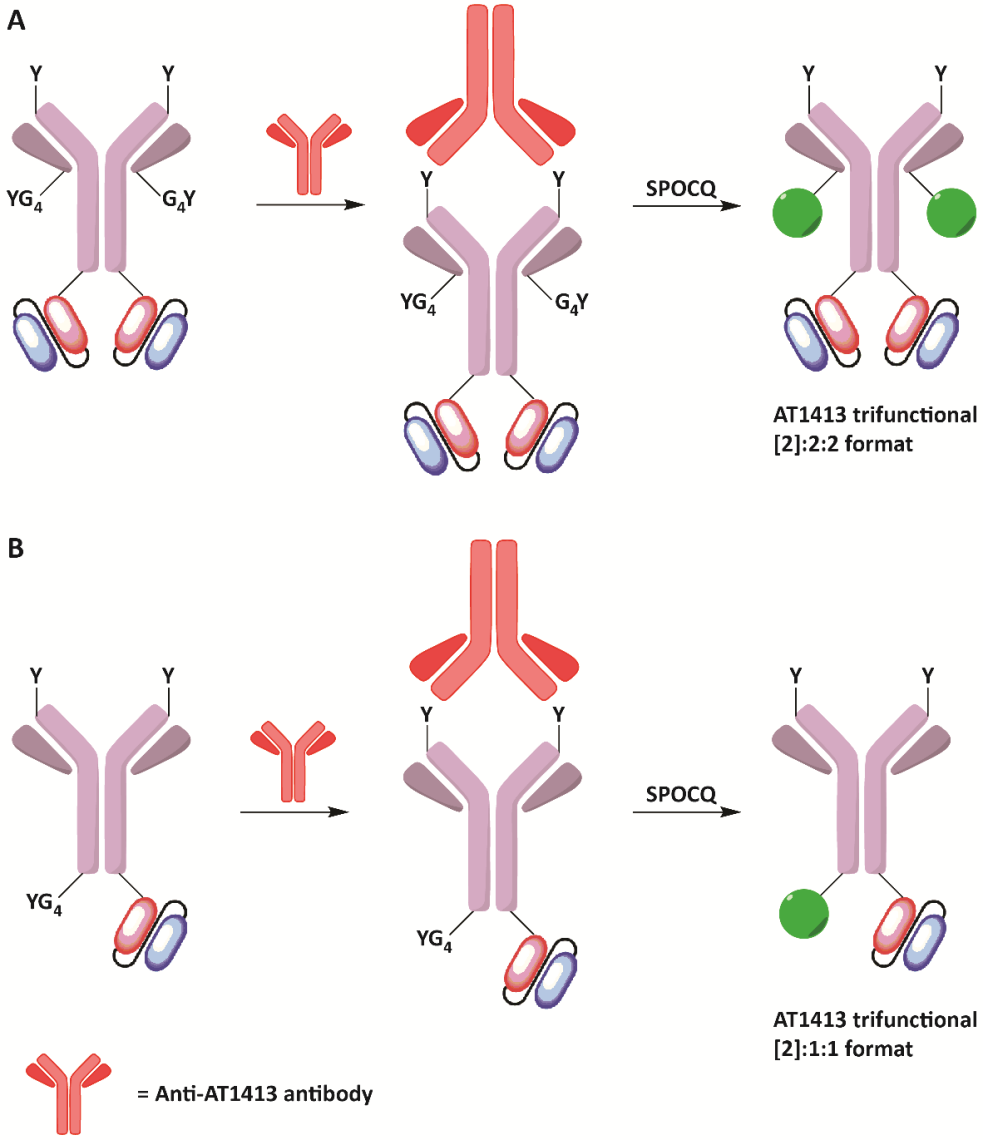



**Figure 3**. Protection of CDR-H3 tyrosine by anti-AT1413 antibody (AIA) to generate (A) [2]:2:2 trifunctional constructs and (B) [2]:1:1 trifunctional constructs.

Of more relevance is to consider potential fields of application of SPOCQ, such as the production of antibody-drug conjugates (ADCs), radiolabelled antibodies, and even trispecific antibodies. The latter trispecific antibodies were effectively generated in chapter 5, but not evaluated for biological activity yet. Specifically, we generated trifunctional variants of the antibody AT1413,

an antibody targeting a sialylated epitope on CD43,[15] a transmembrane cell surface protein expressed on all cell lines of acute myeloid lymphoma (AML).[16] For functionalization, we employed the scFv of UCHT1, an α-CD3 antibody,[17] and cytokine IL-2,[18] known to increase T-cell recruitment and activation, respectively. As hypothesized in chapter 5; by combination of SPOCQ and sortase to conjugate UCHT1 and IL-2, a system would be generated capable of efficiently recruiting and activating the immune system, which as a concept obviously could be extended to other antibodies. Interestingly, SPOCQ on a $G_4$Y-tag on AT1413 was found not to be selective, due to the presence of a cystine knob containing a tyrosine residue in the CDR-H3 loop (chapter 6).

However, by blocking the CDR with an anti-idiotypic antibody, effectively functioning as a temporary protective group for the tyrosine-bearing cystine knob, the $G_4$Y-tag expressed on the light chain could be selectively modified (chapter 6, Figure S1). This was achieved with only a one equivalents of AIA compared to AT1413, resulting in a 1:1 binding of AIA to AT1413 (Figure 3). To the best of our knowledge, the use of antibodies for protection of a protein region during bioconjugation at a distinct site is unprecedented and warrants further investigation. For example, it may pave the way for generation of the AT1413–UCHT1–IL-2 trifunctional antibody, either in a [2]:2:2 format or a [2]:1:1 format (Figure 3).

The generation of solvent-exposed loops for internal tyrosine expression (chapter 6) potentially presents an avenue towards stable SPOCQ conjugates not conjugated at either protein terminus. However, stability turned out to be an issue when a peptide tag containing 12 amino acids was introduced in trastuzumab. By introducing cystine knobs in various solvent-exposed loops on an antibody, a tyrosine residue might be introducible without hampering the antibody stability. For example, Grünewald *et*. *al*. reported several stable conjugation sites after insertion of a peptide tag,[19] therefore introduction of a "CG**Y**SSC" peptide tag might provide internal tyrosine residues for conjugation without hampering antibody stability.

