Technology-Mediated Functionalization of Proteins and Antibodies Using a Continuous Flow Microreactor

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Thesis directed by

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Dedication

To my family and friends, for their unwavering support and constant encouragement.
Acknowledgments

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To Meaghan Sebeika: Thank you for being there consistently, providing the help that I initially needed to assimilate into the lab and everyday thereafter. You do an amazing amount for the Jones lab and we are all appreciative for it.

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Abstract

The therapeutic index of conventional chemotherapeutic techniques is narrow and treatment results in a variety of systemic issues. Antibody-drug conjugates represent a targeted delivery method of highly potent cytotoxic payloads to cell surface antigens overexpressed on cancer cells. In recent years, a large number of antibody-drug conjugates have entered clinical trials for the treatment of an assortment of cancers. One of the major limitations in the search for ideal antibody-drug conjugates is the inability to consistently conjugate cytotoxic payloads to antibodies. Conventional conjugation techniques result in heterogeneous mixtures that can aggregate or cause other toxicity. Design of the synthetic linker that joins the toxin and antibody is also crucial; the payload must selectively cleave once internalized by the cell and not in the bloodstream or other organs. The results of the investigation into linker chemistry and the applicability of continuous flow microreactor technology as a viable alternative for the functionalization of proteins is reported herein, including the coupling of antibody-drug conjugates.
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<th>Description</th>
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<tbody>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>ADC</td>
<td>antibody-drug conjugate(s)</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N'-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DAR</td>
<td>drug-to-antibody ratio</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>EEDQ</td>
<td>N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FSB</td>
<td>N-succinimidyl-4-fluorobenzoate</td>
</tr>
<tr>
<td>HC</td>
<td>heavy chain</td>
</tr>
<tr>
<td>HER2+</td>
<td>human epidural growth factor receptor 2</td>
</tr>
<tr>
<td>HIC</td>
<td>hydrophobic interaction chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing gel electrophoresis</td>
</tr>
<tr>
<td>LC</td>
<td>light chain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>MCC</td>
<td>(N-maleimidomethyl)cyclohexane-1-carboxylate</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>matrix assisted laser desorption ionization-time of flight</td>
</tr>
<tr>
<td>MMAE</td>
<td>monomethyl auristatin E</td>
</tr>
<tr>
<td>MMAF</td>
<td>monomethyl auristatin F</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PABA</td>
<td>para-aminobenzyl alcohol</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission topography</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>ToF</td>
<td>time of flight</td>
</tr>
<tr>
<td>UAA</td>
<td>unnatural amino acid</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>UV/VIS</td>
<td>ultraviolet/visible light spectroscopy</td>
</tr>
<tr>
<td>val-cit</td>
<td>valine-citrulline</td>
</tr>
</tbody>
</table>
General Experimental Information

All reagents and solvents were purchased from Fisher Scientific or Sigma Aldrich. All continuous flow reactions were performed using the Chemtrix Labtrix® Start system (Chemtrix BV, NL) utilizing T-mixer glass microreactors, which are commercially available through Chemtrix BV. All reagents were dissolved, loaded into 1 mL glass gas-tight syringes (SGE) and dispensed through the microreactor into the autosampler loaded with HPLC vials. $^1$H NMR spectra were recorded at 400 MHz, and $^{13}$C NMR were recorded at 100 MHz on a Varian NMR instrument, and spectra was prepared using ACD/Labs (Toronto, Canada). High-resolution mass spectroscopy was obtained on a Waters 70-VSE (EI) or a Waters Q-ToF Ultima mass spectrometer (ESI) at the UIUC Mass Spectrometry Facility for analysis of synthesized linkers. For ADC-linker analysis by LC-MS, samples were desalted using a Zebra (Thermo Scientific, Rockland, IL) spin desalting column (7 kDa MWCO). The LC-MS analysis was performed on a micro-LC 200 system (Eksigent, Framingham, MA) coupled with a 5600 ESI Triple ToF mass spectrometer (AB Sciex, Framingham, MA). Samples were injected on an Eksigent MicroLC ChromXP C4, 5 μm, 300Å, 50 × 0.5 mm column (Eksigent, Framingham, MA) set at 35 °C. Data analysis was performed with PeakView 1.0 and MagTran 1.03.
Chapter 1
Introduction

1.1.1 Antibody-Drug Conjugates

Cancer is one of the leading causes of mortality worldwide, with an expected 70% increase in the number of cases over the next two decades.[1] A defining aspect of cancer is uninhibited proliferative potential coupled with sustained angiogenesis.[2] In the twentieth century, some focus was placed on treating cancer via chemotherapeutic means, but the mid-1960s saw a breakthrough: an increased remission of untreated Hodgkin’s disease from 0% to 80% using a combination of nitrogen mustard with vincristine, procarbazine, and prednisone.[3] A significant amount of research was then directed towards the development and discovery of synthetic and natural chemotherapeutic agents.

However, conventional cancer treatment programs using chemotherapeutic drugs were, and still are, plagued by a lack of specificity. Most chemotherapy relies on the rapid division of cancerous cells, as seen in the use of drugs that interfere with deoxyribonucleic acid (DNA) synthesis, like the nucleoside analogues thioguanine and 5-fluoruracil, and the inhibition of tubulin, such as paclitaxel. These drugs perform their expected jobs very well – but lack the specificity to solely affect cancerous cells. What results is the unintentional killing of rapidly proliferating healthy cells, such as those found in hair follicles and the gastrointestinal tract.[4] Clinical techniques and academic efforts to combat this issue involve optimization of the therapeutic index: lower the minimum effective dose of the drug and increase the maximum tolerated dose to reduce systemic toxicity.

Targeted therapies were quickly recognized as a potential method for selectively eradicating tumor cells without the off-target effects noted in other chemotherapeutic methods.
One of the major targeted therapy approaches that have been investigated in research settings is antibody-drug conjugates (ADCs), stemming from Nobel Laureate Paul Ehrlich’s thoughts on the development of a “magic bullet.”[5] ADCs are composed of three major components: a monoclonal antibody (mAb), a linker, and a cytotoxic payload. The mAb of choice should have a paratope that recognizes an overexpressed epitope, typically on the surface of a tumor cell, which ensures specific delivery. The linker is responsible for physically linking the cytotoxic payload to the site-directing antibody and can attach to the mAb through a number of sources, notably via free amines on lysine side chains and via thiolates liberated from the reduction of disulfide bonds.

Upon epitope recognition and antigen (Ag) binding, antibodies are able to enter the cell through two endocytotic pathways: clathrin-mediated endocytosis and caveolae-mediated uptake, with clathrin-mediated endocytosis playing the major role. Internalized ADCs then undergo endosomal transportation to the lysosome, where they are exposed to stimuli that encourage payload liberation, such as a low pH environment, proteolytic cleavage, and high glutathione concentrations.[6] Cytotoxic drugs liberated from their antibodies are then able to diffuse through the lysosomal membrane and perform their task inside the cell. Figure 1 illustrates the internalization and degradation processes.[6]
1.1.2 Construction of ADCs

One of the most important aspects in the design of safe and effective ADCs is the rational selection of mAb, linker, and cytotoxic payload. The significance of building block optimization can be seen from the distinct lack of clinical success by early ADCs. These three components each play an integral role in the overall efficacy of an ADC and are discussed in detail below.

