Bioorthogonal Antigens

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Summary and future prospects

The research described in this thesis offers an initial exploration of bioorthogonal chemistry as a tool to study antigen cross-presentation. Furthering the understanding of this process is crucial as it is an important mechanism to elicit specific cytotoxic T cell response necessary for clearance of cancers and pathogenic infections. It is also crucial regarding vaccinations with protein antigens as the aim is to apply the knowledge obtained here to the design of new peptides for anti-cancer vaccines.

In **chapter 1**, the general principles of antigen processing and presentation in MHC-I, -II, as well as the rudimentary details of cross-presentation are described. Antigen cross-presentation pathways are central to the work described in this thesis. Therefore, **Chapter 2** presents an overview of the various antigen cross-presentation pathways as well as molecular approaches for studying them. A few examples of the current methods are described in detail together with their limitations and potential applications. Also, bioorthogonal antigens as novel tools to study cross-presentation process are introduced in this chapter.

The initial development of a new strategy to quantify specific peptide–MHC-I complexes (pMHC-I) on cell surface using bioorthogonal chemistry is described in **chapter 3**: A library of peptides containing different bioorthogonal handles (azides and alkynes) within the epitope were synthesized and the MHC-I binding[1] and stability of these modified peptides in the RMA-S cell line assay[2] were optimized. In

order to obtain the most efficient bioorthogonal ligation reaction, various types of bioorthogonal ligation reactions were tested and assessed. The most optimal condition, type of fluorophore and bioorthogonal ligation reaction[3] were established. The requirement for the most efficient epitope quantification is the alkyne modification in non-anchor residues in solvent-accessible epitope positions using CalFluor-488 in combination with a Cu(I)-catalyzed Huisgen cycloaddition reaction (Figure 1).

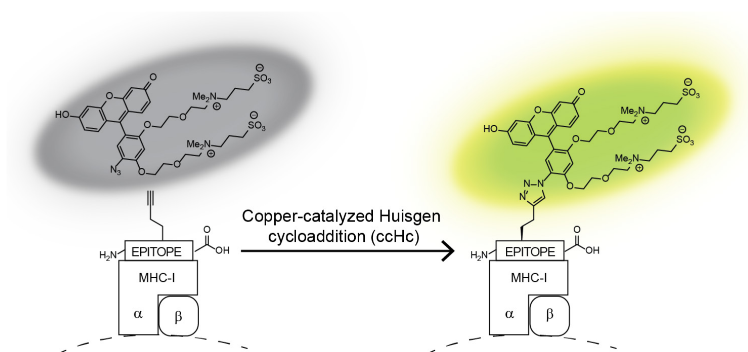

Figure 1. *Schematic representation of the bioorthogonal ligation strategy using CalFluor-488 in ccHc reaction.*

The major limitation of this approach is the requirement of copper in the ccHc reaction. Alternative to this approach, the use of click chemistries not requiring catalysis could be applied. For example, the inverse electron-demand Diels-Alder (IEDDA) reaction (Figure 2) between cyclopropene as a dienophile and tetrazine as a diene can, in principle, allow *in vivo* labeling without the need of copper and fixation[4]. However, the background reactivity of this chemistry has not been fully explored in a system as stringent as antigen presentation.

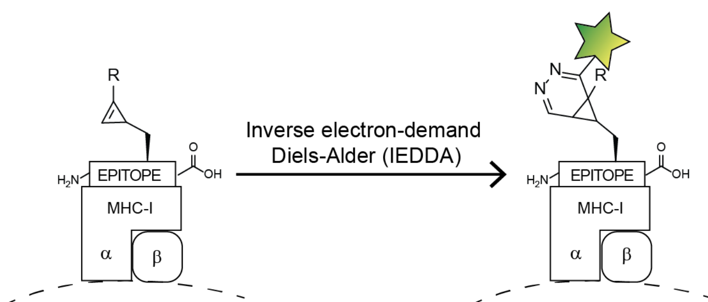

Figure 2. *Proposed* schematic *representation of the bioorthogonal ligation strategy using tetrazine-fluorophore in IEDDA reaction.*

When studying antigen processing and presentation, techniques that can label the cell surface in isolation would be very valuable. Hence, such a method was developed based on a three- step labeling procedure. To circumvent the permeability to small molecules caused by the use of copper, a three step labeling approach was developed in the second part of **chapter 3**. This three-step labeling consists of three labeling steps: first step is accomplished by modifying all bioorthogonal groups with Alexa Fluor 488 in the ccHc reaction, the second step is executed by applying the anti-Alexa Fluor 488 antibody and the final third step by applying protein A conjugated to Alexa Fluor 647 (Figure 3). The steric bulk of the antibody minimizes intracellular labeling to allow imaging of the surface pool of the bioorthogonal epitope.