From a commercial standpoint, using mushroom tyrosinase, an enzyme that can be gathered from the common mushroom, would allow for cheap fabrication of antibody conjugates. Other proteins beside antibodies are also candidates for modification by SPOCQ. By expressing a $G_4$Y-tag on either terminus of enzymes, introduction of a chemical moiety for immobilization of enzymes onto supports such as nanoparticles, microbeads, or surfaces can be achieved.[20-23] This approach allows for improved rate of catalysis, recyclability, enzyme stability, and easier product purification for many industrial processes.[24-26] Therapeutic proteins can also be modified with SPOCQ for site-specific PEGylation with little effort for an enhanced stability of the protein, and longer in vivo half-lives.[27]

Tyrosine oxidation is also applicable to proximity based labeling. By introducing the photoreactive moiety diazirine (Figure 4A) via SPOCQ on an N-terminal tyrosine tag, proximity-

based labeling can be insured after the antibody binds the target (Figure 4B).[28] In the case of antibodies with an exposed tyrosine residue on the CDR-H3, as with AT1413, this can be more easily achieved via the selective oxidation to the DOPA-moiety via mTyr and ascorbic acid.[29] The DOPA-moiety can then selectively be oxidized to the quinone by electrochemistry[30] or sodium periodate,[7] allowing for nucleophilic amino acids to perform Michael addition on the CDR-located quinone (Figure 4C). By doing either the photoaffinity labeling, or the electrochemistry labeling, detailed information about the epitope of these antibodies can be achieved.

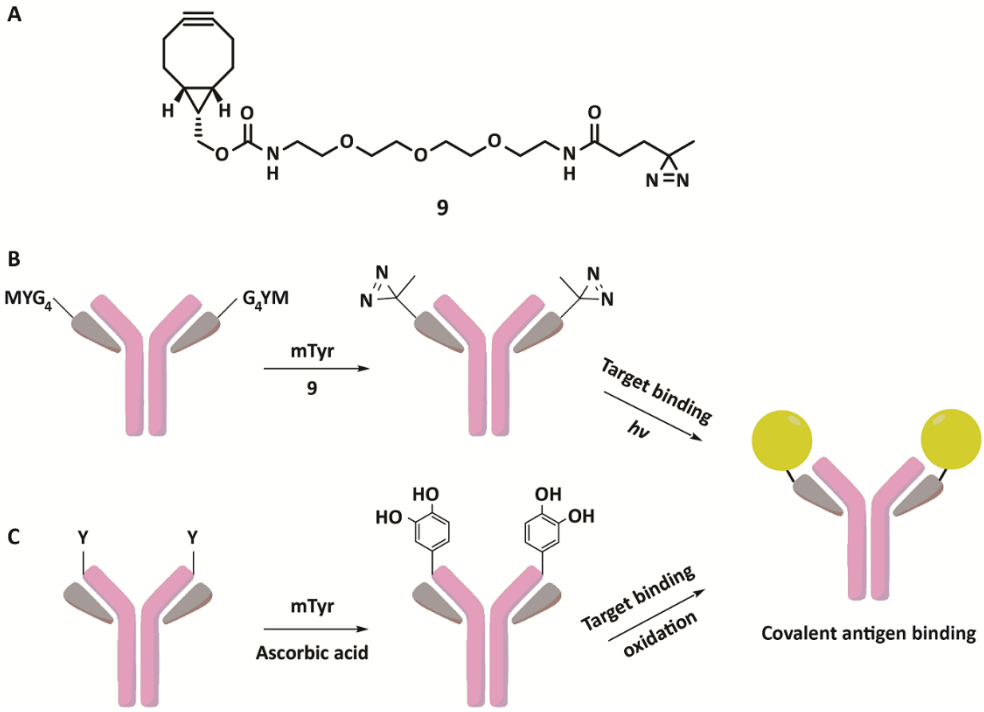



**Figure 4**. (A) Proposed structure of BCN-diazirine bifunctional molecule (**9**). (B) Covalent antigen binding via introduction of diazirine and subsequent photolabeling. (C) Covalent antigen binding via DOPA-formation and subsequent oxidation.

Altogether, there are many applications for SPOCQ to contribute in the field of bioconjugate chemistry. Its mild conditions and selective nature allows for the modification of proteins in an inducible fashion, and it has proven capable of creating conjugates for the next generation of antibody-drug conjugates and immunotherapies.

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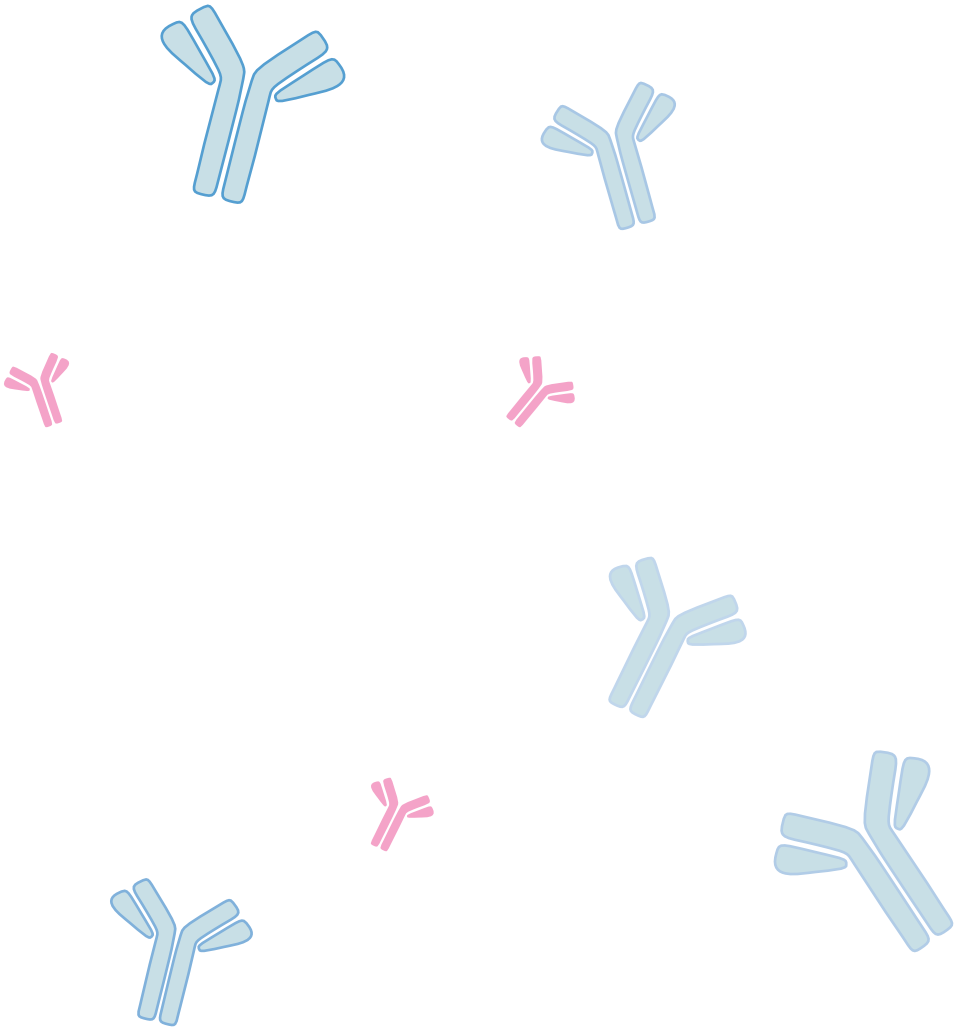
# Appendix

