Repurposing ubiquitination for innovative antibody conjugation

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Introduction and scope of the thesis

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Monoclonal antibodies and antibody fragments

The concept of using monoclonal antibodies as therapies can be tracked back to the late 19th century, when Ehrlich and Behring discovered that injecting animals with foreign material resulted in the production of natural antitoxins, now known as antibodies. These antibodies were proposed to serve as highly specific "magic bullets" against disease. However, it was more than 80 years later that antibody-based therapy truly started to unravel to its potential as a "magic bullet". Particularly, following the revolutionary discovery of both the hybridoma technology[1], enabling larger scale clonal production of antibodies, and the development of humanization approaches[2–4] to minimize antibody immunogenicity.

Antibodies have a Y-shaped symmetric structure consisting of two identical heavy chains (~50 kDa) and two identical light chains (~25 kDa), connected by interchain disulfide and non-covalent bonds[5]. The structure is divided into two main regions: the variable region (Fab) and the constant region (Fc) (Fig. 1a)[6]. The Fab region, located at the tips of the "Y," contains antigen-binding sites and is responsible for the antibody's specificity. The Fc region, forming the stem, mediates effector functions, such as binding to immune cells and complement activation. Antibody fragments are engineered derivatives of full-length antibodies that retain functional properties while being smaller and more versatile. Common fragments include Fab (fragment antigen-binding)[6], ScFv (single-chain variable fragment)[7], and VHHs[8,9], which consist of the variable heavy-chain domain derived from the unique heavy-chain-only antibodies found in camelids (Fig. 1b). These fragments are valuable for therapeutic and diagnostic applications due to their smaller size, improved tissue penetration, and ease of production in recombinant systems[6–9].

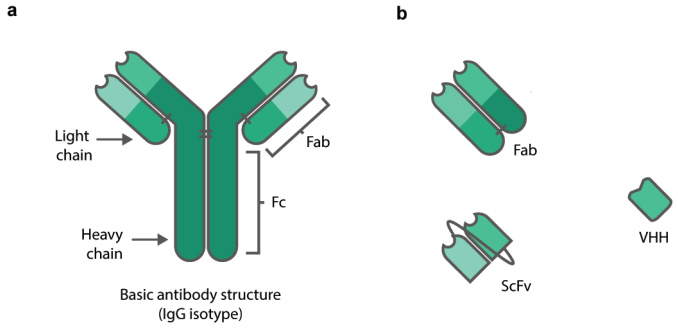

Figure 1| Antibodies and antibody fragments. (a) Schematic illustration of the basic antibody (IgG) structure. (b) Schematic illustration of commonly used antibody fragments.

Antibody engineering and approaches to multispecificity

Antibody engineering is a rapidly advancing field that leverages the specificity and adaptability of antibodies for various therapeutic, diagnostic, and research applications. This field involves the modification and optimization of antibodies to enhance their performance, improve their stability, and tailor their functionality to meet specific needs. The development of multi-specific antibodies represents a significant advancement in

antibody engineering, with many different formats of bi- and multi-specific antibodies developed in the last two decades[10]. While antibodies are naturally monospecific, recognizing one epitope, bi- and multi-specific antibodies are designed to recognize two or more different epitopes[11]. This offers several therapeutic advantages including enhanced targeting, improved efficacy, and synergistic effects. Their ability to integrate diverse functions, such as targeting cancer cells while activating immune responses, has made them valuable in therapeutic applications[11]. Various methods have been developed to generate these innovative molecules, each with distinct advantages and challenges.

One of the earliest approaches for the generation of a bispecific antibody, is using hybrid hybridomas[12]. This classical approach involves the fusion of two hybridoma cell lines producing distinct monoclonal antibodies. The resulting hybrid produces bispecific antibodies naturally but often suffers from low yield and instability, making it less practical for large-scale applications. Similarly, chemical crosslinking of antibody fragments such as Fab or ScFv from different monoclonal antibodies, offers a straightforward method for creating bispecific antibodies[13,14]. However, the lack of precision in chemical crosslinking often results in a heterogeneous product.