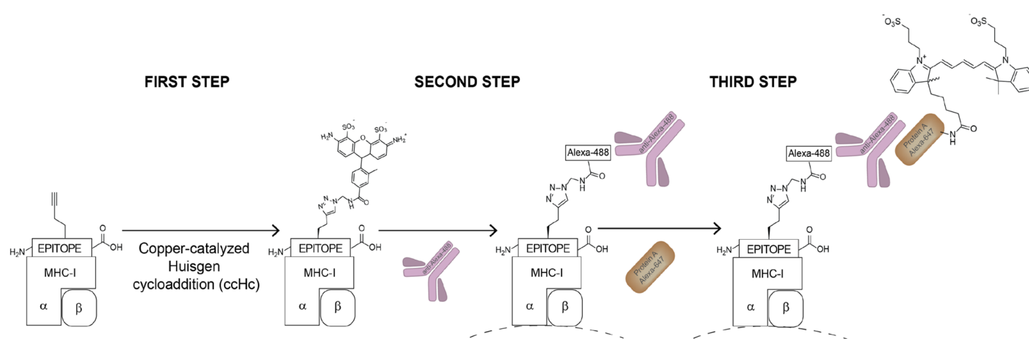

Figure 3. *Schematic representation of the three-step labeling.*

This three step labeling protocol was used to perform a super resolution (stochastic optical resolution microscopy or STORM[5]) imaging experiments of peptides in MHC-complexes. The preliminary results of the STORM imaging of bioorthogonal epitopes using the three-step labeling revealed a potential to localize and later quantify the epitopes on the cell surface. In the future, this approach could be applied to quantify the number of epitopes per cell and – once T cells against bioorthogonal groups become available – to quantify the correlation of surface peptide quantity to T cell response strength.

Chapter 4 describes the exploration of bioorthogonal chemistry to the study of longer antigens that – unlike the minimal epitopes of chapter 3 – do require intracellular processing prior to their presentation. A series of bioorthogonal synthetic long peptides (SLPs) were designed and the use of click chemistry to study their uptake, routing and surface presentation was assessed. The labeling and imaging of a herpes virus vaccine candidate HSV-Gp$_{488\text{-}505}$-Pg-7 with Alexa Fluor-488 azide revealed a patchy pattern of fluorescent signal on the cell membrane indicating that the peptide aggregated and that these aggregates are either slowly internalized or not at all. This was confirmed using correlative-light electron microscopy (CLEM)[6] of the bioorthogonally introduced fluorophores. Due that reason to selectively label

epitopes on the cell surface, the switch was made to a more soluble SLP that requires proteasome-dependent processing on both the *N* and *C*-terminus (HSV-$Gp_{488\text{-}505}A_5K$-Pg-7)[7].

The rate of uptake of this HSV-$Gp_{488\text{-}505}A_5K$-Pg-7 was assessed using click chemistry and it was attempted to use the three-step labeling described in chapter 3 to quantify the cell surface appearance of the processed epitope. The quantification turned out to be troublesome. The weak extracellular signal (due to only a small fraction of the bioorthogonal SLP reaching the surface for MHC-loading and instead mostly remaining in endo-lysosomal[8] like compartments) prevented robust labeling. Even the use of the three-step labeling approach did not give enough signal over background.

In summary, the use of the three-step labeling in combination with a Cu(I)-catalyzed Huisgen cycloaddition reaction for the bioorthogonal long peptides allowed for imaging of the cellular uptake however the cell surface labeling still requires further research.

A possible alternative approach to achieve this would require live-cell compatible chemistry and/or a signal enhancement step. For example one alternative could be the recently reported 'DNA-click-PAINT' method[9]. Here an azide or tetrazine moiety can be attached to a single-stranded DNA and used in a click reaction (ccHc or IEDDA). A complementary DNA strand equipped with a fluorophore can be annealed to the docking DNA strand. This approach has as the advantage that the fluorophore is more water soluble reducing background signal. Mismatching of the two strands can also be used to induce fluorophore blinking (Figure 4), where a correctly chosen DNA strand can give on/off rates optimal for STORM imaging[10].

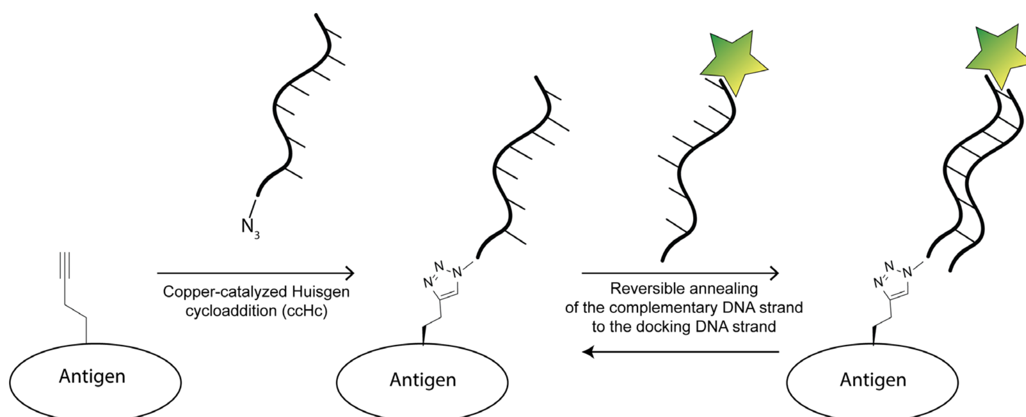

Figure 4. *Schematic representation of the 'DNA-click-PAINT' method. The fluorescently labeled complementary DNA strand (imager strand) anneals to the 'docking' DNA strand inducing fluorophore blinking needed for high-precision single-molecule localization.*