# Inducible, Selective Labelling of Proteins *via* Enzymatic Oxidation of Tyrosine



**Abstract**

*Proteins can be labelled site-specifically and in inducible fashion by exposing a small peptide tag ($G_4Y$) on any of its termini and activating the newly exposed tyrosine residue with the enzyme mushroom tyrosinase. The enzyme generates a quinone by oxidizing the tyrosine, which in turn can perform strain-promoted oxidation-controlled ortho-quinone cycloaddition (SPOCQ) with strained alkynes and alkenes, generating a stable conjugation product. Here, we describe a protocol to perform SPOCQ reaction on proteins, along with notes to optimize yield and reaction rates. Conjugation efficiencies of over 95% to antibodies have been reported using this protocol.*

**This work was published as:**

Bruins, J. J., van de Wouw, C., Keijzer, J. F., Albada, B., and van Delft, F. L. (2019) Inducible, Selective Labeling of Proteins via Enzymatic Oxidation of Tyrosine, in *Enzyme-Mediated Ligation Methods* (Nuijens, T., and Schmidt, M., Eds.) pp 357-68, Springer New York, New York, NY.

## Introduction

Strain-promoted oxidation-controlled *ortho*-quinone cycloaddition (SPOCQ) is a reaction based on the facile (4+2) cycloaddition of an *ortho*-quinone (also known as 1,2-quinone) with a strained alkyne or strained alkene (Figure 1).[1-3] Within the class, cyclopropanated versions of cyclooctyne (bicyclo[6.1.0]nonyne, BCN).[2, 4] and trans-cyclooctene (cpTCO)[3, 5] were found to undergo particularly fast cycloaddition, similar to accelerated cycloaddition with azido groups, by virtue of the additional strain imparted by the fused cyclopropane ring.

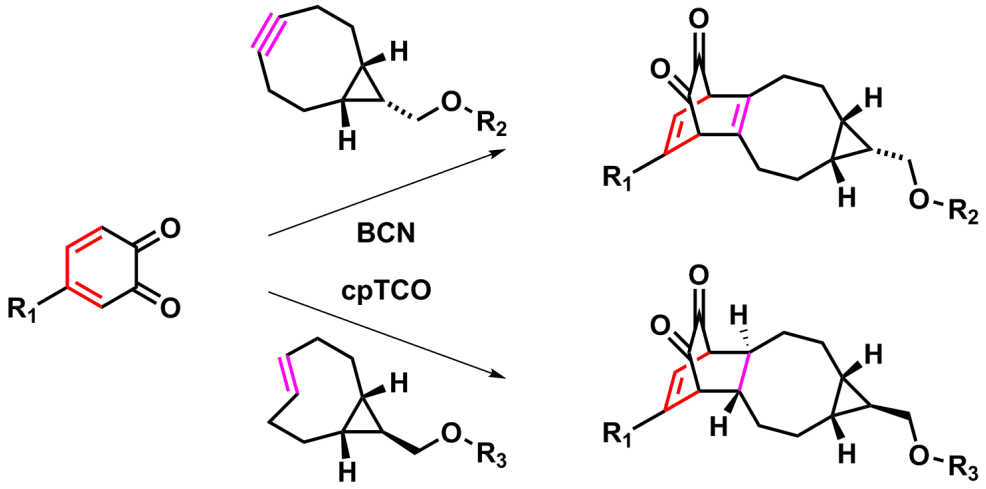



**Figure 1.** Concept of SPOCQ of an ortho-quinone with strained alkyne BCN or strained alkene cpTCO.

While of general interest to the field of metal-free click chemistry, a particularly useful aspect of an *ortho*-quinone is that it can be readily generated from a (substituted) phenol group by oxidation. Moreover, as the phenol moiety is present in the canonical amino acid tyrosine, *in situ* generation of an *ortho*-quinone moiety provides an excellent opportunity for application in the field of protein labelling. However, it is also apparent that global oxidation of tyrosine side-chains in a protein, for example by means of Fremy's salt, will result in a heterogeneous product with varying amounts of conjugated label. One elegant approach to site-selective labelling entails the genetic encoding of L-DOPA (3,4-dihydroxyphenylalanine) followed by mild selective oxidation.[6] However, genetic encoding of non-canonical amino acids often provides relatively modest protein titers.

A promising approach to directed protein labelling based on canonical amino acids involves the introduction of an exposed tyrosine residue in a *C*- or *N*-terminal peptide fusion, *e.g.* tetra-glycyltyrosine ($G_4Y$), which enables the chemoselective oxidation of the exposed tyrosine moiety exclusively upon exposure to the enzyme mushroom tyrosinase (mTyr).[7, 8] Thus, in the presence

of oxygen, tyrosine is first oxidized to the corresponding 3,4-dihydroxyphenyl derivative, and subsequently to the *ortho*-quinone (Figure 2). Since the active site of mushroom tyrosinase is embedded in a large protein structure (120-130 kDa), endogenous tyrosine residues are typically not a suitable substrate for mTyr, resulting in selectivity for exposed tyrosine residues, such as the one located on an engineered $G_4Y$-tag. While the $G_4Y$-tag can be fused conveniently to the protein's *C*-terminus as such, it is recommended to add an additional amino acid in case of *N*-terminal fusion (*e.g.* methionine, leading to $MYG_4$) as the *in situ* generated quinone may otherwise undergo intramolecular nucleophilic addition of the terminal amino group, thereby forming a stable 5-membered ring.[9]

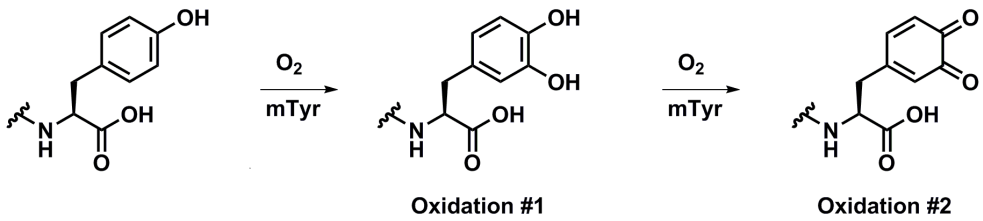



**Figure 2**. Oxidation of the side-chain of a *C*-terminal tyrosine residue under the action of mushroom tyrosinase (mTyr) in the presence of oxygen provides ortho-quinone in two consecutive steps.