Sophisticated designs of multi-specific antibodies can be generated by genetic engineering and expression in various host systems such as CHO cells and *E. coli*[15–17]. Examples include single-chain formats like diabodies, triabodies, and tetrabodies[18], or dual-variable-domain immunoglobulins (DVD-Ig)[19], which utilize two sets of variable regions. Another widely used approach involves single-chain variable fragments (ScFvs), where the variable regions of heavy and light chains are linked to form small, functional antibody fragments[7]. These can be used to create bispecific T-cell engagers (BiTEs), which enhance immune-mediated killing by linking T cells to target cells[20].

Technologies such as knobs-into-holes engineering and CrossMAbs provide additional solutions for bispecific antibody production. Knobs-into-holes engineering introduces complementary mutations in the Fc region of antibodies, promoting heterodimerization and ensuring proper assembly[21–23]. CrossMAbs achieve stability by swapping domains of heavy and light chains, allowing precise pairing of bispecific components[24,25]. These methods represent significant advancements in antibody engineering, enabling the development of tailored therapies for complex diseases like cancer, autoimmune disorders, and infectious diseases.

Approaches for the generation of antibody conjugates

Another rapidly expanding area in the field of antibody engineering is that of antibody conjugates, where continuous efforts are made for the attachment of one or multiple payloads, such as toxins, fluorophores or radioisotopes, to antibodies in a controlled and homogeneous manner [26]. These conjugates combine the specificity of antibodies for targeting specific epitopes on cells with the potency of the attached agents, making them highly effective in therapeutics and diagnostics. The generation of antibody conjugates involves various strategies aimed at achieving stable, efficient, and site-specific attachment while preserving the biological activity of the antibody.

Chemical conjugation is one of the earliest and most commonly used approaches. It

involves modifying specific amino acid residues, such as lysines or cysteines, to attach payloads[27–29]. Lysine-based conjugation is widely used due to the high abundance of lysines on antibody surfaces, while cysteine-based conjugation is slightly more specific, targeting the sulfhydryl groups of reduced cysteines. These approaches often produce heterogeneous products because of the limited control over the site and number of lysines or cysteines that are modified. This also often results in compromised stability, functionality and pharmacokinetics[30–33].

In recent years, site-specific conjugation methods have gained prominence due to their ability to produce homogeneous conjugates with well-defined stoichiometry. Techniques such as engineered glycosylation sites[34–36] or the incorporation of unnatural amino acids[37–41] to introduce unique reactive sites enable precise attachment of payloads to antibodies at designated sites, minimizing off-target effects and enhancing therapeutic efficacy.

Enzyme-mediated conjugation methods have emerged as highly specific approaches for generating antibody conjugates. These methods use enzymes such as sortase, transglutaminase, or glycosyltransferases to catalyze the covalent attachment of payloads to specific amino acid sequences on antibodies. Sortase recognizes a unique pentapeptide sequence (LPXTG) engineered into the antibody, cleaving at the threonine residue and attaching the payload via a covalent bond to an incoming substrate with a glycine residue[42–45]. Transglutaminase targets glutamine residues, forming stable amide bonds with primary amines in the payload, offering flexibility in conjugate design[31,46]. Glycosyltransferases modify antibody glycosylation sites to attach payloads selectively, ensuring stability and functionality[47]. These enzyme-mediated approaches introduce site-specific modifications while preserving antibody integrity and functionality however, the yields are often low, and these approaches often require extensive optimization. As technology evolves, enzyme-mediated strategies continue to expand the possibilities for designing next-generation antibody conjugates with enhanced safety and efficacy profiles.

Antibody-antigen conjugates for DC-targeting vaccines

Antibody-antigen conjugates have emerged as an effective strategy for targeting dendritic cells (DCs) in vaccine development[48]. By leveraging the specificity of monoclonal antibodies for DC surface receptors, these conjugates enable the direct and efficient delivery of antigens to DCs, the primary antigen-presenting cells responsible for orchestrating adaptive immune responses[49–52]. Given the pivotal role of DCs in antigen uptake, processing, and subsequent T cell activation, targeted delivery strategies using antibody-antigen conjugates have the potential to significantly enhance the immunogenicity and therapeutic efficacy of vaccines, particularly in cancer immunotherapy and infectious disease control[48,52].

