Repurposing ubiquitination for innovative antibody conjugation

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Summary and future prospects

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Antibody conjugation techniques have revolutionized the development of multifunctional antibody-based therapeutics and diagnostics. Despite the advancements in antibody conjugation technologies, several challenges remain. Achieving site-specific conjugation while preserving antibody functionality is crucial, though often challenging. Developing improved conjugation strategies is vital to broaden the applications and effectiveness of antibody-based therapies, diagnostics, and research.

Chapter 1 provides an overview of approaches for the generation of multi-specific antibodies and antibody conjugation strategies for the generation of various antibody formats. In particular, antibody-antigen conjugates are discussed in the context of targeted antigen delivery to DCs. In addition, ubiquitin is discussed, touching upon the enzymes involved in ubiquitination and different ubiquitin chains. The chemical synthesis of ubiquitin-based tools is highlighted to emphasize the versatility of the chemical synthesis of ubiquitin.

In **Chapter 2**, we introduce ubi-tagging, a ubiquitin-based modular antibody conjugation platform, establish its feasibility, and explore the potential of this conjugation method for the generation of antibody conjugates and complexes of various formats. This conjugation method exploits the site specificity of the enzymatic process of ubiquitination to covalently attach antibodies to payload or to other antibodies through ubiquitin chain formation. By fusing ubiquitin to an antibody or antibody fragment, it can be used as a conjugation tag. To ensure the controlled conjugation of one ubi-tag to another, it is essential to use ubiquitin as a tag in two complementary forms, which we named the donor ubi-tag and acceptor ubi-tag. In the Donor ubi-tag, the C-terminal glycine residue is free and available for conjugation to another ubi-tag, whereas the lysine residue specific for the E2 and E3 pair used in the reaction is mutated. In contrast, the acceptor ubi-tag has this specific lysine residue available for conjugation, while its C-terminal glycine is either lacking or blocked by the presence of a fused short peptide. Thus, during the ubiquitination reaction using the corresponding E2 and E3, the C-terminus of a donor-ubitag can only covalently attach to the lysine residue of an acceptor ubitag and no other conjugate can be formed. In this chapter, we first generated Fab-ubitag fusion proteins and used them to establish the feasibility of this conjugation method for the generation of fluorescently labelled Fab fragments, Fab-multimers, and a Fab heterodimer that can be site-specifically elongated for fluorescent labeling or trimer formation. We also demonstrated the recognition and processing of the formed conjugates by deubiquitinating enzymes (DUBs). We then demonstrated that ubi-tagging can also be applied to the conjugation of mAbs, where each mAb carries two ubi-tags, one fused to each heavy chain. We used ubi-tagging to generate fluorescently labelled monoclonal antibodies (mAbs) and a bivalent bispecific antibody conjugate.

Ubi-tagging is further expanded in **Chapter 3**, where it is applied for the generation of ubi-tagged antibody-peptide conjugates for dendritic cell (DC)-targeted antigen delivery. We conjugated anti-mDCE205 to the ovalbumin antigenic peptide SIINFEKL by both ubi-tagging and the current state-of-the-art sortagging. When tested in vitro, the ubi-tagged conjugates induced significantly higher T cell activation and cytokine secretion compared to the sortagged conjugates. Building on these results, we assessed the *in vivo* efficacy of both conjugates where the mice treated with the ubi-tagged conjugates exhibited robust OT-I cell proliferation, whereas the sortagged conjugates induced minimal proliferation at the same concentration. Biodistribution studies revealed preferential uptake of ubi-tagged conjugates by CD11c+ dendritic cells, suggesting that this increased uptake underlies the enhanced T cell activation.

In **chapter 4**, we translate ubi-tagged DC-targeting vaccines to human applications. Here, we use ubi-tagged VHHs for the generation of VHH-peptide conjugates for the targeted antigen delivery to human DCs. Considering the relatively large size of ubi-tag conjugation with regard to the molecular weight of VHHs, we first demonstrate that ubi-tag conjugation does not interfere with the antigen-binding capacity of VHHs. We then proceed to successfully conjugate ubi-tagged VHHs to a library of notoriously insoluble epitopes, showing that the presence of the ubi-tag fused to these hydrophobic epitopes enhanced their solubility. We demonstrated the functionality of the generated anti-DC-SIGN VHH-Ub_2-$gp100_p$ *in vitro*, showing dose-dependent T cell activation.

In **chapter 5** we move from an enzymatic approach of antibody conjugation to a synthetic approach using native chemical ligation (NCL) to generate a synthetic GFP nanobody (Nb) primed for on-demand functionalization. We successfully modified the GFP-Nb using CuAAC to selectively attach either a biotin or a fluorophore, and used them in pull-down and confocal microscopy experiments, respectively. We demonstrate that the labelling process does not compromise the antigen-binding site, enabling its application for nanobodies with a cysteine residue in the CDR regions, which can be disrupted by conventional maleimide-based modification.