The electrophilic nature of quinones and their tendency to undergo Michael addition has been widely reported.[9] For example, the free thiol residue of cysteine can react rapidly with *ortho*-quinones, which also applies to amino groups present on lysine side-chain or at the protein *N*-terminus, or the imidazole ring of histidine. Obviously, any such nucleophilic addition must be fully nihilated or outcompeted in case SPOCQ is desired.[9]

As mentioned above, strained unsaturated C-C bonds, like the triple bond in BCN and the double bond in cpTCO, readily undergo SPOCQ (Figure 1).[2, 3] It must be noted that an additional advantage of BCN is its ability to rapidly undergo cycloaddition with azides in a process known as SPAAC (strain-promoted alkyne-azide cycloaddition), in contrast to cpTCO which is inert to azides.[10] As a result, dual labelling of proteins containing both an azide (*via* SPAAC) and an exposed tyrosine (*via* SPOCQ) is also possible.[3] However, the scope of this chapter is limited to the use of SPOCQ for protein conjugation, as this method allows for easy, selective and nearly quantitative introduction of a range of functionalities of choice based on the canonical amino acid tyrosine. Here, we exemplify the usage of SPOCQ by modifying anti-HER2 antibody trastuzumab with BCN- or cpTCO-bearing fluorophores by oxidizing C-terminal tyrosine residues with mTyr.

## Materials

Prepare stock solutions of buffers in 18 MΩ·cm water (MilliQ). Protein stock solutions can be stored for short-term usage at 4 °C, or for long-term storage at –80 °C. Specific cpTCO-probes and BCN-probes should be stored long-term at –20 °C in dimethyl sulfoxide (DMSO), N,N-dimethylformamide (DMF) or N,N-dimethylacetamide (DMA). BCN containing an alcohol functionality (BCN-OH; CAS 1263166-90-0) can be synthesized[4] or purchased from commercial vendors. Functionalities can be introduced *via* standard organic chemistry methods. cpTCO containing an alcohol functionality (cpTCO-OH) can be synthesized[5, 11] and functionalities can also be added *via* standard organic chemistry methods. Mushroom tyrosinase can be purchased from several vendors (CAS Number 9002-10-2).

## Materials - SPOCQ reaction

1. Phosphate Buffered Saline (PBS) buffer: 50 mM sodium phosphate,150 mM NaCl, pH 5.5 (*see* **Notes 1,2**).
2. Phosphate buffer: 50 mM sodium phosphate, pH 6 (*see* **Note 2**).
3. Protein solution: Protein of interest constituted in PBS buffer (*see* **Notes 3**) (*e.g.* trastuzumab containing a $G_4$Y-tag on its heavy chain; Tras[HC]$G_4$Y).
4. Stock solution of cpTCO-probe: ≥0.4 mM solution of any cpTCO probe (with attached fluorophore, toxin or other molecule; *e.g.* cpTCO-Lissamine) in DMSO (*see* **Notes 4–6**).
5. Stock solution of BCN-probe: ≥0.4 mM solution of any BCN probe (with attached fluorophore, toxin or other functional group; *e.g.* BCN-Lissamine) in DMSO (*see* **Notes 4–6**).
6. Mushroom tyrosinase solution: 0.5–10 mg/mL of mushroom tyrosinase in 50 mM phosphate buffer, pH 6 (*see* **Note 7,8**).
7. A tabletop thermoshaker for controlling the reaction temperature (*e.g*. Eppendorf ThermoStat™ C).

## Materials - Purification

1. Amicon® ultra-spin filtration units with the appropriate molecular weight cut-off (MWCO) for the selected protein (*see* **Note 9**).
2. Phosphate Buffered Saline (PBS) buffer: 50 mM sodium phosphate, 150 mM NaCl, pH 7.2
3. For further purification, depending on the protein of interest, affinity based columns can be used, *e.g.* Protein A columns for antibodies, Nickel-NTA columns for proteins with a histidine tag.

### Materials - SDS-PAGE

For SDS-PAGE (polyacrylamide gel electrophoresis) analysis a suitable system is required, *e.g.* Bio-Rad Mini-PROTEIN® Tetra Vertical Electrophoresis Cell. Either cast SDS-PAGE gels by hand using Bio-Rad protocol bulletin 6201 or purchase pre-cast gels from commercial vendors. Bio-Rad dual colour ladder standard is an advised reference protein ladder.

1. Sample buffer (2x) SB: Mix 4 mL glycerol (100%), 0.66 mL 1.0 M *Tris(hydroxymethyl)aminomethane (*Tris).HCl pH 6.8, 0.2 gram sodium dodecyl sulfate (SDS), 1 mg bromophenol blue and 5.44 mL MilliQ. Before use, add β-mercaptoethanol (BME) for the reduction of disulphide bonds (19:1 (v/v) SB:BME).
2. Staining solution: dissolve 1 g of Coomassie Brilliant Blue R-250 in 1 L solution of 5:4:1 (v/v/v) methanol (MeOH):water:acetic acid (AcOH).
3. Destaining solution: 5:4:1 (v/v/v) MeOH:Water:AcOH.
4. SDS-gel scanner, *e.g.* BioRad ChemiDoc™ system.

### Materials - HPLC/MS

High-performance liquid chromatography (HPLC) (and LC-MS) analysis can be used for the analysis of products obtained from the SPOCQ reaction, though protocols can vary for each different protein with regards to the used machine, eluents and columns. A full overview is beyond the scope of this protocol, only antibody analysis will be described in detail as an example.

1. HPLC system, *e.g.* Agilent 1220 HPLC Infinity system with in-line diode-array detector (DAD) (*see* **Note 10**).
2. RP-HPLC column for antibody analysis, *e.g.* ThermoScientific™ MAbPac™ reversed phase (RP) column (3.0 x 50 mm, 4 μm) column.
3. HPLC buffer A: 95% MiliQ, 5% MeCN with 0.1% trifluoroacetic acid (TFA).
4. HPLC buffer B: 95% MeCN, 5% MiliQ with 0.1% TFA.
5. Tris base for DTT denaturation: 0.1 M tris base buffered to pH 8.0 with 1.0 M HCl.
6. Freshly prepared 0.2 M dithiothreitol (DTT) in 0.1 M tris pH 8.0.

### Methods - SPOCQ

SPOCQ should be performed at low temperature (4 °C), preferably in PBS buffer pH 5.5 and can be performed at any volume necessary. Consider the final volume to be 10 volume equivalents.

1. Add 8 volume equivalents of 10-200 μM of a *C*-terminal $G_4Y$-expressed protein in PBS (pH 5.5) to a 1.5 mL Eppendorf tube and cool to 4 °C.

2. Add 1 volume equivalent of 0.4-4.0 mM label (*e.g.* BCN covalently attached to a polyethylene glycol (PEG) group (BCN-PEG)); cpTCO covalently attached to a lissamine group (cpTCO-lissamine) in DMSO (resulting in 3-10 equivalents of label compared to $G_4Y$) and mix properly by pipetting up and down 3 times. (*See* **note 3)**.
3. Finally, add 1 volume equivalent of 0.5-10 mg/mL mushroom tyrosinase (resulting in 2.5-10 mol % mushroom tyrosinase) and mix properly by pipetting up and down 3 times (*See* **notes 11, 12**).
4. After the appropriate reaction time (0.5-5 hour), the reaction is available for purification, workup (*e.g.* Dithiothreitol (DTT) denaturation) and/or analysis (*e.g.* HPLC, LC-MS) (*See* **note 13**).