The underlying mechanism of antibody-antigen conjugates centres on their ability to bind selectively to well-characterized DC surface molecules, such as DEC-205[53–55], DC-SIGN[56], or CLEC9A[57,58]. This receptor-mediated targeting facilitates enhanced antigen internalization, processing, and presentation via major histocompatibility complex (MHC) molecules, leading to efficient T cell priming and activation[50,51]. By concentrating

antigen delivery specifically to DCs, antibody-antigen conjugates reduce off-target effects and optimize immune modulation, which is critical for achieving potent and sustained immunological responses with minimal toxicity.

Ubiquitin (Ub)

As key player in the process of proteasome-mediated protein degradation and antigenic peptide presentation in the context of immune response initiation, the role of the small protein ubiquitin (Ub) has gained lots of attention. As such, as well as the interesting potential of Ub to be incorporated into poly-Ub chains we explored the potential of employing Ub as means of generating a diverse portfolio of Ab-conjugates. Ubiquitin is a 76-amino acid post-translational modifier fundamental to cellular homeostasis. Cellular processes regulated by ubiquitin modification range from classically known Ub-mediated proteasomal degradation to DNA repair, cell division, endoplasmic reticulum-associated degradation (ERAD), mRNA stability and even regulation of the innate immune system[59]. The post-translational modifier can be covalently attached to substrate proteins at the ε-amino group of lysine residues or at the protein's N-terminal residue[60,61]. Since ubiquitin harbors seven intrinsic lysine residues, it can also be conjugated to another Ub moiety. In this manner, homotypical ubiquitin chains of a single linkage type consisting of M1, K6, K11, K27, K29, K33, K48 or K63 can be formed, all of which are known to

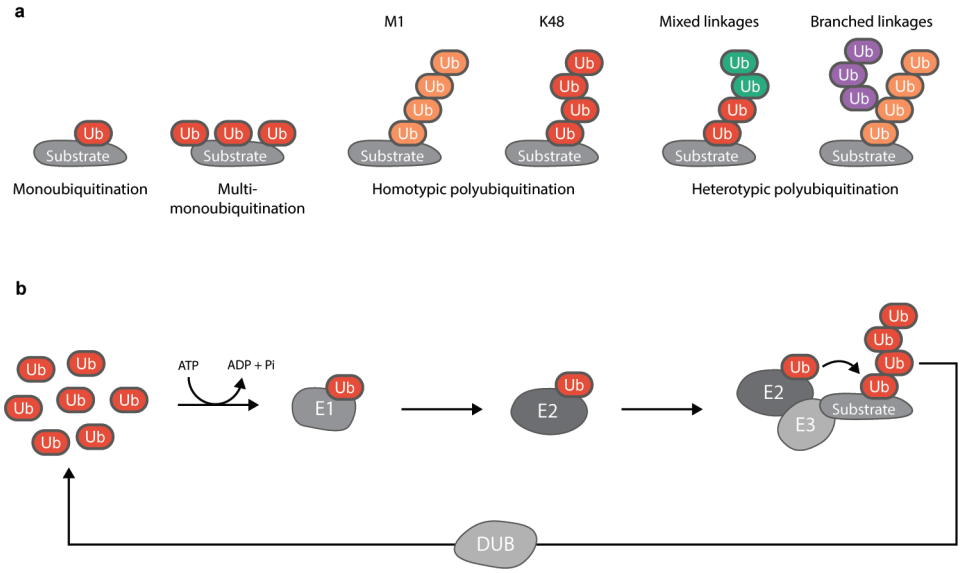

Figure 2| The ubiquitin system. (a) Types of ubiquitin conjugation: ubiquitin (Ub) can be conjugated as a monomer on one site, or on multiple sites of the substrate protein (multi-monoubiquitination). It can also form homotypical ubiquitin polymers through its N-terminus (M1-linked) or either one of its seven lysine residues (e.g. K48-linked). Mixing of different linkage types gives rise to heterotypic polyubiquitin chains. (b) General overview of ubiquitin conjugation and deubiquitination by E1, E2, E3 enzymes and deubiquitinating enzymes (DUBs). Ubiquitin is activated by an E1 enzyme, transferred to a specific E2 enzyme and conjugated to a substrate protein with the help of an E3 ligase.

