Application Note
SEP 2024
AN-850-0145

Structural Characterization of Antibody Drug Conjugates as Formulated and Under Multiple Conjugation Paradigms

- ○ Biosimilars
- ✓ mAbs
- ✓ ADCs
- ○ AAVs
- ○ Ligand Binding
- ✓ Protein/Peptide Analysis
- ○ VLPs
- ○ Nucleic Acid
- ○ Fusion Proteins
- ○ Enzyme Analysis

- ⬢ Aggregation
- ⬢ Quantitiation
- ✓ Structure
- ✓ Stability
- ✓ Similarity

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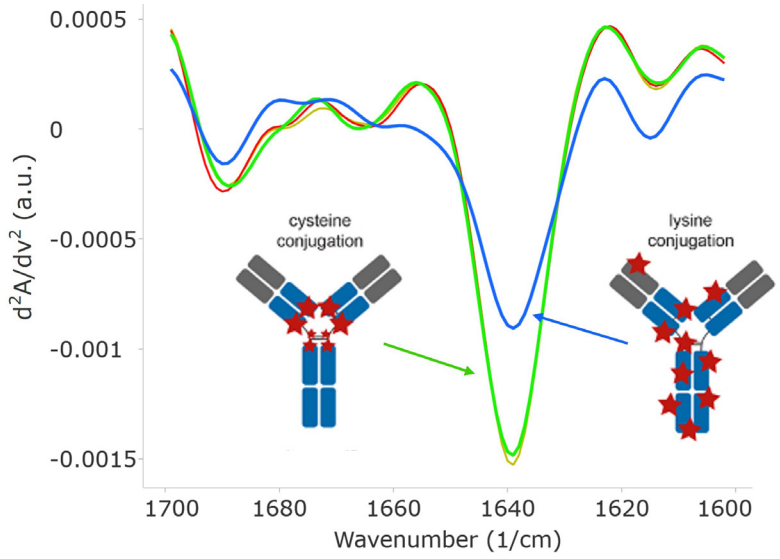
Abstract

Antibody Drug Conjugates (ADCs) combine the specificity of a monoclonal antibody (mAb) with the cytotoxicity of small molecule therapeutics. Biophysical characterization, including the analysis of the conformation and higher-order structure (HOS) of the mAb before and after drug conjugation, is a critical aspect to understanding the stability and eventual efficacy of the ADC. In this study, Trastuzumab-based ADCs are studied using MMS, a novel infrared-based biophysical technique. ADCs produced by two different conjugation methods are structurally compared using the MMS data. Results reveal significant structural discrepancies from the different conjugation methods. These structural differences ultimately contribute to a change in thermal stability of the ADCs measured by Differential Scanning Calorimetry (DSC).



Introduction

Antibody Drug Conjugates (ADCs) are an emerging class of biopharmaceutical drugs for cancer treatment due to their high specificity and targeting capability. Because drug delivery is directed to the cancer cells, dosages can be low and systemic toxicity is minimized. As a result, ADCs enable the use of more potent drugs than can be delivered by conventional chemotherapy with a more acceptable side effect profile for patients.

Maintenance of the native structure of the monoclonal antibody (mAb) within the conjugate is critical to the success of an ADC since this underpins the targeting efficiency and stability of the full construct. Effective technology for the characterization of protein structure is therefore vital for development. Microfluidic Modulation Spectroscopy (MMS) is a novel infrared-based biophysical technique, developed and commercialized by RedShiftBio. MMS combines a quantum cascade laser (QCL) with a microfluidic optical flow cell to provide robust, hands-free protein secondary structure analysis with much greater sensitivity than traditional technologies, over a wide concentration range (0.1 to over 200 mg/mL) and under otherwise challenging conditions for spectroscopic techniques. [1,2]

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Introduction, continued

In this study, we characterized a commercial mAb, Trastuzumab, directly in its formulation buffer at 0.825 mg/mL utilizing MMS to understand the higher order structure for this important mAb therapeutic. In addition, we characterized Trastuzumab at a variety of different drug antibody ratios utilizing both non-specific labeling and click chemistries for the drug conjugation, to understand their effect on the mAb structure. Finally, we compared the MMS results to an orthogonal biophysical technique, Differential Scanning Calorimetry (DSC), to evaluate the impact of any structural changes associated with the different conjugation methods on the thermal stability of these ADCs.