Example: 100 µL reaction of labelling Trastuzumab containing a $G_4Y$-tag on its heavy chain (Tras[HC]$G_4Y$) with fluorophore cpTCO-lissamine[3]

1. Add 80 µL of 10 mg/mL (67 µM) Tras[HC]$G_4Y$ solution (in PBS, pH 5.5) to a 1.5 mL Eppendorf tube and cool to 4 °C in a thermostat machine.
2. Add 10 µL of a 5 mg/mL (5.8 mM) cpTCO-lissamine stock solution to the Tras[HC]$G_4Y$ solution and mix the sample by pipetting up and down 3 times.
3. Add 10 uL of the 10 mg/mL mushroom tyrosinase solution to the mixture and mix the sample by pipetting up and down 3 times.
4. React the sample for 1.5 h at 4 °C.
5. Purify the sample according to section 5.2 and analyse using SDS-PAGE (section 5.3) or HPLC (section 5.4).

In this example, the Tras[HC]$G_4Y$ had a final concentration of 8 mg/mL (53 µM), the mushroom tyrosinase a concentration of 1.0 mg/mL (1000 units/mL), and the label a final concentration of 0.5 mg/mL (580 µM). This would equal 10.9 equivalents of label, but because the symmetrical antibody has two $G_4Y$ tags, the excess label is halved to 5.5 equivalents. This method is generally applicable for any protein with the aforementioned tyrosine tag.

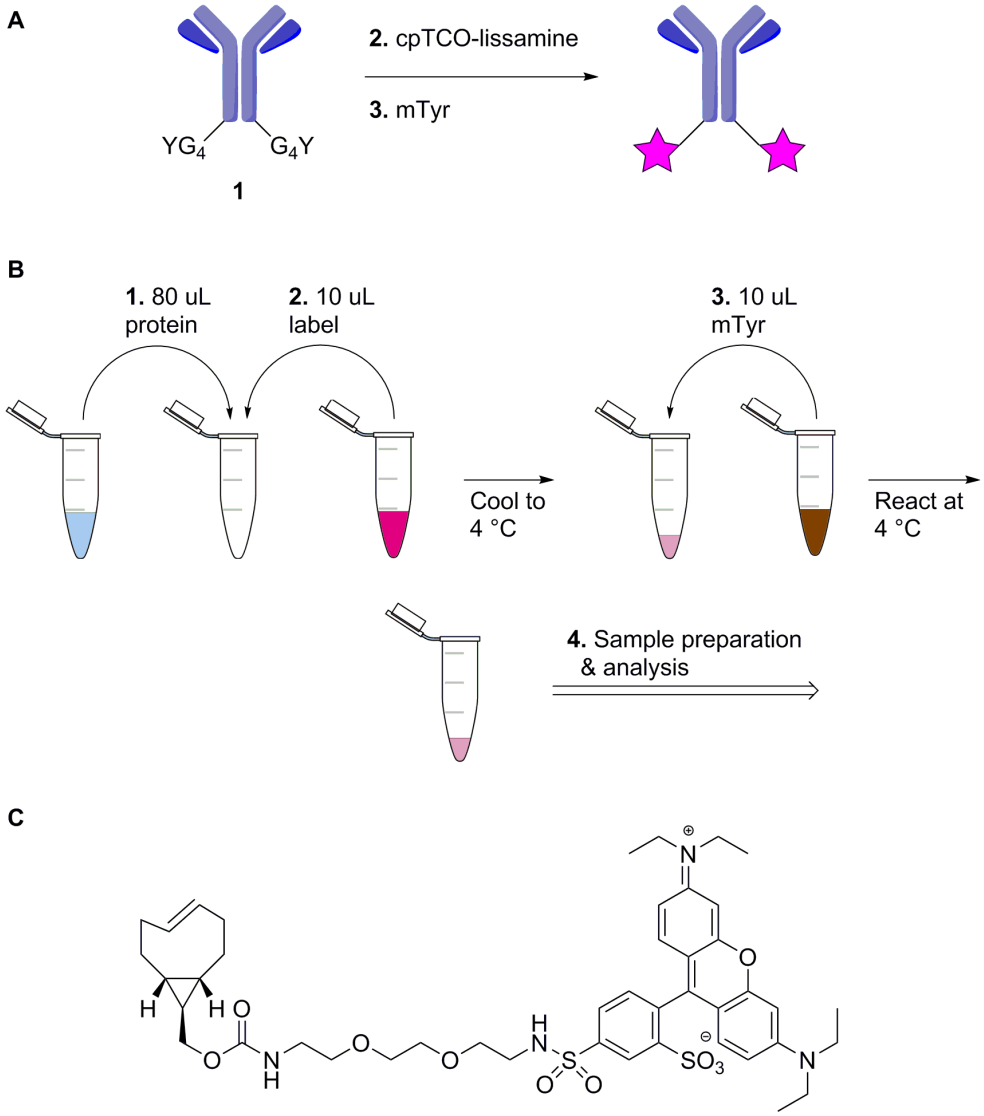



**Figure 3**. (A) Example reaction, Tras[HC]G4Y labelling with cpTCO-lissamine, (B) Simplified overview of the practical steps to perform SPOCQ with 100 µL reaction volume, (C) Structure of cpTCO-lissamine.

## Methods - Purification

To prepare the sample for further use, size-exclusion and subsequent purifications can be performed.

Size-exclusion is most easily performed using Amicon® ultra-spin filtration units with the appropriate MWCO (*see* **Note 9**). This will remove any residual label and allows for concentration of the sample and/or buffer exchange.

1. Pre-wash the ultra-spin filtration unit by adding 500 µL of the buffer of choice (*e.g.* PBS pH 7.2) and centrifuge at 14.000 x g to a final volume of 25-50 µL.
2. Pipet the sample into a pre-washed 0.5 mL ultra-spin filtration unit and dilute to 500 µL.
3. Centrifuge at 14.000 x g to a final volume of 25-50 µL. The time depends on the MWCO. Please consult the specifications of the selected spin filtration unit.
4. Wash and exchange buffer to the buffer of choice by diluting to 500 µL with the buffer of choice and concentrate by centrifuging identical to step 2. Repeat this step twice to a total of three times and concentrate by centrifugation until the desired concentration is achieved. Determine concentration *via* UV/Vis spectrophotometry.
5. Further purification can be performed, *e.g.* to remove mushroom tyrosinase *via* a nickel-NTA column (if a histidine-tag is available), or in the case of an antibody, protein A affinity chromatography. For small volumes, ThermoFisher Scientific NAb™ Protein A Plus Spin Columns and comparable products are suitable.