However, still only a single fluorophore per bioorthogonal handle is introduced which can impinge on the signal to noise ratios of this approach. To increase the signal-to-noise ratios a technique called rolling circle amplification (RCA) could be applied[11]. RCA is an efficient isothermal enzymatic process conducted at a constant temperature where a short DNA or RNA primer in the presence of fluorophore conjugated nucleoside triphosphates containing deoxyribose (dNTPs), is amplified using a circular DNA template and DNA or RNA polymerases to form a long single stranded DNA or RNA containing multiple fluorophores[12]. Antigens functionalized with complementary DNA sequences and equipped with the fluorophore could be potentially visualized by docking to the long single stranded DNA at various locations resulting in a presence of multiple fluorophores and thus an enhanced signal-to-noise[13].

Chapter 5 focused on different uses of bioorthogonal antigens. During the work leading to chapters 3 and 4, it was discovered that the 2-3 atom alterations of the epitope obliterated recognition by the cognate T cells. This led to the development of a new method that allowed for chemical control over T cell activation[14]. The chemical deprotection strategy was used to study the activation of cytotoxic T cells by antigen presenting cells: by substituting the key lysine in the H2-K^b-restricted epitope SIINFEKL for an azidonorleucine, the peptide was rendered unresponsive to its cognate T cell. By then performing a Staudinger reduction[15] (from the azidonorleucine back to a lysine) on the surface of the cell, more than 80% of the original T cell reactivity was recovered[16] (Figure 5A).

The chemical uncaging strategy worked well *in vitro,* but the required reaction conditions were not compatible with *in vivo* use. In the future the IEDDA-based elimination reaction could serve as an *in vivo*-compatible deprotection reaction. In this reaction a strained alpha-substituted trans-cyclooctene[17] (TCO)-modified antigen reacts with certain tetrazines to result in the elimination of the alpha-positioned substitute.[17-18] This reaction has been used *in vivo* for the release of drugs from antibodies[19] and the unblocking of enzyme active sites[20]. In the context of these experiments, it would allow for deprotection and chemical control over T cell activation *in vivo* (Figure 5B), which in turn would allow the study of T cell activation kinetics and the role they have on their activation.

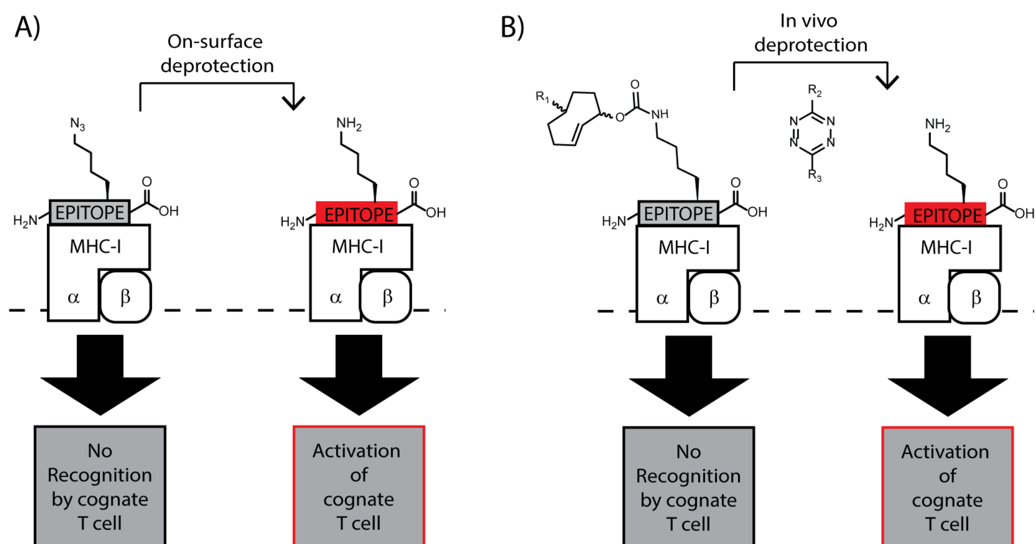

Figure 5. *A) Schematic representation of the on-surface deprotection using Staudinger reduction reaction. B) Proposed schematic representation of the in vivo deprotection using trans-cyclooctene-modified epitope and tetrazine as a reaction partner in inverse electron-demand Diels-Alder reaction.*

In conclusion, the results from this thesis show that bioorthogonal antigens exhibit potential as reagents for the study of antigen cross-presentation. However, limitations with regards to signal-to-noise and the use of metal-based catalysts need to be addressed to truly allow them to fulfill their potential.

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