exist *in vivo*[62]. In addition, heterotypical chains of multiple ubiquitin linkage types can be formed, opening up an even more complex layer of post-translational modification (Fig. 2a). Conjugation of Ub to a substrate protein is carried out by a cascade of three enzymatic activities: E1 ubiquitin-activating, E2 ubiquitin-conjugating and E3 ubiquitin-ligating activities (Fig. 2b). To date, 2 human E1s, about 40 E2s and over 600 E3 enzymes are known. The combination of E2 and E3 enzymes dictates what type of ubiquitin chain is formed and which substrate protein becomes ubiquitinated. In addition, the ubiquitination status of a protein can be regulated by removal or editing of ubiquitin chains, which is carried out by a family of approximately 100 deubiquitinating enzymes (DUBs)[63]. For some of these DUBs, linkage specificity has also been observed. To study ubiquitin signals, much effort has been put into making differentially linked ubiquitin derivatives through synthetic and semi-synthetic methods, since these methods allow site specific incorporation of a specific chemoselective ligation handle. In addition, ubiquitin-based DUB probes and fluorescent ubiquitin-based enzyme substrates have seen an enormous boost, producing ubiquitin-based tools in all sorts of different flavors[64].

Chemical synthesis of Ubiquitin-based tools

Native chemical ligation (NCL) has been an extremely useful tool to make ubiquitin, ubiquitin–peptide conjugates, ubiquitin dimers and ubiquitin tetramers, as reviewed by Pham *et al*.[65]. Chemical synthesis of ubiquitinated peptides was first established by Muir and co-workers and utilizes a ligation auxiliary where the auxiliary group is removed under photolytic conditions, yielding a natively linked Ub-peptide conjugate[66]. Today, most reported methods rely on the incorporation of a γ-thiolysine or δ-thiolysine moiety at a designated lysine residue to allow NCL with a thioester moiety. The thiol containing ubiquitin module can be synthesized with total, linear synthesis[67], or from two fragments[68]. During the total, linear fluorenylmethyloxycarbonyl (Fmoc)-based solid phase peptide synthesis (SPPS) approach, the growing peptide chain is stabilized by the incorporation of special building blocks that prevent the formation of aggregates as the ubiquitin chain grows. In the two segment approach, an N-terminal Ub(1–45)-SR fragment is synthesized and ligated to a synthetic C-terminal Ub[(46–76)-A46C] fragment. In the latter fragment, alanine 46 is replaced by N-methylcysteine to allow NCL with the first fragment and is afterwards converted into the native alanine residue through a desulphurization step. To make ubiquitin dimers and other conjugates, a thioester needs to be introduced at the ubiquitin C-terminus for NCL with the Ub thiolysine-containing module. The thioester functionality can be incorporated by E1-mediated enzymatic conversion with sodium 2-mercaptoethane sulfonate (MESNa) [67] or during Fmoc-based SPPS[69]. Next, NCL can be performed and a subsequent desulphurization step results in a ubiquitin conjugate that bears the native isopeptide linkage. In addition, other strategies yielding a non-native isopeptide linkage have been reported and include oxime-based ligation for non-hydrolysable ubiquitin–conjugate synthesis[70] and thioether based ligation to prepare diubiquitin[71], branched tri-ubiquitin[72] and polyubiquitin[73] modules that retain a sulfur atom in the forged isopeptide bond. It is clear that synthetic protein chemistry has much to offer to the biological community,

but one must keep in mind that the chemistry is not always straightforward. In addition, protein folding must always be checked, although this is less of an issue with the very stable ubiquitin protein.