### Methods - SDS-PAGE

General SDS-PAGE workup for SPOCQ performed on antibodies (See **note 14**):

1. Prepare acrylamide gel according to standard protocol from Bio-Rad or other source.
2. Dilute 5 µg of antibody solution 1:1 with SDS-PAGE sample buffer SB (2×) including 5% BME.
3. Heat for 5 minutes at 95 °C.
4. Apply the denatured sample for SDS-PAGE analysis (12% acrylamide gel; with Bio-Rad dual colour standard is an advised reference protein ladder).
5. If a fluorescent probe is added, measure fluorescence with the required equipment (*e.g.* BioRad ChemiDoc™ systems).
6. Stain SDS-PAGE gel with staining solution for 30 minutes.
7. Destain SDS-PAGE gel with destaining solution for 60 minutes.
8. Re-equilibrate SDS-PAGE gel in demiwater for at least 2 hours. Analyse gel with a scanner or gel documentation system.

Example: After performing the SPOCQ reaction according to the procedure described in section 3.1, SB + BME denaturation and SDS-PAGE analysis can be performed as described earlier. After the SPOCQ reaction, the heavy chain band of Tras[HC]$G_4$Y at 50 kDa shifts up slightly (lane II and III) and can be measured to have fluorescence (absorbance at λ= 565 nm and excitation at λ=

586 nm for Lissamine, *i.e.* sulphorhodamine B; Figure 4A). No labelling of the light chain is detected.

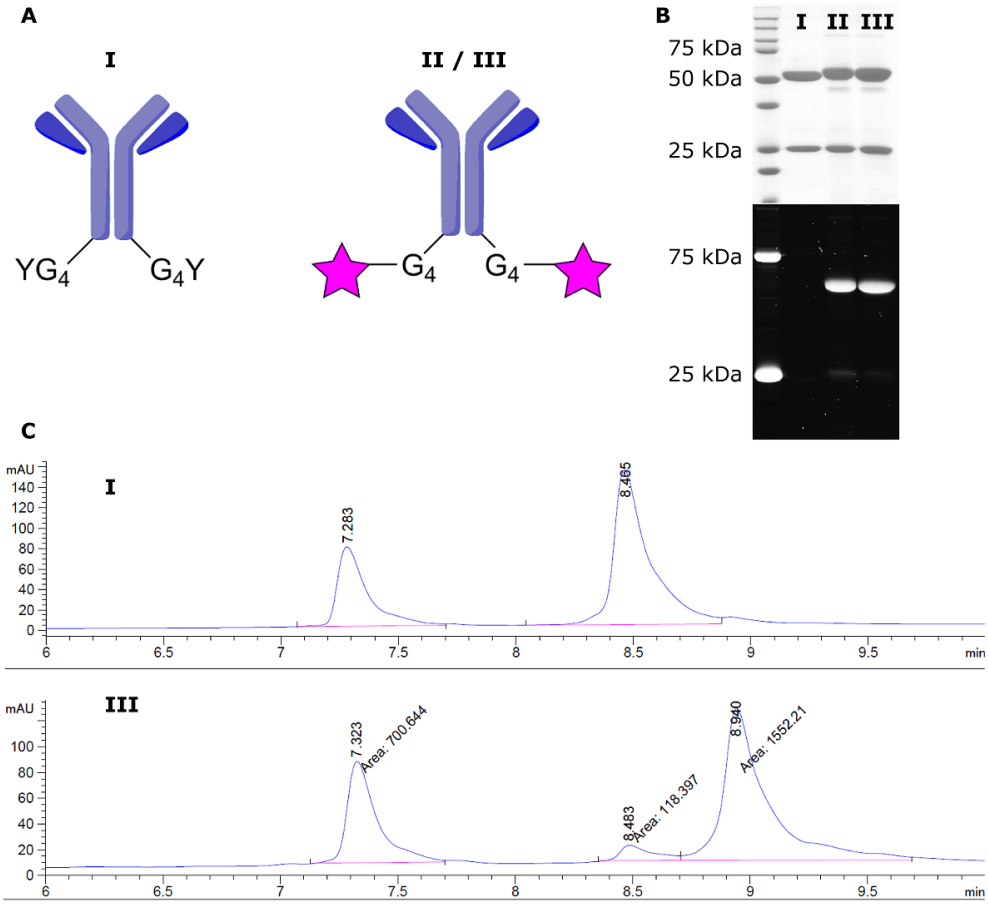



**Figure 4**. (A) Schematic representation of Tras[HC]G4Y before (**i**) and after (**ii**) labelling with BCN-Lissamine or (**iii**) labelling with cpTCO-Lissamine. (B) SDS-PAGE analysis and (C) HPLC analysis after the SPOCQ reaction performed according to protocol.[i]

## Methods - HPLC Analysis

General HPLC (or LCMS) workup for SPOCQ performed on antibodies (See **note 14**):

[i] *Bruins, J. J., Blanco-Ania, D., van der Doef, V., van Delft, F. L., and Albada, B., Orthogonal, dual protein labelling by tandem cycloaddition of strained alkenes and alkynes to ortho-quinones and azides. Chem. Commun.* ***2018****, 54 (53), 7338-41.* ***Reproduced by permission of The Royal Society of Chemistry.***

1. Prior to HPLC analysis, freshly prepare a 0.2 M DTT solution in 0.1 M Tris pH 8.0.
2. Dilute antibody samples to 1 µg/µL antibody and add 2.5 µL DTT stock solution per 10 µg antibody.
3. Incubate the mixture for 30 minutes at 37 °C in order to reduce the interchain disulfide bonds. After which the reaction was quenched by adding 30 uL of a mixture of 49:49:2 (v/v/v) MilliQ:acetonitrile (MeCN): FA per 10 µg antibody.
4. HPLC analysis was performed by injecting 10-25 µL of reduced antibody. Analysis was performed with a flow rate of 0.6 mL/min at 80 °C using a 10 minute linear gradient from 20% to 45% buffer B (with buffer A = 95% MilliQ, 5% acetonitrile, 0.1% trifluoroacetic acid (TFA) and buffer B = 95% acetonitrile, 5% MilliQ, 0.1% TFA).
5. Protein absorption spectra were measured at λ= 215 nm and λ= 280 nm. Another wavelength can be selected if the attached probe has a specific absorbance (*e.g.* λ= 586 nm for Lissamine *i.e.* sulphorhodamine B).

Example: After performing the SPOCQ reaction on Tras[HC]$G_4$Y according to the procedure described in section 3.1, DTT denaturation and HPLC analysis can be performed as described in section 2.3.2. This results in a shift in retention time for the heavy chain from 8.47 min to 8.94 minutes (Figure 4C) in case of a conjugation of BCN-lissamine to Tras[HC]$G_4$Y.