1.1.2.1 The mAb and Target Ag

The antibody chosen should target a well-characterized and well-internalized Ag that is highly expressed on tumorous cells relative to normal cells. The mAb confers its specificity and delivers the cytotoxic payload to the cancerous cells, resulting in a concentration of ADC on the
tumor surface and eventual internalization via endocytosis. Table 1 shows some of the target Ags that are currently being probed in preclinical or clinical ADCs.[7]

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Target Ags</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-Hodgkin’s Lymphoma</td>
<td>CD19, CD20, CD21, CD22, CD37, CD70, CD72, CD79a/b, and CD180</td>
</tr>
<tr>
<td>Hodgkin’s Lymphoma</td>
<td>CD30</td>
</tr>
<tr>
<td>Acute myeloid leukemia</td>
<td>CD33</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>CD56, CD74, CD138, and endothelin B receptor</td>
</tr>
<tr>
<td>Lung</td>
<td>CD56, CD326, CRIPTO, FAP, mesothelin, GD2, 5T4, and alpha v beta6</td>
</tr>
<tr>
<td>Colorectal</td>
<td>CD74, CD174, CD227 (MUC-1), CD326 (Epcam), CRIPTO, FAP, and ED-B</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>CD74, CD227 (MUC-1), nectin-4 (ASG-22ME), and alpha v beta6</td>
</tr>
<tr>
<td>Breast</td>
<td>CD174, GPNMB, CRIPTO, nectin-4 (ASG-22ME), and LIV1A</td>
</tr>
<tr>
<td>Ovarian</td>
<td>MUC16 (CA125), TIM-1 (CDX-014), and mesothelin</td>
</tr>
<tr>
<td>Melanoma</td>
<td>GD2, GPNMB, ED-B, PMEL 17, and endothelin B receptor</td>
</tr>
<tr>
<td>Prostate</td>
<td>PSMA, STEAP-1, and TENB2</td>
</tr>
<tr>
<td>Renal</td>
<td>CAIX, and TIM-1 (CDX-014)</td>
</tr>
<tr>
<td>Mesothelioma</td>
<td>Mesothelin</td>
</tr>
</tbody>
</table>

Table 1: Target antigens for ADCs in preclinical or clinical development.

Early ADCs contained murine antibodies, which elicited immunogenic responses in human patients. In the late 1990s, the antibody-doxorubicin conjugate BR96-Dox advanced to phase II clinical trials to treat metastatic breast cancer. The randomized trial consisted of one group receiving the conjugated antibody and a second group receiving free doxorubicin. The results of this trial showed an immunogenic response in 50% of the group that received the conjugated antibody. However, this trial also showed a difference in the toxicity profile of conjugated and free doxorubicin, suggesting that an ADC can change the biodistribution of the drug, and propelling research on the subject forward.[4, 8]
mAbs chosen for ADCs today should take advantage of the readily available techniques that produce “humanized” antibodies to prevent an immunogenic response and increase the circulation retention time in vivo.[9] Chimeric antibodies, which consist of the constant region of a human immunoglobulin fused with the variable region of murine mAbs, can be chosen as the targeting agent. These mAbs are advantageous because they retain antigen specificity from the large repertoire of mouse mAbs available, but still maintain the characteristics of a human mAb during circulation (e.g., longer circulating half-lives, reduced immunogenicity, and improved biological activity).[10] Currently, the most common antibodies used in ADC development are all IgG isotypes, with IgG1 being the most common.[7] Additionally, choosing a clinically relevant antibody that has already been approved for use in humans by the Food and Drug Administration (FDA) can help to ensure that there will be a benchmark to verify how well the conjugate is acting compared to the individual mAb.

1.1.2.2 The Linker

The design of an ADC linker must ensure safe circulation of the ADC, allow for effective release of the cytotoxic payload, and mediate clean and efficient conjugation to the mAb. A linker needs to be sufficiently stable to enable the ADC to circulate in the blood stream for a long period of time before reaching the tumor site. A linker that suffers hematological instability has the potential to release the cytotoxic payload prematurely, resulting in systemic toxicity. Additionally, upon internalization, the linker should be labile enough to release the active payload. Finally, the linker should rely on chemistry that allows for facile and safe conjugation reactions during the ADC synthesis.

Linkers can be categorized into two main classes: cleavable and noncleavable. Cleavable linkers act in response to physiological stimuli, such as low pH, high glutathione concentrations,
and proteolytic cleavage. Conversely, noncleavable linkers rely on mAb degradation within the lysosome following ADC internalization.[11]

Linkers that are sensitive to proteases, such as cathepsin B, represent one example of enzymatically degradable linkers. Cathepsin B recognizes and cleaves an amide bond between a dipeptide linker and the conjugated payload, releasing free drug that is then able to diffuse through the lysosomal membrane. This method of linker technology is gaining in popularity due to superior plasma stability and release mechanisms. Another method for linker cleavage capitalizes on the relatively high concentration of thiols, such as glutathione, within the cell relative to the bloodstream. Reducing thiols cleave disulfide bonds within the linker and release the free drug. Often, methyl groups are added to the drug side of the disulfide bond to discourage premature cleavage in the bloodstream.[7]

A third type of cleavable linker is the acid-sensitive linker. This chemistry takes advantage of the low pH environment found within the lysosome and results in the hydrolysis of hydrazone linkers or the reduction of disulfide linkers.[7, 11] Acid-sensitive linkers are hindered by short plasma stability times and the fact that nonspecific drug release can be spurred by acidic conditions that are found in various other parts of the body.[12]

Noncleavable linkers require the degradation of the conjugated mAb for drug release. These linkers have shown increased plasma stability when compared with cleavable linkers in vivo. They are a viable route if the cytotoxic payload is still active despite being chemically modified (e.g., with the linker and degraded amino acid attached).
1.1.2.3 The Cytotoxic Payload

Important considerations in the choice of cytotoxic payload involve amenability to conjugation, stability, solubility, and potency. The drug’s potency must be in the sub-nanomolar range due to the fact that approximately only 1-2% of an administered ADC dose reaches its target, resulting in a low concentration of liberated drug.\footnote{13} Conjugated cytotoxic payloads, therefore, must demonstrate 100-1000-fold greater potency when compared with conventional chemotherapeutic options.\footnote{14} These drugs are often unable to be used as stand-alone treatment, but are given new life with ADC technology.

Currently, ADC payloads fall into two major categories: antimitotic agents and DNA damaging agents. Antimitotic agents disrupt microtubule assembly and alter the cytoskeletal architecture of cells, resulting in cell death. This mechanism is especially toxic to cells that have a high rate of proliferation, such as tumorous cells, the cells of the intestinal lining (resulting in nausea), cells in hair follicles (resulting in hair loss), and myeloid cells (resulting in myelosuppression). There are two classes of antimitotic agents currently being investigated for use in ADC design: maytansinoid and auristatin agents. The structures of potential maytansinoid derivatives are highlighted in Figure 2.
Maytansinoid agents cause apoptosis by strongly binding tubulin near the vinblastine-binding site and suppressing microtubule dynamics.[15] These agents have very high cytotoxicity, but fail to display a therapeutic benefit at tolerable doses, making them promising ADC payloads.[16] Maytansinoid agents are more amenable to conjugation when linked through a semisynthetic route beginning from the maytansine-derivative ansamitocin P-3. Controlled reduction of ansamitocin P-3 cleaves the ketone and yields a hydroxyl functional group, which is esterified with a carboxylic acid that contains a disulfide in the presence of a Lewis acid and a coupling agent to provide maytansinoid disulfides. These maytansinoid disulfides can then be reduced to provide thiol-bearing maytansinoids (such as DM1, DM3, or DM4).[4]

The small linear peptide dolastatin 10 and derivatives thereof have shown pronounced antitumor activity in vivo and in vitro on numerous malignant cell lines via microtubule-inhibition and the resulting apoptotic effects.[17-18] Dolastatin 10 analogues (shown in Figure 3), such as monomethyl auristatin E (MMAE) and monomethyl auristatin F (MMAF), are currently being investigated as ADC payloads. MMAE exhibits 100 times greater cytotoxicity when compared with MMAF and can be linked to an antibody through a dipeptide attached to a para-
aminobenzyl alcohol moiety. The N-terminal of the dipeptide can be attached to a maleimide linker that will interact with a free thiol group at the conjugation site.