Methods

Trastuzumab (0.825 mg/mL), T-Cys-MMAE (0.842 mg/mL), T-Cys-Geld (0.736 mg/mL), and T-Lys-DM1 (0.785 mg/mL) in 5 mM histidine buffer at pH 6 with 2% w/v trehalose and 0.009% w/v polysorbate-20 were analyzed at room temperature using the AQS^{3}pro system. Samples were run in triplicate at a modulation rate of 1 Hz and backing pressure of 5 psi. Higher order structure (HOS) components of the samples were determined using the delta analytical software package.

Thermal stability analysis was performed using the MicroCal VP-Capillary DSC (Malvern). All samples were analyzed at 0.5 mg/mL in the formulation buffer. The scan range of the analysis was 15-95 °C at a heating rate of 60 °C/h. Data were buffer subtracted and baselined corrected and the temperature corresponding to the apex of endothermic transitions was defined as apparent T_m.

Results and Discussion

There are two general routes to conjugate the therapeutic payload to the antibody for ADC synthesis (Figure 1). The lysine-based conjugation relies on the nucleophilic amines of the lysine residues to react with the carboxyl group on a drug after activation by reagents such as N-hydroxysuccinimide (NHS). The cysteine-based conjugation relies on the thiols of the cysteine residues to react directly to maleimides after the disulfide bonds are reduced. LC-MS results showed that the lysine-based conjugation produced a heterogeneous population of ADCs with a wide range of drug-to-antibody ratios (DAR).[3] The cysteine-based conjugation, on the other hand, showed a much narrower DAR distribution. Despite having a wider range of DAR distribution, the lysine-based conjugates are still the most common ADCs in both clinical trials and the market due to the simpler synthetic route.

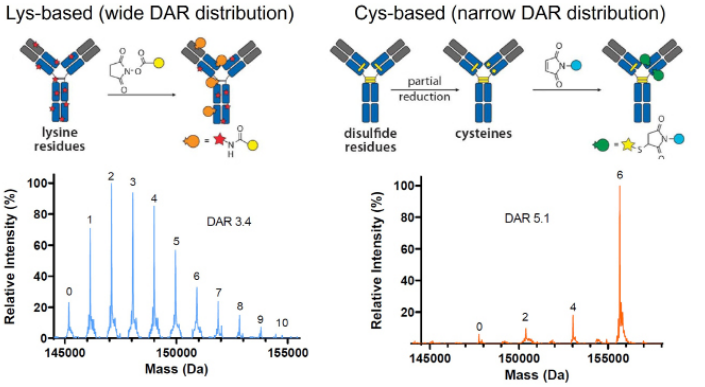

Figure 1.. Two common antibody-drug conjugation methods and their resulting drug to antibody ratio (DAR) measured by LC-MS. Left: lysine-based ADC conjugation produces a heterogeneous population with a wide range of DAR. Right: cysteine-based ADC conjugation requires partial reduction of the inter-chain disulfide bonds and thus also produces heterogeneous mixture of ADC species but with narrower range of DAR.

To investigate the structural impacts of these two conjugation methods on the antibody itself, a clinically relevant mAb, Trastuzumab, was studied here under multiple conjugation conditions. Naked Trastuzumab (Tras), a cysteine-based ADC made using MMAE (T-Cys-MMAE), a cysteine-based ADC made using Geld (T-Cys-Geld), and a lysine-based ADC made using DM1 were analyzed using MMS and their spectra are shown in Figure 2. Both T-Cys-MMAE and T-Cys-Geld have very similar spectra compared to the naked Tras. The lysine-based ADC, T-Lys-DM1, shows significant spectral difference from the other samples. Specifically, the peak at ~ 1640 cm^{-1}, assigned to beta-sheet structures, decreases in intensity in T-Lys-DM1 compared to the naked Tras. This weakened signal indicates a loss or possible unfolding of beta-sheets in the structure of T-Lys-DM1. At the same time, an increase in intensity can be observed at 1650 cm^{-1}, suggesting more unordered structures present in the structure.