## Notes

1. Generally, PBS is used for the SPOCQ reaction, which consists of 50 mM sodium phosphate (monobasic) and 150 mM of sodium chloride and is buffered to the desired pH using 1.0 M NaOH. Alternatively, other non-nucleophilic bases such as HEPES or MES can be used.
2. While pH 5.5 is optimal, SPOCQ has been performed at pH ranges from 5.5–8.0 (mushroom tyrosinase is deactivated below pH 5.0).[12] Lowering the pH to 5.5 will increase the yield due to a higher level of protonation of the lysine, cysteine and histidine side-chains, which lowers their reaction rates.
3. It is advised to have the protein stock solution fairly concentrated (100–200 µM), with 10 µM as a minimum concentration. Stock solutions can easily be diluted with PBS before reaction if necessary. Some proteins might require a higher concentration to obtain higher yields, as higher concentrations help outcompete intramolecular Michael additions by nucleophilic amino acid residues. A SPOCQ reaction consisting of 50–200 µM protein with 3–10 equivalents of cpTCO or BCN generally results in optimal conversion. If multiple $G_4$Y tags are present on the protein, the equivalents of cpTCO or BCN are calculated versus the amount of $G_4$Y-tags present. For example; two $G_4$Y-tags are present on the Tras[HC]$G_4$Y in section 5.1, and 10 equivalents of cpTCO-lissamine compared to the antibody results in 5 equivalents per $G_4$Y-tag.[3]

4. Either cpTCO-bearing or BCN-bearing probes can be used for SPOCQ. For encompassing all possible variations, we simply call them probes. Probe activity can be tested with mass spectrometry, NMR or other methods. Chemical testing of probe activity can be performed by dissolving *tert*-butyl quinone in a mixture of 1:1 (v/v) MeOH/water, then adding a slight excess of BCN or cpTCO label. The yellowish colour should fade within seconds.
5. Due to the fact that not all probes dissolve well in water, it is advisable to maintain a concentration of approximately 10 vol.% DMSO. Therefore, different protein concentrations might require different concentrations of functional probes to be added. It is advisable to make a stock solution of maximum concentration. As is the case with the protein, more concentrated stock solutions of probes are also advisable.
6. Generally, DMSO is chosen as the co-solvent for the reaction. While it is not necessary for the reaction to proceed, it improves the solubility of more apolar probes. DMF or DMA can also be used as a co-solvent and likely other non-reactive solvents can be used (*e.g.* propylene glycol). However, a high concentrations of co-solvent (>20%) can affect mushroom tyrosinase's reaction rate and stability.[13] In the case of water-soluble probes the co-solvent can be omitted altogether.
7. Mushroom tyrosinase should be stored in buffer without sodium chloride, due to chloride ions slowly inhibiting its catalytic activity.[12] Generally, phosphate buffer is used, which consists of 50 mM sodium phosphate (monobasic) adjusted to the desired pH using 1.0 M NaOH. Mushroom tyrosinase activity can be tested by dissolving L-tyrosine in 9:1 (v/v) water/DMSO, followed by adding some of the mushroom tyrosinase stock solution. The mixture should turn visibly brown within a few minutes.
8. Mushroom tyrosinase can be purchased from Sigma-Aldrich and can be used without any purification, *i.e.* 10 mg/mL of mushroom tyrosinase corresponds to 10 mg of lyophilized enzyme dissolved in 1 mL buffer.
9. Generally select a membrane with a MWCO that is 2-3 times smaller than the molecular weight of the protein.
10. A mass spectrometer can be attached to this system to allow LC-MS. In that case, TFA is swapped out for formic acid (FA) for the two HPLC buffers as TFA can interfere with the MS signal.
11. Mushroom tyrosinase should be added to the reaction last, since the oxidation is initiated upon addition, since the presence of reactive quinone species can lead to side-reactions with the side-chains of lysine, histidine and cysteine residues.
12. Mushroom tyrosinase stoichiometry is important for the reaction rate. Although ~1% of enzyme is sufficient for complete conversion, generally 5–10% of mushroom tyrosinase is added to limit the reaction time and minimize side-reactions.
13. The reaction time of SPOCQ varies significantly from protein to protein, depending on the accessibility of the $G_4Y$-tag for the oxidation by mushroom tyrosinase. It is advised to execute a time-resolved reaction series and measure the conversion by HPLC or SDS-PAGE.

It is possible that the installed tyrosine tag might be poorly accessible for mushroom tyrosinase, it is then advised to elongate the tag with an additional $G_4S$-spacer sequence preceding the $G_4Y$-tag, effectively yielding a $G_4SG_4Y$-tag. This can be repeated with several $G_4S$ units to further increase the spacing and improve the steric accessibility. This can lead to increased reaction rates and improved overall conjugation efficiencies.

14. Depending on the size of the protein a different amount of protein may be used for denaturation, as well as a different acrylamide concentration and protein ladder. Depending on the protein stability, different incubation times may be applied. The same holds true for DTT denaturation and subsequent HPLC analysis. Different denaturation times and different columns might be necessary.

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# Acknowledgements

Allright, here we go. Over the last four years so many people, from both inside and outside academia, have made a huge impact on my life. I cannot express my gratitude for you enough, I would need a second booklet for that. But, let's give it a shot.

First my promotor, Floris. I am incredibly glad I did this PhD under your supervision. You ensured that I was always critical of my work, both in quality and quantity. I valued our weekly brainstorm sessions where we came up with dozens of ideas, not many PhD students have that luxury. What I appreciate most however, is that you always listened to my ideas, suggestions, and advice. This helped me so much in becoming very invested in my work, and I have not been as proud about my work as I did during my PhD. Thank you for that, sincerely.

My co-promotor, Bauke. We 'adopted' you on our project just a few months in, and I could not have asked for a better daily supervisor. Countless times you have given me invaluable advice about research, and anything related to being a researcher. Your open-door policy, which I might have used a bit too much, meant that I always had somebody to discuss problems and opportunities with. I hope the follow-up project with Yuri (good luck man!) will be just as fruitful, pleasant and educational!

About talking too much, office 8038 & lab 8039… "Het sfeer in dit kantoor" was generally a sentence used for when things got downright silly, which were the best of times. My paranyphms Ian and Jordi, and the other office-mates Daniele, Dieuwertje, Jorin, Sjoerd, and Tjerk, thank you for the help, discussions, and allowing me to ventilate when something incredibly silly bothered me way too much. It truly was the right amount of crazy for me. I also want to explicitly thank Judith for helping me with so many lab and teaching issues, as well as joining in on 'ff lekker klagen over dingen".

My fellow PhD'ers and post-docs. Alexandre, Alyssa, Ana, Andrada, Andrii, Annemieke, Ariadni, Bas, Caroline, DJ, Dongdong, Ellen, Esther, Esther, Fatima, Florine, Fred, Ghazal, Gina, Jay, Jorge, Kaustub, Kushal, Lucas, Medea, Michel, Milou, Nathalja, Pepijn, Qiang, Rafael, Rick, Rui, Satesh, Sevil, Si, Simon, Sidhu, Steffi, Sybren, Swecha, Vincent, Wei, Wilco. Thank you for the Karaoke-nights, game-nights, go-karting, dinners, coffee-runs @ plaza, October fests, PhD-trips, partying at chains, tour- and footballpoules, FSB-borrels, maternity/paternity visits, Efteling visit, friday lunches, and sooo much more. You guys rock!