![Dolastatin 10 and Auristatin Analogues]

Figure 3: Selected analogues of dolastatin 10 that have been used in ADCs

DNA damaging agents represent the second major class of cytotoxic payloads currently being investigated in ADC development. Calicheamicins are a class of enediyne-containing agents that are considered some of the most potent antitumor agents discovered. The structures and mechanisms of action of these compounds are complex. For the most prominent member of this group, calicheamicin γ1, an aryl tetrasaccharide directs the drug to bind tightly within the minor groove of DNA, positioning the enediyne unit within the DNA double helix. From here, a free thiol is generated via nucleophilic attack, which spontaneously cyclizes into the α, β-unsaturated ketone bridgehead of the enediyne. The newly formed angular strain of the enediyne is relieved by a Bergman type reaction which forms a diradical benzene species. This diradical can then abstract hydrogens from double-stranded (ds) DNA, producing diradical dsDNA, and eventual DNA cleavage followed by cell death.[4]

Other DNA damaging agents include the duocarmycins and the camptothecins. Duocarmycins display very high potency for a five-base pair AT-rich sequence of DNA, resulting in alkylation of DNA and subsequent cell death. Early duocarmycins showed delayed
toxicity in mice, with later derivatives displaying little therapeutic activity.\textsuperscript{[19]} Camptothecins exert apoptosis by arresting DNA replication in the S-phase via inhibition of DNA topoisomerase I.\textsuperscript{[20]} This class initially suffered from poor solubility, and later attempts to rectify this resulted in a better solubility profile but reduced potency.

The physiochemical properties of the chosen payload have the potential to significantly alter ADC efficacy. Conjugation of a foreign cytotoxic agent to a mAb can significantly increase protein aggregation and reduce conjugate solubility. Typically, a drug-to-antibody ratio greater than 4 should be avoided for these reasons. Additionally, the payload should be amenable to conjugation. Modification of the drug’s structure to accommodate “functional handles” can have a damping effect on the drug’s potency. Therefore, considerations must focus on using a linker that facilitates the release of active and potent drugs upon internalization of the ADC.

1.1.3 Food and Drug Administration approved ADCs

The first ADC to gain approval in the United States (US) was gemtuzumab ozogamicin (Mylotarg) for the treatment of acute myeloid leukemia (AML). Gemtuzumab ozogamicin (shown in Figure 4) is composed of the humanized mAb hP67.6 directed against CD33 overexpressed on myeloblasts in patients with AML, and the semisynthetic calicheamicin N-acetyl-$\gamma$ calicheamicin 1,2-dimethyl hydrazine, both linked by an acid labile acetylphenoxybutanoic linker. Following internalization of the ADC, the calicheamicin and linker are hydrolytically cleaved by the low pH environment in the lysosome, yielding the active calicheamicin that then interacts with the minor groove of DNA to exert an apoptotic pathway.\textsuperscript{[21]}
Gemtuzumab ozogamicin was the first drug to prove the idea of ADCs in the clinic. Phase II studies showed thirty percent of patients that received the ADC obtained remission, as characterized by ≤5% blasts in the marrow, recovery of neutrophils to at least 1,500/µL, and transfusion independence.[22] Gemtuzumab ozogamicin was granted FDA marketing approval under accelerated conditions, indicated for the treatment of patients with CD33-positive AML in first relapse who were over 60 years of age and not able to be considered for chemotherapy.[21] Several follow-up Phase II studies noted an acceptable safety profile, opportunity for first-line therapy, and remarkable activity in relapsed acute promyelocytic leukemia.[22] However, in June 2010, after a decade on market, gemtuzumab ozogamicin was voluntarily withdrawn due to a narrow therapeutic window and a lack of target-dependence, resulting in a high rate of fatal toxicity.[23-24] Despite its withdrawal from the US market, gemtuzumab ozogamicin is still available in Japan with full regulatory approval. In light of the controversy surrounding this early
ADC, researchers found themselves well equipped with the knowledge necessary to design safer and more effective therapies.

Currently, there are two ADCs that have full approval by the FDA: Adcetris and Kadcyla. In 2011, brentuximab vedotin (Adcetris, Figure 5) gained FDA approval and is shown to be a selective and potent ADC against CD30+ anaplastic large cell lymphoma and Hodgkin’s disease.[25] Brentuximab vedotin was given accelerated FDA approval following a small but promising phase II clinical trial. In the study, 102 Hodgkin lymphoma patients were treated with the drug and 32% of patients showed complete remission within two years with an additional 40% of patients experiencing partial remission complete with 50% shrinkage in tumor size.[23-24]

![Figure 5: A schematic representation of brentuximab vedotin.](image)

The dipeptide linker, spacer, and MMAE are bound to brentuximab via a Michael type addition between a reactive maleimide moiety and the thiolate of a free cysteine residue. Due to the fact that the linker relies on a reduced disulfide bond, the drug-to-antibody ratio (DAR) for
brentuximab vedotin is observed in multiples of 2 from 0-8, with an average DAR of 4. Cleavage of the amide bond between the valine-citrulline (val-cit) dipeptide linker and the aromatic amine of the para-aminobenzyl alcohol (PABA) spacer by a protease, such as Cathepsin B, allows for the subsequent self [1,6]-elimination of the spacer, releasing the active MMAE (Scheme 1).[4]

Scheme 1: Mechanism of 1,6 Immolation of PABA spacer and consequential release of active MMAE.

Less than two years after FDA approval of brentuximab vedotin, trastuzumab emtansine (Kadcyla, Figure 6) gained approval for advanced human epidermal growth factor receptor 2 positive (HER2+) metastatic breast cancer. A phase I and three phase II trials produced promising results and lead to the development of a pivotal phase III trial that evaluated 991 patients. The results of this trial significantly favored trastuzumab emtansine over lapatinib (tyrosine kinase inhibitor) plus capecitabine (a prodrug that eventually acts as a suicide inhibitor via irreversible inhibition of thymidylate synthase) in the areas of objective response rate, progression-free survival, and overall survival.[23, 26-27]

Trastuzumab emtansine consists of the previously FDA approved mAb Trastuzumab (under the trade name Herceptin) attached to a (N-maleimidomethyl)cyclohexane-1-carboxylate (MCC) linker, a thioether, and a cytotoxic maytansinoid drug, DM-1. The conjugation occurs via succinimide displacement by the free amine of a lysine residue. Due to the large number of solvent-exposed lysines on a given mAb (84), a heterogeneous mixture of conjugations exist: from a DAR of 0 to a DAR of 9, with an average of 3.5 DM-1 molecules per antibody. Upon
cellular uptake of the ADC, lysosomal degradation of the antibody liberates the active payload: DM-1 conjugated to the thioether, MCC linker, and an added lysine residue.\textsuperscript{[23]}

![Diagram of trastuzumab emtansine and lysosomal degradation](image)

Figure 6: A schematic representation of trastuzumab emtansine and the released active drug, DM-1 with attached linker and lysine.

In addition to the biologics mentioned above, as many as 30 independent ADCs are currently undergoing clinical trials.\textsuperscript{[4, 7, 23]} For example, AMG 172 is being investigated in phase I trials to treat renal cell carcinoma.\textsuperscript{[28]} AMG 172 consists of a mAb specific for CD27L, a member of the tumor necrosis factor (TNF) family that is overexpressed in some tumor cell types, and the drug DM-1 linked via a non-cleavable linker, similar in construction to trastuzumab emtansine. ADCs represent a widely varied class of targeted therapies and offer the potential for a substantial improvement in how we face and treat diseases like cancer.
1.1.4 Characterization of ADCs

Due to the large heterogeneity and complexity of mAbs and ADCs, properly characterizing ADCs represents a major challenge. As ADCs represent a more complex structure than their corresponding mAb, analytical techniques employed must take into account the linker, drug, and site of attachment (e.g., lysines, reduced disulfides, or glycans). Methods of analyzing a parent mAb may not work for its corresponding ADC, and different techniques must be employed between ADCs. A further consideration that is important when analyzing ADCs is the type of information being analyzed. Major ADC analytical techniques include protein mass spectrometry, chromatography, and ultraviolet/visible light spectroscopy (UV/VIS) for the determination of DAR and drug distribution.