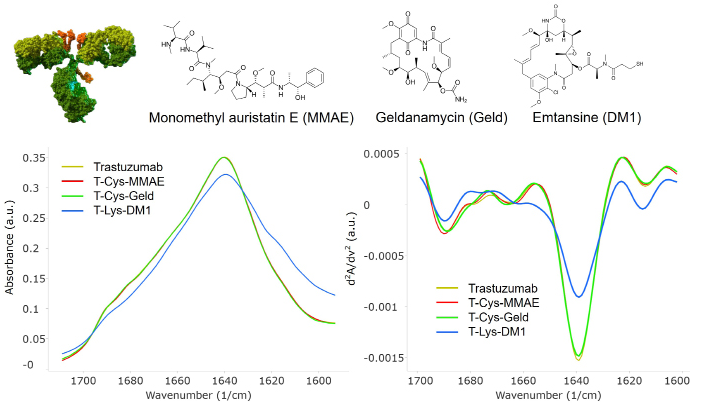

Figure 2.. Top: crystal structure of an ADC (credit: Waters®) and the chemical structures of the conjugated drugs used in this study. Bottom: absolute absorbance (left) and second derivative spectra (right) of the tested samples.

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Results and Discussion, continued

To quantify the amount of secondary structure in these samples, Gaussian curve fitting was performed on the similarity spectra (inversed and base-lined second derivative spectra, not shown) as reported previously.[4] This process generated the higher order structure (HOS) graph shown in Figure 3. Tras, T-Cys-MMAE, and T-Cys-Geld all shared similar structures in terms of their relative amounts of secondary structures. However, there is a significant difference in the amount of secondary structure in T-Lys-DM1 compared to the other samples, which agrees with the spectral data in Figure 2. Specifically, there is a 2% drop in beta-sheet structure, 2% drop in turn structure, and 3% increase in unordered structure. Although they appear small, these changes in secondary structures are significant and may lead to a change in the stability of the antibody.

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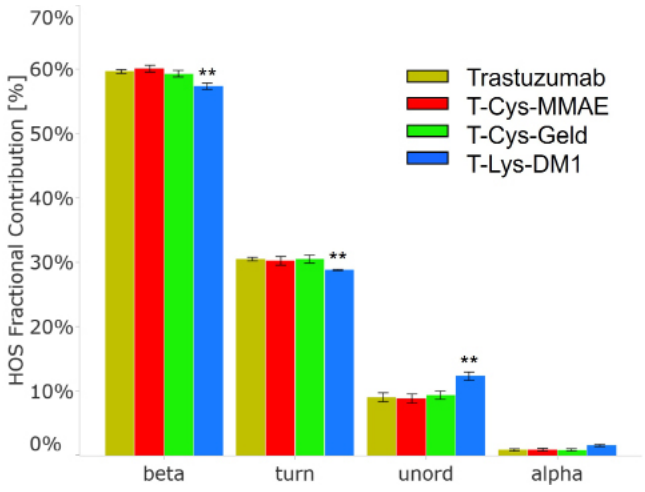

B

Samples	Beta	Turn	Unord	Alpha
Trastuzumab	59.6 ± 0.3	30.5 ± 0.3	9.0 ± 0.7	0.8 ± 0.2
T-Cys-MMAE	60.1 ± 0.5	30.2 ± 0.7	8.8 ± 0.7	0.9 ± 0.2
T-Cys-Geld	59.3 ± 0.5	30.5 ± 0.6	9.4 ± 0.6	0.8 ± 0.2
T-Lys-DM1	57.4 ± 0.4	28.8 ± 0.1	12.3 ± 0.6	1.5 ± 0.2

Figure 3. (A). Higher order structure (HOS) analysis showing the relative abundance of the secondary structural motifs in each sample. (B) Significant differences in beta, turn, and unordered structures are observed in T-Lys-DM1.