Discussion and future prospects

In this thesis, we present new broadly applicable approaches for the generation of antibody conjugates. We successfully applied both (chemo)enzymatic and chemical approaches for the efficient generation of chemically defined, site-specifically modified, homogenous antibody conjugates in various formats (Fig. 1).

For the ubi-tagging antibody conjugation technique presented in chapters 2, 3, and 4, we use the E2-E3 pair gp78RING-Ube2g2, which is specific for K48, and the donor ubi-tags used were K48R mutants. We used a gp78RING-Ube2g2 chimera, in which the c-terminus of the RING domain of gp78 was engineered to be fused to the N-terminus of Ube2g2 separated by linker[1]. This fusion results in a much higher enzyme activity of the pair compared to when used as separate proteins (Fig. 2). This enhanced activity

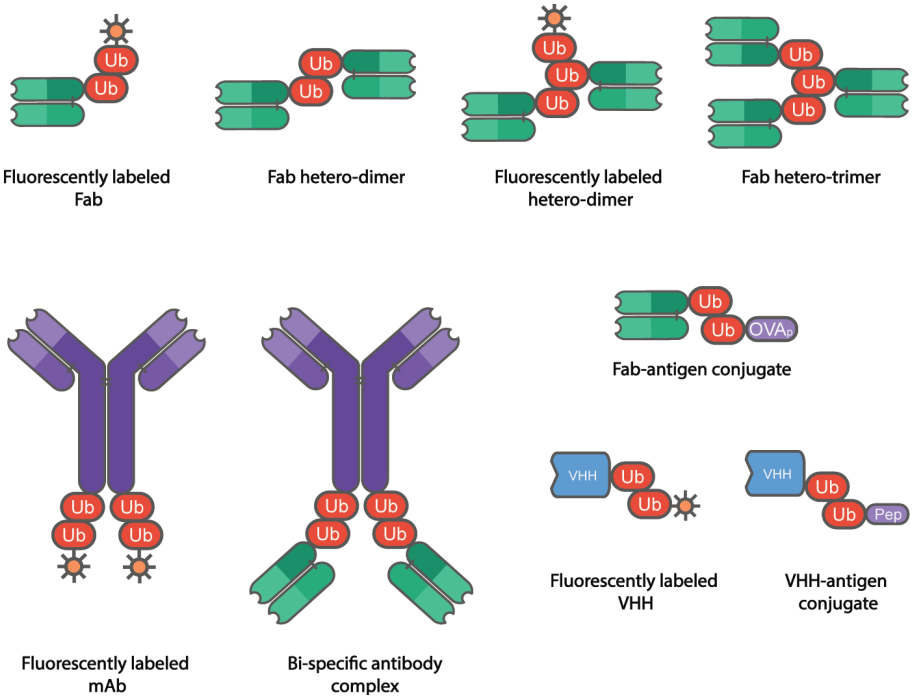

Figure 1| Schematic illustration of various antibody conjugates and complexes generated within the scope of this thesis.

largely contributed to the efficiency of the ubi-tag reactions in this thesis (Fig. 3). Although this E2/E3 pair is K48 specific, ubi-tagging is not restricted to this linkage type. Considering the variety of linkage-specific ubiquitination enzymes and the resulting ubiquitin chains, it would be interesting to further explore ubi-tagging using different ubiquitin linkage types. In particular, because differently linked ubiquitin chains are known to have very different conformations, which in the context of ubi-tagging could be exploited to gain control over the spatial orientation of the antibodies or proteins to be conjugated to each other. Another level of flexibility provided by ubi-tagging as an antibody conjugation technique, is its reversibility by DUBs. Although very lightly touched upon in this study, it was established that the ubi-tagged conjugates are recognized and processed by DUBs such as UCHL3 and OTUB1. This could be interesting for further exploration in applications where the conditional cleavage for the disassembly of the conjugate is desired.

Another aspect that adds to the versatility of this platform is the plethora of chemical modifications that can be synthetically incorporated in ubiquitin as its full chemical synthesis is well established and the ubiquitin toolbox is continuously expanding.[2] One of the synthetic ubiquitin variants that might be interesting to in the context of ubi-tagging is DOTA-Ub.[3] Conjugating it to an antibody or antibody fragment could