The simplest method of ADC analysis employs UV/VIS spectroscopy. The maximum absorbance values of the unconjugated mAb and the unconjugated drug must not be the same for this method to yield meaningful results. DAR can be calculated using the Beer-Lambert law with the applicable extinction coefficients and measured absorbance values. Despite the simplicity of this method, it is limited in applicability due to the fact that it can only determine the average DAR for the whole sample.

Isoelectric focusing gel electrophoresis (IEF) is an electrophoretic technique that separates species by charge based on their isoelectric point (pI). Conjugation through lysine side chains will change the positive charge profile of the mAb, thereby shifting the pI and allowing for separation by IEF. Gemtuzumab ozogamicin was found to have the expected average DAR 3 drugs per mAb, but was ultimately generated by approximately 50% unconjugated mAb and 50% conjugated with 6 drug molecules. IEF is a powerful tool, but relies on a change in the pI by the linker-drug moiety.
A powerful chromatography tool for determining drug load, hydrophobic interaction chromatography (HIC), relies on the increased hydrophobicity imparted on a mAb by the conjugated drug and linker. Separation with this method is achieved by decreasing the ionic strength of the buffer and monitoring with UV absorbance. The resulting HIC profile shows a number of peaks – corresponding to intact mAbs conjugated with increasing numbers of bound drug. Additionally, the chromatographic peaks can be weighted to determine the average drug load for the ADC.\[29\] HIC is an advantageous technique that does not degrade the conjugate, however, this technique is hampered by the high salt content of the mobile phases, making direct coupling to mass spectrometry impossible, and thereby limiting the sensitivity.

Mass spectrometry (MS) provides one of the most versatile technologies for studying ADCs at the structural level. Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) MS can detect mass shifts between parent mAb and ADC, confirming conjugation, but lacks the resolution to resolve individual species with different numbers of conjugated drugs.\[11\] Native MS allows for the intact analysis of ADCs while retaining noncovalent interactions and folded native conformation, while providing a smaller charge-state envelope that simplifies the spectra.\[32\] High mass resolution and high sensitivity systems employing electrospray ionization (ESI) MS technology makes characterization and structure elucidation studies of intact mAbs more efficient and speedy processes. Additionally, ESI-MS preserves the native protein structure and is amenable to intact disulfide-linked ADC analysis.\[11, 33-34\] Chromatographic techniques can be coupled to the MS to separate conjugates with various DARs and help increase resolution.\[11, 33\]

A second important piece of information that can be learned about ADC conjugation efficiency is drug-load distribution. In 2005, ESI-TOF-MS and peptide mapping was used to
assess the drug distribution and the individual drug conjugation sites of the maytansinoid DM1 to the mAb huN901.\textsuperscript{[35]} In this case, a mixture of conjugation sites was found spanning 20 different lysines, with approximately a double incidence of conjugation found on the heavy chain over the light chain.

1.2.1 Flow Chemistry

Conventional chemical synthesis relies on well-defined techniques and the use of standardized glassware (e.g., round-bottomed flasks and Erlenmeyer flasks). These conventional reactions are subject to a number of limitations, such as inefficient mixing of the reaction vessel and inconsistent temperature distribution. Many reactions also face challenges in the “scaling up” process.\textsuperscript{[36]} An ingenious breakthrough made to combat these issues was the invention of continuous flow microreactors, first introduced in 1990 by Strauss and co-workers.\textsuperscript{[37]} Continuous flow microreactors represent a method for consistent reproducibility. High surface area to volume ratios of reaction channels result in efficient mixing and heating as a reaction progresses. As a result, reaction times and reagent volumes are often reduced to a fraction of those needed for conventional reactions. Another advantage comes in terms of reaction scalability. Reactions in flow are “scaled out” rather than scaled up, in that a series of reactors can be stacked in parallel to afford larger quantities of a product.

Conventional chemistry techniques are still a hallmark of chemical synthesis, but flow chemistry is rapidly gaining popularity, especially at the industrial level.\textsuperscript{[38]} Some microreactor systems consist of a glass reactor chip, smaller in size than a credit card, with small channels that allow for solvent/reagent movement and equipped with a number of inlets and an outlet. An example of a continuous flow microreactor from Chemtrix ® is shown in Figure 7.
Labtrix Start ® system, syringe pumps are connected to the chip via inlets with PEEK tubing and dispense reagents through at a controllable flow rate (typically between 0.5 and 100 µL/min).[39] The chip holder is placed on top of a heating/cooling block that allows for even temperature control throughout the entire chip. A quench inlet is also available on many chip models to allow for reaction termination prior to reaching the collection vessel. More advanced models, such as the Labtrix S1® system is run by software that allows the user to set up recipes for automated collection. Collection directly into LC or GC vials by the autosampler simplifies transition to analytical platforms.

![Chemtrix Labtrix® Start system](image)

Figure 7: A Chemtrix Labtrix® Start system. (A) The chip sits in the black holder, with a product collection off to the right and the (B) inlets from the syringe pumps connected. (C) Represents the optional temperature regulation device.

Flow chemistry also has the distinct advantage of representing a shift in chemistry from “dirty” to “green.” The waste generated by a conventional chemical reaction is significantly greater than that produced in flow because flow chemistry allows for reactions to be optimized on a small scale. Flow reaction parameters can be tightly controlled, allowing for a higher product purity profile and an increased atom economy. Additionally, exposure to hazardous chemicals is greatly reduced in flow reactions – in the quantity (volume), time exposed, and the
number of potentially dangerous side-products generated. Finally, flow chemistry is inherently safer in regards to accident prevention.\textsuperscript{[40]}

Flow chemistry also is not limited in its applicability, evidenced by the following published reactions: Claisen re-arrangement, Diels-Alder, Mannich, Heck, C-N cross-coupling, Murashasi, as well as various catalytic and enantioselective reactions.\textsuperscript{[41-46]} The field of continuous flow chemistry has been growing over the past two decades and that growth is expected to continue, bolstered by the numerous advantages discussed above.

1.3.1 Application of Flow Chemistry in Antibody Drug Conjugates

Despite the ability for ADCs to optimize the therapeutic index, the heterogeneous nature of small molecule conjugation to the significantly larger mAbs offers clinical challenges. Site-specific conjugation techniques are currently still lacking, evidenced by the nonspecific acylation of lysine residues with activated esters in trastuzumab emtansine and the nonspecific alkylation of cysteine thiols with maleimides in brentuximab vedotin. Heterogeneous conjugations result in a distribution of species with varying DAR, each of which have distinct pharmacokinetic, toxicity, aggregation, Ag affinity, and drug release profiles.\textsuperscript{[47]}

Current methods for site-specific conjugation of ADCs include glycoconjugation and unnatural amino acid (UAA) incorporation. Glycoconjugation involves the conjugation of the cytotoxic payload to N-glycans located in the CH2 domain at the N297 residue. This location is far enough away from the variable region such that Ag binding is not affected and the conserved glycosylation site represents a convenient site-specific conjugation choice. However, glycosylation is a post-translational modification that results in heterogeneous mixtures, which poses challenges in the functionalization and conjugation of proteins.
Unnatural amino acid incorporation is another popular option for site-specific conjugation, relying on the ribosomal incorporation of UAAs into proteins. Choosing to conjugate through an UAA opens up new options for linker chemistry as the traditional lysine and cysteine conjugation methods are no longer limiting factors. However, a low DAR is seen with most UAA incorporation methods and the production of mAbs engineered to contain UAAs is technically difficult and costly.\cite{4, 47}

Equipped with this knowledge, our lab has investigated alternative bioconjugation techniques, specifically using continuous flow chemistry. Because of our previous success applying flow conditions to a variety of processes, we investigated the hypothesis that flow chemistry could yield consistent, homogeneous protein and antibody conjugations, as well as speed up the respective processes.\cite{48-49} This thesis focuses on the application of continuous flow chemistry in ADC conjugation reactions, including linker synthesis, flow reactions and optimization reactions, and ADC analytical techniques.
Results and Discussion

1.4.1 Proof of Principle with N-succinimidyl-4-fluorobenzoate (FSB)

As a first proof of principle study, several protein standards were conjugated with the small molecule probe N-succinimidyl-4-fluorobenzoate (FSB). FSB is an organofluorine compound that can act as a positron emission topography (PET) imaging agent by the incorporation of the fluorine-18 radioisotope.\[50\] The short half-life of fluorine-18 (~120 minutes) necessitates an expeditious synthetic route which is satisfied by the fast conjugation times of continuous flow reactions. Protein standards examined in these proof-of-principle conjugation reactions include human serum albumin, insulin, interferon, and myoglobin. Reaction conditions were modified to study the effect of various times, temperatures, and the presence of the base triethylamine (TEA). Scheme 2 shows an example conjugation reaction, with a solvent accessible lysine residue randomly chosen and shown in green.