Thermal stability assessment was conducted using DSC to compare the melting temperatures of T-Lys-DM1, the lysine-based ADC with significant structural changes, with the unconjugated Tras. The thermal ramps were conducted from 15 °C to 95 °C, resulting in two recorded melting temperatures for each sample. As shown in Figure 4, both T_m1 and T_m2 of T-Lys-DM1 are lower compared to the unconjugated Tras, but the difference in T_m1 values is significantly larger (~3°C). In mAbs, the T_m1 peaks correspond to the unfolding of the CH2 domain of the Fc region. Thus, this result is a direct indication of decreased thermal stability caused by a small change in structural integrity of the mAb, due to the lysine drug conjugation causing an increase in unordered structure.

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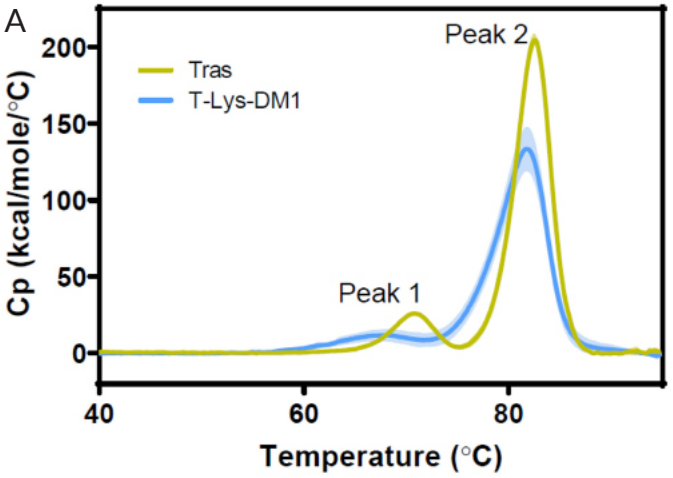

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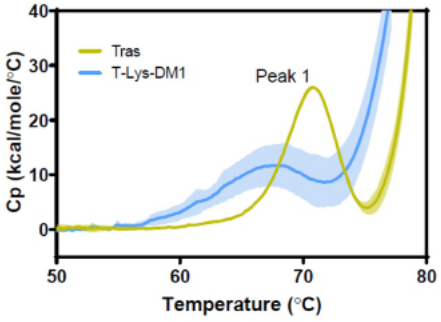

	T_m1 (°C)	T_m2 (°C)
Tras	70.6 ± 0.1	82.6 ± 0.1
T-Lys-DM1	67.7 ± 0.3	81.7 ± 0.0

Figure 4. (A). Melt curves of Tras and T-Lys-DM1 assessed by DSC, showing range from 40 °C to 95 °C. (B). The first melting point is highlighted showing a lower melting temperature in T-Lys-DM1 compared to Tras.

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Conclusions

In this study, we used MMS to characterize the structures of Trastuzumab and its ADCs under different conjugation methods. Our results revealed that cysteine-based conjugation did not affect the secondary structure of Trastuzumab. Using this conjugation method, the two different payloads, MMAE and Geld, did not exhibit structural impacts on the mAb either. On the other hand, the lysine-based conjugation caused significant changes in the secondary structure of the mAb. Specifically, beta-sheet and turn structures dropped by 2% each and the unordered structures increased by 3%. These subtle changes in secondary structure gave rise to a decrease in thermal stability compared to the unconjugated mAb. Overall, this app note demonstrates that MMS is an ideal tool to evaluate the structure as well as the stability of ADCs after payload conjugation. MMS can potentially serve as a stability prediction tool thanks to its high sensitivity to small structural changes.

Contributors

Richard Huang, Ph.D.
Valerie Collins, Ph.D.

RedShiftBio
80 Central St.
Boxborough, MA 01719

Christine Nervig, Ph.D.
Shawn Owen, Ph.D.

University of Utah,
201 Presidents Circle
Salt Lake City, UT 84112

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