Scope and outline of this thesis

Conjugated antibodies are critical tools across research, diagnostics, and therapeutic applications. These conjugates range from fluorescently labelled antibodies used in imaging, to bispecific antibodies for dual antigen targeting, and antibody-drug conjugates (ADCs) for the targeted delivery of cytotoxic agents. However, current antibody conjugation strategies face several key challenges, notably the efficiency of the conjugation process and the heterogeneity of the resulting conjugates. These factors can significantly influence the stability, pharmacokinetics, toxicity profiles, and batch-to-batch consistency of the conjugated products. Together with the growing interest in multispecific antibodies and antibody complexes for dual or higher-order targeting, this presses for advances in conjugation techniques.

This thesis aims to develop and optimize covalent, site-specific conjugation technologies to enhance the efficiency, reproducibility, and functional performance of antibody conjugates. Additionally, it explores the application of synthetic peptide chemistry to overcome current limitations, offering better control over conjugation sites, improved reproducibility, and enhanced functional performance. By addressing these challenges, the work sets out to contribute to the development of next-generation antibody-based tools with broad implications for diagnostics, research, and therapeutic interventions.

In **chapter 2**, we introduce a novel modular antibody conjugation platform, based on the highly regulated yet versatile ubiquitin biochemistry. We exploit the site-specificity and efficiency of the enzymatic process of ubiquitination, by using ubiquitin as a conjugation tag fused to antibodies or antibody fragments, and hence provide a site-specific conjugation tag for the attachment of fluorescent labels to antibodies or for antibody multimerization. We establish the feasibility and efficiency of this method for the generation of fluorescently labelled Fabs, Fab di-, tri- and multimers, fluorescently labelled mAbs, and for the generation of bivalent, tetrameric antibody complexes.

The versatility of this platform, together with the involvement of ubiquitin in the cellular signalling for proteasomal degradation led us to explore the possible benefit of using ubi-tagging for the generation of antibody-based DC targeting vaccines presented in **chapter 3**. Here, we use our ubi-tagging technology for the conjugation of chemically synthesized Ub-OTI peptides to the DC targeting anti-DEC205 Fab-Ub. We then compare the ubi-tag based anti-DEC205 Fab-OTI conjugates to the state of the art anti-DEC205 Fab-OTI conjugates generated through sortagging. We demonstrate the superiority of ubi-tag-based Fab-OTI conjugates over the sortag-based conjugates *in vitro* and *in vivo*. Results obtained in this chapter provide a foundation for follow-up studies exploring the mechanism underlying the superiority of the ubi-tagged conjugates and further expanding the use of ubi-tagging for the targeted delivery of antigens to DCs.

Ubiquitin is known for its solubility and stability even when exposed to extreme

conditions[74,75]. It is often used as a solubility-tag to enhance the solubility of proteins during their expression in bacterial expression systems[76,77]. This led us to hypothesise that ubi-tagging could enhance the solubility of antigenic-epitopes known for their hydrophobicity such as NY-ESO-1 and 2W1S. In **Chapter 4**, we test the solubility of chemically synthesized Ub-peptides known to be highly hydrophobic and conjugate them to VHH-Ub. In this chapter we also establish the feasibility of using ubi-tagging for smaller antibody fragments as VHHs and demonstrate their functionality in the context of targeted vaccination to human DCs in vitro. We hereby provide a proof-of-concept of using ubi-tag conjugates for targeted antigen delivery in a human setting.

In **Chapter 5**, we present the total chemical synthesis of a 123 amino acid nanobody targeting GFP. We synthesized this nanobody modified with a propargyl functionality at its C-terminus for on-demand functionalization. We demonstrate that the nanobody is properly folded ad functionalize it with either a Biotin or a sulfo-Cyanine 5 dye and demonstrate the functionality of the resulting probes in a pull-down assay and using confocal microscopy. The total chemical synthesis of a nanobody shown here allows the generation of homogenous batches of nanobodies of defined quality and could potentially be applied for the streamlined preparation of nanobody-drug conjugates and multimers. In our opinion, this work underscores the importance of chemical synthesis in expanding the utility of nanobody-based tools across diverse scientific and medical fields.

In **Chapter 6**, we summarise the findings described in this thesis, discuss them in the context of existing literature and contemplate on the future prospects of the work presented in this thesis.

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