![Scheme 2: FSB conjugation to equine myoglobin (PDB ID: 4NS2).](image)

Analysis of the number of FSB tags per protein was performed on a LC-ESI-MS and Table 2 shows the results of these preliminary conjugation reactions. We initially hypothesized that the addition of a base, such as TEA, would increase the rate of reaction. However, no positive change in the rate of the reaction was seen in the presence of TEA, and it was determined that the slightly basic nature of the buffer (pH=8) was sufficient for the reaction to
proceed. Temperature seems to play a major role in accelerating the conjugation, but care must be maintained to ensure protein structural integrity. The addition of microwave irradiation has been found to increase the rate of chemical conversion while working with other sensitive substrates in our lab.\textsuperscript{[51]} Equipped with this knowledge, we investigated microwave accelerated conjugation reactions and noted a positive effect on reaction times and conversions.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Protein</th>
<th>Temperature (°C)</th>
<th>Time (minutes)</th>
<th>TEA</th>
<th>FSB tags added</th>
<th>% Labeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HSA</td>
<td>25</td>
<td>30</td>
<td>-</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>HSA</td>
<td>25</td>
<td>30</td>
<td>+</td>
<td>0</td>
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<td>3</td>
<td>INS</td>
<td>25</td>
<td>30</td>
<td>-</td>
<td>2</td>
<td>42</td>
</tr>
<tr>
<td>4</td>
<td>INS</td>
<td>25</td>
<td>30</td>
<td>+</td>
<td>1</td>
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</tr>
<tr>
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<td>INS</td>
<td>50</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>53</td>
</tr>
<tr>
<td>6</td>
<td>INS</td>
<td>50</td>
<td>5</td>
<td>-</td>
<td>2</td>
<td>56</td>
</tr>
<tr>
<td>7</td>
<td>INT</td>
<td>25</td>
<td>30</td>
<td>-</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>8</td>
<td>INT</td>
<td>25</td>
<td>30</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>MYO</td>
<td>25</td>
<td>30</td>
<td>-</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>10</td>
<td>MYO</td>
<td>25</td>
<td>30</td>
<td>+</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>11</td>
<td>MYO</td>
<td>25</td>
<td>360</td>
<td>-</td>
<td>4</td>
<td>95</td>
</tr>
<tr>
<td>12</td>
<td>MYO</td>
<td>25</td>
<td>360</td>
<td>+</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>13</td>
<td>MYO</td>
<td>25</td>
<td>5</td>
<td>-</td>
<td>3</td>
<td>61</td>
</tr>
<tr>
<td>14</td>
<td>MYO</td>
<td>50</td>
<td>5</td>
<td>-</td>
<td>2</td>
<td>42</td>
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<tr>
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<td>1</td>
<td>-</td>
<td>3</td>
<td>62</td>
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<td>MYO</td>
<td>50</td>
<td>5</td>
<td>-</td>
<td>3</td>
<td>62</td>
</tr>
<tr>
<td>17</td>
<td>MYO</td>
<td>25</td>
<td>&lt;1</td>
<td>-</td>
<td>2</td>
<td>&gt;99</td>
</tr>
<tr>
<td>18</td>
<td>BSA</td>
<td>25</td>
<td>&lt;1</td>
<td>-</td>
<td>2</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

Table 2: FSB conjugation to several protein standards under various conditions. Note: all reactions carried out with 3 equivalents of FSB. \textsuperscript{a}These reactions were performed under microwave irradiation. \textsuperscript{b}These reactions were performed under continuous flow conditions. HAS=human serum albumin, INS=insulin, INT=interferon, MYO=myoglobin, BSA=bovine serum albumin.

To ensure protein compatibility with our continuous flow system, conjugation reactions were performed on myoglobin and bovine serum albumin under continuous flow conditions. Within 1 minute at room temperature, derivative protein was found for both proteins investigated. These promising results showed that continuous flow chemistry can be used to
derivatize proteins, while elucidating the applicability of continuous flow in the expeditious synthesis of time-sensitive products, such as fluorine-18 containing PET imaging agents. **Figure 8** shows a representative deconvoluted mass spectrum and a full mass spectrum for the flow synthesis of myoglobin with FSB.

![Mass spectrum](image)

**Figure 8:** Representative deconvoluted mass spectrum (top panel) and full mass spectrum (bottom panel) for the flow synthesis of myoglobin with FSB
1.4.2 Comparative studies using BSA and fluorescein isothiocyanate (FITC)

With the feasibility of small-molecule derivatization of proteins established under continuous flow conditions, we examined the conjugation of the highly fluorescent probe FITC to the reagent protein BSA. Performing these reactions in both conventional and continuous flow conditions was hypothesized to allow for a greater understanding of the advantages that continuous flow held in regards to conjugation efficiency and simplicity. FITC couples to BSA via lysine conjugation to the isothiocyanate group, represented below in Scheme 3. Additionally, FITC displays a high fluorescence intensity, allowing for reaction monitoring via spectrophotometric methods and application of the Beer-Lambert Law.

![Scheme 3: FITC conjugation to BSA (PDB ID: 3V03).](image)

Under both conventional and continuous flow conditions, reaction time, temperature, and the number of equivalents of FITC were varied. In an attempt to prevent protein degradation, temperatures were maintained between 4°C and 37°C. Flow reactions were performed on a
Chemrix ® #3223 microreactor with two inlets, one quench, and one outlet. A representative display of the chip used can be seen in Figure 9. The average number of FITC tags added to each protein was calculated using UV/VIS spectrophotometry following reaction purification. All reactions were performed in triplicate and the results can be seen in Table 3.

Figure 9: A representation of the microreactor used in the FITC-BSA conjugation reactions.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Temp (°C)</th>
<th>Equiv. FITC</th>
<th><strong>Conventional</strong></th>
<th><strong>Flow</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Time (minutes)</td>
<td>Avg. dye per protein (mol)</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>3</td>
<td>30</td>
<td>1.35</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>5</td>
<td>30</td>
<td>1.29</td>
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<tr>
<td>3</td>
<td>4</td>
<td>10</td>
<td>30</td>
<td>1.55</td>
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<tr>
<td>4</td>
<td>3</td>
<td>3</td>
<td>240</td>
<td>0.91</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>5</td>
<td>240</td>
<td>1.06</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>10</td>
<td>240</td>
<td>1.48</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>3</td>
<td>720</td>
<td>0.00</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>5</td>
<td>720</td>
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<tr>
<td>9</td>
<td>4</td>
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<td>720</td>
<td>1.74</td>
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<tr>
<td>10</td>
<td>23</td>
<td>3</td>
<td>30</td>
<td>1.31</td>
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<tr>
<td>11</td>
<td>23</td>
<td>5</td>
<td>30</td>
<td>1.45</td>
</tr>
</tbody>
</table>
The results of these experiments were encouraging; continuous flow techniques show better correlations and provided an accelerated method for protein conjugation when compared with conventional reaction conditions. Although increases in the residence time did not uniformly result in increases in protein tagging under flow conditions, it was shown that increasing the temperature slightly increased tagging in general. Interestingly, the most pronounced effect was seen upon changing the ratio of FITC to BSA (as observed in Entries 19, 20, and 21). The degree of conjugation is less dependent on reaction time and more dependent on a sufficient ratio of small-molecule probe to protein. This experiment once again suggested that continuous flow chemistry provides a more facile and timely route to protein derivatization when compared with conventional reaction conditions.