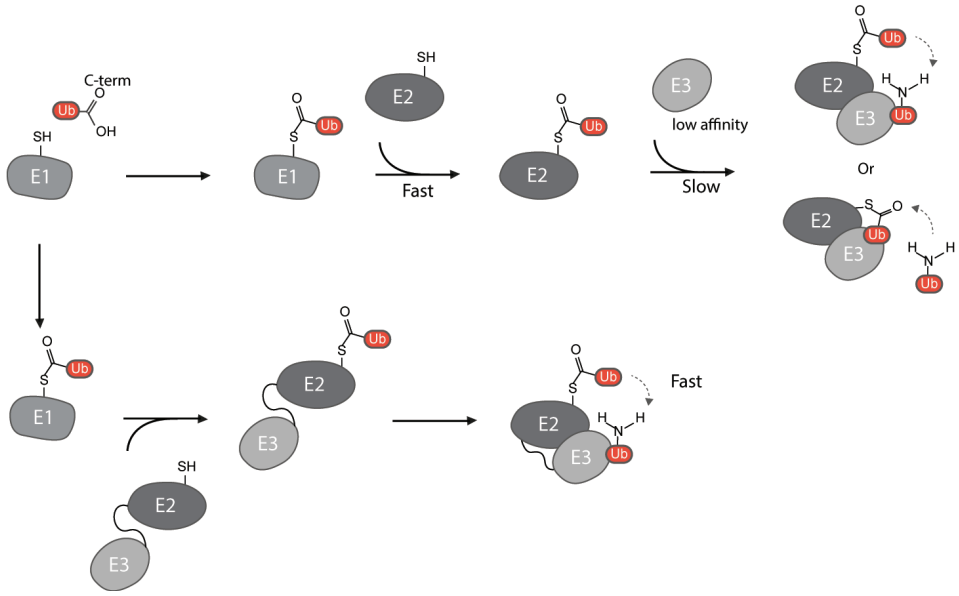

Figure 2|Schematic illustration showing the mechanism of the enhanced activity of the fusion of the E2/E3 pair gp78RING-Ube2g2 compared to the reaction in which the enzymes are not fused.

result in an antibody-DOTA conjugate that can be used as a lanthanide-based contrast agent in diagnostic applications. It is also interesting to explore the attachment of multiple chemical handles to ubiquitin with the idea of generating a scaffold that can be functionalized on demand with multiple payloads, such as multiple cytotoxic drug moieties, for the generation of ubi-tag-based antibody-drug conjugates. Such a scaffold could also be used to attach multiple fluorophores in research and diagnostic applications, where a high detection sensitivity is required. Additionally, since we showed that the ubi-tag enhanced the solubility of antigenic peptides in the context of targeted antigen delivery to DCs, it would be interesting to generate chemically synthesized libraries of ubiquitin fused to antigenic peptides that can be readily conjugated to antibodies for the generation of DC-targeted vaccines.

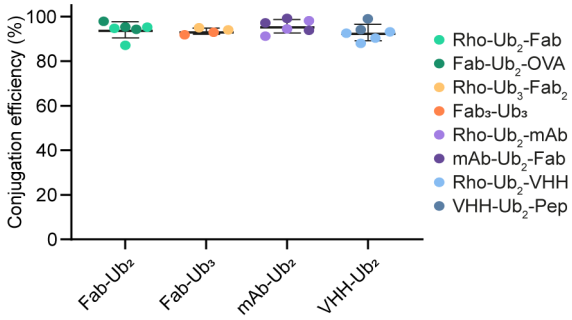

Figure 3|The Efficiency of ubi-tag conjugation reactions conducted in this thesis. Conjugation reactions involving ubi-tagged Fab fragments forming di-ubiquitin chains showed an average reaction

efficiency of 94.2%, whereas conjugation reactions involving ubi-tagged Fabs forming tri-ubiquitin chains showed an average efficiency of 93.4%. Conjugation of ubi-tagged mAbs showed an average reaction efficiency of 95.7%, whereas ubi-tagged VHHs reacted with an average efficiency of 92.8% within 60 mins.

Combining ubi-tagging with other approaches to generate multimeric antibody complexes may also be worth exploring. For instance, combining Fab-arm exchange[4] with ubi-tagging could be an approach to generate a trispecific antibody complex where only one Fab-arm would carry a ubi-tag or even a tetraspecific antibody complex where the both Fab-arms are ubi-tagged and separately conjugated to two different ubi-tagged Fabs before mixing for exchange.

Future prospect of synthetic nanobodies: Synthetic antibody fragments, such as fully synthetic nanobodies, offer exciting prospects for preclinical research. Their completely synthetic nature allows for precise design and rapid development, enabling highly targeted drug delivery, diagnostics, and therapeutic applications. These synthetic constructs can be easily modified for use in sensitive immunoassays, diagnostic platforms, and imaging techniques, without the limitations of traditional antibodies. With the ability to reduce immunogenicity, improve tissue penetration, and facilitate high-throughput screening, synthetic antibody fragments are poised to revolutionize drug discovery, diagnostic tools, and targeted therapies in preclinical settings.

Collectively, in this thesis a novel modular ubiquitin-based antibody conjugation method is presented. While we successfully applied this method for the generation of a range of antibody conjugates, limitless other antibody formats and conjugates can be generated using this method. We also present the chemical synthesis of a nanobody that can be functionalized on-demand. The implementation of the work presented here empowers researchers with new strategies for the generation of antibody -based tools and therapeutics.

References

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