No words can describe the amount of patience and friendliness the staff has with us PhDs. Aleida, Bep, Elly, Esther, Meta, thank you for helping me with literally ANY problem I had, be it from asking how a TSP works, salary questions, or making sure I am mistaken for Pepijn. ☺ Performing research would be impossible without Barend, Elbert, Frank, Hans, Henny, Jelmer, and Pepijn, as

we would have exactly 0 working equipment, let alone the expertise to run said equipment. Thank you for keeping the department afloat! I would also like to thank the rest of the staff. Ai, Albert, Anne-marie, Carel, Cees, Erik, Hans-Gerd, Gert, Hendra, Jacob, Louis, Maarten, Maurice, Michel, Teris, and Tom, as well as the Surfix-people for all the good discussions and pleasant conversations. I want to give a final big thank you to head of our department, Han, for always trying to get the best out of us and our research!

My students, Criss, Eduard, Mirthe, Robin, Vincent and Wouter. I wish I could have put all your work in this thesis. Unfortunately, not every project is destined to succeed, despite your endless input, patience, and perseverance. I am grateful for your enthusiasm, honesty, your desire to learn, and the lessons you taught me on the way. Sometimes I might have been a bit strict, but please know that I wish nothing but the absolute best for you guys (and gal)!

There were also loads of people outside of ORC helping me, starting with Koen and Lina. I really liked our bimonthly(-ish) discussions, and I have learned a lot from you guys in fields I was fully unaware of before this journey. I am, however, grateful that we do not have to make any more shared posters/presentations anymore! ☺ Thank you, and I am looking forward to your defence Lina!

I would also like to thank Daniel from the Radboud University for the nice collaboration on our cpTCO-paper, it really was a smooth project from start to finish! Also a big thank you to the amazing people from SynAffix, who helped me before, during and even after my PhD. A special thanks to Remon for explaining me many antibody-related issues, as well as helping me with analysis on multiple occasions! A final thank you to Adrie and Willem from the Wageningen biochemistry department. Your help early in my PhD really helped me understand how to handle proteins and the analysis thereof, and we still use some of your equipment with lots of gratitude!

Besides having amazing people at work, I am also blessed with having the best people outside of WUR:

Ik heb gelezen… dat ik de beste vrienden in de wereld heb. Ik wil jullie bedanken voor de ontelbare discord-game sessies, LAN-parties, Dungeons and Dragons games, vakantie naar Nieuw-Zeeland (prachtige schapen!), kroeg-sessies, etentjes en eindeloze goede herinneringen. Zonder jullie had ik het niet vol gehouden. Homies 4 life!

Pap en mam, Robin en Loes (+ Domien en Elsie) en ook de rest van mijn familie. Jullie hebben het grondwerk gelegd voor de afgelopen jaren, door altijd in mij te hebben geloofd. Ondanks dat jullie soms niet begrepen waar ik het over had, ging dat jullie niet uit de weg om interesse te tonen en tóch nog een keer te vragen hoe het nu allemaal zat. Bedankt voor alles. Bedankt voor alles wat nog gaat komen. Ik hou van jullie allemaal!

Finally, my amore. You understand me better than anybody else, and have been there for infinite love and support in my times of need. You are also the first in line when it is time to mock me, be it my love of hagelslag, my horrible attempts at making pasta, or when I overreact to minor problems. I cannot imagine being remotely as happy as I am now, if you were not a part of my life. Thank you Alice, I love you.

# List of publications

Bruins, J. J., van de Wouw, C., Wagner, K., Bartels, L., Albada, B., and van Delft, F. L., Highly Efficient Mono-Functionalization of Knob-in-Hole Antibodies with Strain-Promoted Click Chemistry. *ACS Omega* **2019,** *4* (7), 11801-7.

Bruins, J. J., van de Wouw, C., Keijzer, J. F., Albada, B., and van Delft, F. L., Inducible, Selective Labeling of Proteins via Enzymatic Oxidation of Tyrosine, in *Enzyme-Mediated Ligation Methods* (Nuijens, T., and Schmidt, M., Eds.) **2019**, 357-68, Springer New York, New York, NY.

Bruins, J. J., Blanco-Ania, D., van der Doef, V., van Delft, F. L., and Albada, B., Orthogonal, dual protein labelling by tandem cycloaddition of strained alkenes and alkynes to ortho-quinones and azides. *Chem. Commun.* **2018,** *54* (53), 7338-41.

Bruins, J. J., Albada, B., and van Delft, F., ortho-Quinones and Analogues Thereof: Highly Reactive Intermediates for Fast and Selective Biofunctionalization. *Chem. - Eur. J.* **2018,** *24* (19), 4749-56.

Bruins, J. J., Westphal, A. H., Albada, B., Wagner, K., Bartels, L., Spits, H., van Berkel, W. J. H., and van Delft, F. L., Inducible, Site-Specific Protein Labeling by Tyrosine Oxidation-Strain-Promoted (4+2) Cycloaddition. *Bioconjugate Chem.* **2017,** *28* (4), 1189-93.

van Kalkeren, H. A., Bruins, J. J., Rutjes, F. P. and van Delft, F. L., Organophosphorus-Catalysed Staudinger Reduction. *Adv. Synth. Catal*. **2012**, 354, 1417-1421

# Overview of completed training activities

| Discipline-specific activities | Organizer | Year |
|---|---|---|
| CHAINS | NWO | 2015-2018 |
| Wageningen SOC Symposium 2016 | KNCV | 2016, 2018 |
| Institute for Chemical Immunology (ICI) Meetings | ICI | 2016-2019 |
| OxiZymes | VLAG | 2016 |
| FIGON Dutch Medicin Days 2018 | FIGON | 2018 |
| EFMC-YMCS 2019 (Athens) | EFMC | 2019 |
| | | |
| Advanced Organic Chemistry | ORC | 2015-2019 |
| Advanced Chemistry | VLAG | 2016 |
| Summer Course Glycosciences | VLAG / GGB | 2016 |
| Applied Biocatalysis | VLAG / ENTEG / GGB | 2017 |
| | | |
| **General courses** | | |
| Essentials of Scientific Writing and Presenting | WGS | 2016 |
| Institute for Chemical Immunology (ICI) Module 3 | ICI | 2017 |
| Scientific Artwork | WGS | 2019 |
| Career Perspectives | WGS | 2019 |
| Writing Grant Proposals | WGS | 2019 |
| | | |
| **Optionals** | | |
| Preparation of Research Proposal | ORC | 2015 |
| Weekly Group Meetings | ORC | 2015-2019 |
| Colloquia | ORC | 2015-2019 |
| Bimonthly ICI Project Meetings | ICI | 2015-2019 |
| PhD Study Tour to Denmark and Sweden | ORC | 2017 |
| PhD Study Tour to Israel | ORC | 2019 |

The research and training described in this thesis was financially supported by NWO Gravity Program Institute for Chemical Immunology (ICI).

Financial support for printing this thesis was provided from Wageningen University & Research, and is gratefully acknowledged.

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Printed by GVO drukkers & vormgevers, Ede
Cover design by Loes van de Kraats - Kema