1.4.3 Conjugation of FSB to infliximab under conventional and flow conditions

Following the promising results from the FITC-BSA conjugation reactions, continuous flow methodology was applied to the derivatization of a therapeutic antibody. Infliximab
(Remicade ®) is a therapeutic, chimeric antibody used in the treatment of rheumatoid arthritis and inflammatory bowel disease, specifically Crohn’s disease.[52] Conjugation of FSB to infliximab was assessed under both conventional and continuous flow reactions, as shown in Scheme 4 and Figure 10.

**Scheme 4:** The conjugation of FSB to infliximab.

**Figure 10:** A representation of the microreactor used in the FSB-infliximab conjugation reactions.
Early attempts to conjugate infliximab to FSB were met with difficulty. Due to acetonitrile’s miscibility with water, we thought that combining the mAb in PBS and FSB in acetonitrile would be a viable option for reaction conditions. However, the microreactor quickly became clogged when the protein mixed with the acetonitrile and precipitated out of solution. We decided that to avoid aggregation we needed to investigate a solvent that was organic enough to reconstitute the FSB while retaining miscibility with water. DMSO was chosen and the reaction details and results are discussed below.

Under continuous flow conditions, infliximab dissolved in PBS and FSB dissolved in DMSO were combined on the microreactor at equal flow rates under various conditions, including residence time, temperature, and equivalents of FSB. Similar reaction parameters were investigated for conventional methods. Upon reaction completion, the samples were purified and the degree of conjugation was determined by reduction of the intact mAb to the heavy chain (HC) and light chain (LC), and subsequent analysis using LC-MS with electrospray ionization. Table 4 shows the results for conjugation reactions performed under conventional methods. Conjugation appears to be more dependent on the ratio of FSB to mAb than on the length of the reaction, and an increase in temperature shows efficient tagging on both the HC and LC.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Temp (°C)</th>
<th>Equivalents FSB</th>
<th>Time (hours)</th>
<th>FSB molecules added (LC)</th>
<th>FSB molecules added (HC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>3</td>
<td>1</td>
<td>1</td>
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<td>37</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 4: Conjugation of FSB to infliximab under conventional methodology.
Similarly, conjugation reactions repeated under optimized continuous flow conditions resulted in controlled derivatization within 1 minute as shown in Table 5. Specifically, using 10 equivalents at ambient temperature or 5 equivalents at 37°C resulted in an addition of 2 FSB molecules in 1 minute. A representative deconvoluted mass spectrum and full mass spectrum of the light chain for the flow synthesis of infliximab with FSB is shown in Figure 11.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Temp (°C)</th>
<th>Equivalents FSB</th>
<th>Residence time (seconds)</th>
<th>FSB molecules added (LC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>5</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
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<tr>
<td>6</td>
<td>37</td>
<td>10</td>
<td>60</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 5: Conjugation of FSB to infliximab under continuous flow conditions.
Figure 11: A representative deconvoluted mass spectrum (top panel) and full mass spectrum (bottom panel) of the light chain for the flow synthesis of infliximab with FSB
Methods

1.5.1 Synthetic Procedures and Analytical Techniques

**Analytical methods for Table 2**

After mass tagging, protein samples (200-500 pmols) were injected into an Alltech (Deerfield, IL) analytical in-line guard column, packed with POROS 20-R2 reversed-phase media (PerSeptive Biosystems). The column was connected to a Shimadzu SCL-10A VP HPLC flow water containing 0.05% formic acid, pH 2.6 at 50 μL/min coupled to a Waters LCT Premier mass spectrometer with a standard electrospray interface. Proteins were desalted (with water containing 0.5 % formic acid, pH 2.6) and then eluted directly into the mass spectrometer with a gradient of 15-98% acetonitrile (containing 0.05% formic acid, pH 2.6) in five minutes. Intact mass spectra were deconvoluted, and the ratio of modified to unmodified protein tested was determined using the software MagTran (Zhang, 1998). All intact MS experiments were conducted at least twice.

**Synthesis of N-succinimidyl-4-fluorobenzoate [FSB]**

To a solution of 4-fluorobenzoic acid (2.0 g, 14.0 mmol) and triethylamine (2.89 g, 29.0 mmol) in acetonitrile (100 mL) was added disuccinimidyl carbonate (4.02 g, 16.0 mmol). The reaction was heated to 80ºC for 2 h, at which time the solvent was removed *in vacuo*. The resulting oil was diluted with a 1% sodium bicarbonate solution (100 mL) and extracted with chloroform (4 x 75 mL). The organic extracts were combined, dried over magnesium sulfate, and concentrated *in vacuo*. The reaction mixture was purified by silica gel chromatography (70:30 hexanes: EtOAc) to afford the product as a white solid (3.32 g, 60%). $^1$H NMR (400 MHz, CDCl$_3$): 8.16 (m, 2H), 7.20 (m, 2H), 2.9 (br s, 4H); $^{13}$C NMR (100 MHz, CDCl$_3$): 169.33, 168.18, 165.61, 133.46, 121.44, 116.27, 25.72.
Batch synthesis of FITC-BSA

To a solution of bovine serum albumin in phosphate buffer solution pH 8 (50 μL, 10 mg/mL) was added a 3, 5 or 10 equivalent solution of fluorescein isothiocyanate in ACN (50 μL). The reaction was stirred at 4, 23, or 37°C for 30 min, 4 h, or 12 h. The 27 experiments were immediately diluted with 400 μL of PBS pH 8, and a size-exclusion NAP5 column (Sephadex G-25 DNA grade) was used to remove any unreacted FITC. The sample was eluted with 1.0 mL of PBS to afford 100 μL of the reaction mixture. A spectrophotometer was used to analyze the samples at wavelengths of 288 and 493. The samples were diluted to a total volume of 1 mL for analysis. Calculations were completed based on the Beer-Lambert law.
Flow synthesis of FITC-BSA

To a Labtrix® T-mixer chip (#3223), the following solution was introduced: bovine serum albumin in phosphate buffer solution pH 8 (10 mg/mL) and either a 3, 5, or 10 equivalent solution of fluorescein isothiocyanate in ACN (50 μL). The reaction was performed at 4, 23, or 37°C and 1- 10 μL/min per syringe. Reaction mixtures were collected in 20 μL aliquots and diluted to 100 μL of PBS pH 8. A size-exclusion NAP5 column (Sephadex G-25 DNA grade) was used to remove any unreacted FITC. The sample was eluted with 1.0 mL of PBS to afford 100 μL of the reaction mixture. A spectrophotometer was used to analyze the samples at wavelengths of 288 and 493. The samples were diluted to a total volume of 1 mL for analysis. Calculations were completed based on the Beer-Lambert law.

Synthesis of FSB-myoglobin

To a solution of myoglobin (2 mg, 1 equivalent) in phosphate buffer solution (pH 8, 1 mL) was added N-succinimidyl-4-fluorobenzoate (FSB) (2 mg/mL in ACN, 3 equivalents). The reaction was stirred at room temperature for 5 min, 30 min, or 360 min, and immediately frozen or analyzed by ESI-MS.
Microwave synthesis of FSB-myoglobin

To a solution of myoglobin (2 mg, 1 equivalent) in phosphate buffer solution (1 mL, pH 8) was added \( N \)-succinimidyl-4-fluorobenzoate **FSB** (2 mg/mL in ACN, 3 equivalents). The reaction was heated to 50°C in a CEM Discover SP (Matthews, NC) for 1 and 5 minutes, and immediately frozen or analyzed by ESI-MS.

Synthesis of FSB-myoglobin

To a solution of myoglobin (2 mg, 1 equivalent) in phosphate buffer solution (pH 8, 1 mL) was added \( N \)-succinimidyl-4-fluorobenzoate (2 mg/mL in ACN, 3 equivalents). The reaction was stirred at 50°C for 5 min, and immediately frozen or analyzed by ESI-MS.

Synthesis of FSB-myoglobin with base

To a solution of myoglobin (2 mg, 1 equivalent) in phosphate buffer solution (pH 8, 1 mL) was added \( N \)-succinimidyl-4-fluorobenzoate (2 mg/mL in ACN, 3 equivalents) and triethylamine (2 μL). The reaction was stirred at room temperature for 30 min or 360 min, and immediately frozen or analyzed by ESI-MS.
Synthesis of FSB-insulin

To a solution of insulin (2 mg, 1 equivalent) in phosphate buffer solution (pH 8, 1 mL) was added N-succinimidyl-4-fluorobenzoate (2 mg/mL in ACN, 3 equivalents). The reaction was stirred at room temperature for 30 min, and immediately frozen or analyzed by ESI-MS.

Microwave synthesis of FSB-insulin

To a solution of insulin (2 mg, 1 equivalent) in phosphate buffer solution (1 mL, pH 8) was added N-succinimidyl-4-fluorobenzoate (2 mg/mL in ACN, 3 equivalents). The reaction was heated to 50°C in a CEM Discover SP (Matthews, NC) for 1 and 5 minutes, and immediately frozen or analyzed by ESI-MS.
Synthesis of FSB-insulin with base

To a solution of insulin (2 mg, 1 equivalent) in phosphate buffer solution (pH 8, 1 mL) was added $N$-succinimidyl-4-fluorobenzoate (2 mg/mL in ACN, 3 equivalents) and triethylamine (2 μL). The reaction was stirred at room temperature for 30 min, and immediately frozen or analyzed by ESI-MS.

Synthesis of FSB-interferon-2α

To a solution of interferon-2α (2 mg, 1 equivalent) in phosphate buffer solution (pH 8, 1 mL) was added $N$-succinimidyl-4-fluorobenzoate (2 mg/mL in ACN, 3 equivalents). The reaction was stirred at room temperature for 30 min, and immediately frozen or analyzed by ESI-MS.
Synthesis of FSB-interferon-2α with base

To a solution of interferon-2α (2 mg, 1 equivalent) in phosphate buffer solution (pH 8, 1 mL) was added N-succinimidyl-4-fluorobenzoate (2 mg/mL in ACN, 3 equivalents) and triethylamine (2 μL). The reaction was stirred at room temperature for 30 min, and immediately frozen or analyzed by ESI-MS.

Synthesis of FSB-human serum albumin

To a solution of human serum albumin (2 mg, 1 equivalent) in phosphate buffer solution (pH 8, 1 mL) was added N-succinimidyl-4-fluorobenzoate (2 mg/mL in ACN, 3 equivalents). The reaction was stirred at room temperature for 30 min, and immediately frozen or analyzed by ESI-MS.
Synthesis of FSB-human serum albumin with base

To a solution of human serum albumin (2 mg, 1 equivalent) in phosphate buffer solution (pH 8, 1 mL) was added N-succinimidyl-4-fluorobenzoate (2 mg/mL in ACN, 3 equivalents) and triethylamine (2 μL). The reaction was stirred at room temperature for 30 min, and immediately frozen or analyzed by ESI-MS.

Flow synthesis of FSB-myoglobin

To a Labtrix® T-mixer chip (#3025), the following solution was introduced: myoglobin in phosphate buffer solution pH 8 (2 mg/mL) and either 3 equivalent solution of FSB in ACN. The reaction was performed at RT and 0.5 - 20 μL/min per syringe. Reaction mixtures were collected in 100 μL aliquots and frozen for analysis at Novartis Institutes for Biomedical Research.
Conventional synthesis of infliximab-fluorobenzoate

To a solution of Infliximab (0.005 g, 1 equivalent) in phosphate buffer solution (pH 8) was added a 2 mg/mL solution of N-succinimidyl-4-fluorobenzoate in acetonitrile (12 μL). The reaction was stirred at room temperature for 1 h, and then immediately frozen.

Flow synthesis of infliximab-fluorobenzoate

To a Labtrix® T-mixer chip (#3023), the following solution was introduced: infliximab in phosphate buffer solution pH 8 (2 mg/mL) and either 5 or 10 equivalent solution of FSB in DMSO. The reaction was performed at 23 or 37°C and 0.5 - 5 μL/min per syringe. Reaction mixtures were collected in 50 μL aliquots and frozen for analysis at Novartis Institutes for Biomedical Research.
To a solution of L-lysine (2 g, 13.68 mmol) in water (100 mL) were added allylchloroformate (727 µL, 6.84 mmol) and 1 N sodium hydroxide (until pH reached 11) at 0°C. After stirring for 1 hour and 20 minutes at room temperature, allylchloroformate (727 µL, 6.84 mmol) and 1 N sodium hydroxide (9 drops) were added to the mixture at 0°C once more. After stirring for 6 hours and 30 minutes at room temperature, the reaction mixture was extracted with dichloromethane (100 mL x 3). The aqueous layer was then concentrated in vacuo, reconstituted in ethanol and the insoluble byproduct was filtered. The filtrate was concentrated in vacuo to afford the product as a white solid (600 mg, 19%). $^1$H NMR (400 MHz, D$_2$O): 5.79 (m, 1 H), 5.16 (d, 1H), 5.09 (d, 1H), 4.42 (s, 2H), 3.49 (dd, 1H), 2.84 (t, 2H), 1.74 (m, 2H), 1.55 (m, 2H), 1.32 (m, 2H), 1.01 (t, 2H). HRMS (EI, M$^+$), m/z C$_{10}$H$_{18}$N$_2$O$_4$, calcd. 231.1345, obsd. 231.1343.
Synthesis of 2,5-dioxopyrrolidin-1-yl ((benzyloxy)carbonyl)-D-phenylalaninate [N-Cbz-L-Phe-OSu]

\[
\begin{align*}
\text{phenylalanine} & \quad \text{N-hydroxysuccinimide} \\
\text{THF} & \quad \text{DCC}
\end{align*}
\]

A stirred solution of z-protected phenylalanine (2.5g, 8.3520 mmol) and N-hydroxysuccinimide (1.06 g, 9.1875 mmol) in tetrahydrofuran (25 mL) at 0°C was treated with N,N'-dicyclohexylcarbodiimide (1.81 g, 8.7696 mmol). After 15 minutes at 0°C, the reaction mixture was allowed to warm to room temperature and was stirred for 16 hours. A solid dicyclohexyl urea (DCU) byproduct was filtered and the filtrate concentrated in vacuo. The thick, colorless oil was dissolved in dichloromethane (20 mL) and the mixture was allowed to stand for 1 hour to allow any further byproduct precipitation. The DCU was filtered and the filtrate was concentrated in vacuo. The glassy solid that was dried in vacuo for 3 hours which afforded the product as an off-yellow foam (2.86 g, 86.67%). \(^1\)H NMR (400 MHz, CDCl₃): 7.26 (m, 10H), 5.22 (m, 2H), 5.06 (m, 1H), 3.28 (m, 2H), 2.80 (s, 4H). \(^{13}\)C NMR (100 MHz, CDCl₃):168.788, 167.610, 155.462, 136.015, 134.449, 129.744, 129.554, 128.862, 128.641, 128.383, 128.292, 127.585, 67.407, 53.046, 38.040, 25.671. HRMS (EI, M⁺Na), m/z C₂₁H₂₀N₂O₆Na, calcd. 419.1219, obsd. 419.1214.
Synthesis of $N^6$-(allyloxy)carbonyl-$N^2$-((benzyloxy)carbonyl)phenylalanyl)-D-lysine [Alloc-Lys-Cbz-Phe]

To a solution of N-Cbz-L-Phe-OSu (1.086 g, 2.74 mmol) stirring vigorously in dimethoxyethane (20 mL) at 0°C was added a mixture of Alloc-Lys (600 mg, 2.608 mmol) and sodium bicarbonate (460 mg, 5.477 mmol) in water (10 mL). The mixture was maintained at 0°C for 30 minutes and then was allowed to stir at room temperature for 18 hours. The DCU byproduct was filtered and the filtrate was diluted with water (25 mL), forming a white precipitate. The thick mixture was acidified to pH 3 with 15% aqueous citric acid (~15 mL). Next, the product was extracted with ethyl acetate (100 mL x 3) and the combined organic layers were washed with brine (150 mL x 1), dried over magnesium sulfate, and concentrated in vacuo. The flask was then treated with ether and a white solid precipitated with sonication. The solution was heated to 50°C, allowed to cool, then filtered to collect pure product (340 mg, 26%). $^1$H NMR (400 MHz, CDCl$_3$): 7.28 (m, 10H), 5.68 (d, 1H), 5.21 (q, 2H), 5.09 (2H, s), 4.57 (s, 2H), 4.43 (br s, 2H), 3.09 (m, 2H), 1.91, 1.80, 1.71, and 1.29 (m, 6H). HSMS (EI, H$^+$), m/z C$_{27}$H$_{34}$N$_3$O$_7$, calcd. 512.2397, obsd. 512.2396.
Synthesis of (2R)-2-(2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-5-ureidopentanoic acid [Cit-Cbz-Phe]

To a stirred solution of N-Cbz-L-Phe-OSu (1 g, 2.52 mmol) in dimethoxyethane (10 mL) was added a mixture of citrulline (663 mg, 3.78 mmol) and sodium bicarbonate (317.55 mg, 3.78 mmol) in water (10 mL). The temperature was maintained below 25°C with a cold water bath for the first 30 minutes, and then the reaction was allowed to stir vigorously at room temperature for 16 hours. To quench the reaction, water (20 mL) with 2 mL saturated sodium bicarbonate was added and the aqueous layer was washed with ethyl acetate (70 mL) then acidified to pH 3 with 10% aqueous hydrochloric acid. The resulting suspension was extracted with 10% butanol in ethyl acetate (75 mL x 3). The organic layers were combined, dried over magnesium sulfate, filtered, and concentrated in vacuo to yield the product as a white solid (1.14 g, 99%). 

$^{1}$H NMR (400 MHz, CD$_2$OD): 8.28 (s, 1H), 7.24 (m, 10H), 5.01 (m, 2H), 4.41 (m, 2H), 3.11 (m, 3H), 2.83 (q, 2H), 1.89 (m, 1H), 1.70 (m, 1H), 1.53 (m, 2H). (Butanol impurity at 0.93, 1.36, 1.53; DME impurity at 3.31, 3.54; MeOH impurity at 3.31; water impurity at 4.86). HSMS (EI, H$^+$), m/z C$_{23}$H$_{29}$N$_4$O$_6$, calcd. 457.2087, obsd. 457.2075.
Synthesis of allyl ((5S)-5-(2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-6-((4-(hydroxymethyl)phenyl)amino)-6-oxohexyl)carbamate [Alloc-Lys-Cbz-Phe-PAB]

\[
\text{HN} \quad \text{HN} \quad \text{O} \quad \text{O} \quad \text{OH} \\
\text{HN} \quad \text{HN} \quad \text{O} \quad \text{O} \quad \text{NH} \quad \text{OH} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \\
\text{EEDQ} \quad \text{THF}
\]

A stirred solution of Alloc-Lys-Cbz-Phe (300 mg, 0.587 mmol) and para-aminobenzyl alcohol (75.83 mg, 0.616 mmol) in tetrahydrofuran (8 mL) was treated with N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (152.33 mg, 0.616 mmol). The reaction was allowed to stir in the dark at room temperature for 17 hours. The resultant mixture was concentrated in vacuo and triturated with ether (15 mL) and sonicated. The yellow solid was filtered and collected to afford the product (285 mg, 78.73%). ¹H NMR (400 MHz, CDCl₃): 7.54 (m, 4H), 7.28 (m, 10H), 5.71 (m, 1H), 5.08 (s, 2H), 4.60 (d, 2H), 4.45 (m, 2H), 4.35 (m, 2H), 4.16 (s, 2H), 3.73 (s, 2H), 3.05 (m, 2H), 1.87, 1.69, 1.38, and 1.23 (m, 6H). HSMS (EI, H⁺), m/z C₃₄H₄₁N₄O₇, calcd. 617.2975, obsd. 617.2961.
Synthesis of allyl ((5S)-5-(2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-6-(((4-(((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-6-oxohexyl)carbamate [Alloc-Lys-Cbz-Phe-PAB-PNP]

A stirred solution of Alloc-Lys-Cbz-Phe-PAB (175 mg, 0.284 mmol) in dry tetrahydrofuran (5 mL) was treated with dry pyridine (275.8 µL, 0.341 mmol) and para-nitrophenyl chloroformate (68.6 mg, 0.341 mmol) at room temperature. After 5 hours, thin-layer chromatography indicated formation of a number of side products so the reaction was quenched via the addition of ethyl acetate (25 mL) and 10% aqueous citric acid (25 mL). The organic layer was collected, washed with brine (25 mL) and water (25 mL), dried over magnesium sulfate, filtered, and concentration in vacuo to yield an impure yellow solid. The product was isolated by flash chromatography on silica gel, eluting with 50:1 CH₂Cl₂/MeOH (92 mg, 41.5%). ¹H NMR (400 MHz, CDCl₃): 8.82 (br. s, 1H), 8.23 (d, 1H), 8.07 (d, 2H), 7.55 (d, 1H), 7.27 (m, 10H), 6.87 (d, 4H), 5.96 (m, 1H), 5.84 (m, 1H), 5.63 (m, 1H), 5.29 (s, 2H), 5.22 (q, 2H), 5.04 (s, 2H), 4.49 (d, 2H), 4.26 (m, 2H), 3.06 (m, 4H), 1.84, 1.71, 1.40, and 1.26 (m, 6H). ¹³C NMR (100 MHz, CDCl₃): 172.73, 171.63, 162.93, 155.77, 152.72, 145.57, 141.13, 136.03, 135.80, 132.22, 130.95, 129.89, 129.37, 128.85, 128.17, 127.56, 126.45, 125.57, 122.07, 120.71, 118.74, 115.95, 70.73, 67.80, 66.76, 56.99, 53.75, 38.75, 31.55, 29.94, 28.60, and 22.63. MS (H)⁺, m/z C₄₁H₄₄N₅O₁₁, calcd. 781.30, obsd. 782.13 (MH)⁺, 804.12 (M + Na)⁺.

For NMR spectra please see Appendix.
Conclusions and Future Work

1.6.1 Controlled derivatization of ADCs under optimized continuous flow conditions

Proof of principle protein tagging experiments under continuous flow conditions encouraged us to examine the applicability of conjugations involving the therapeutic antibody infliximab. The expeditious conjugation of FSB, an organofluorine tag with implications in PET imaging, to infliximab via continuous flow methodology provided the required impetus to examine the controlled conjugation of a cytotoxic payload to infliximab.

Currently, we are synthesizing a dipeptide linker (phenylalanine-citrulline and phenylalanine-lysine) equipped with a self-immolative \textit{para}-aminobenzyl spacer attached to the well-characterized drug doxorubicin. Upon completion of this synthesis, controlled conjugations will be performed under continuous flow conditions and the samples will be analyzed by LC-ESI-MS. A representative synthetic route for the dipeptide linker and doxorubicin moiety is shown below in Scheme 5.

![Scheme 5](image-url)

Scheme 5: A representative procedure for the synthesis of a phenylalanine-lysine linker.
References


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Appendix
Chemical Shift (ppm)

(2)
Chemical Shift (ppm)

(3)
(5)
Chemical Shift (ppm)

(6)