High-Throughput and Comprehensive Characterization of Antibody Drug Conjugates (ADCs) by LC-MS and -MS/MS

Andreas Schummer, Lara Blaschke, Thomas Luber, Csilla Vazorka, Jürgen Kast, Burkhard Fleckenstein

ProtaGene GmbH, Heilbronn, Germany

Introduction

Antibody drug conjugates (ADCs) belong to a growing class of highly targeted biopharmaceutical drugs. They combine a monoclonal antibody that specifically binds tumor surface antigen and a highly potent cytotoxic drug, which is attached via a chemical linker (1). ADCs, that employ cysteine or lysine residues as conjugation sites, are highly heterogenous and their characterization presents an analytical challenge (2). Mass spectrometry is the tool of choice for the routine analysis in the ADC development process.

Here we describe two analytical workflows for the characterization of ADCs in combination with a forced degradation analysis. In the first workflow a high-throughput characterization of ADCs allows to analyze up to 48 samples/day using designed SEC-HPLC-MS methods under native and reduced conditions on a Bruker MaXis II™ ETD instrument followed by fully automated data analysis using Biopharma Compass® 3.0. For method setup we used a home-made lysine-conjugated ADC (Dansyl coupled to Trastuzumab) and a commercial cysteine-conjugated ADC (Dansyl-mAb, SigmaMAb ADC Mimic).

In the second established workflow a comprehensive characterization of ADCs was performed using high-resolution LC-MS/MS. This method allows a precise conjugation site determination, quantification of the conjugation site occupancy and the quantification of further posttranslational modifications.

(1) Sievers & Senter (2013). *Annual review of medicine*, *64*, p15-29
(2) Chen *et al*.(2016). *Mabs*, *8* (7), p1210-1223
(3) Bhat & Rabuka (2014). *BioProcess Int, 12* (9))
(4) SigmaMAb ADC Mimic Datasheet (#MSQC8)

Conjugation Types

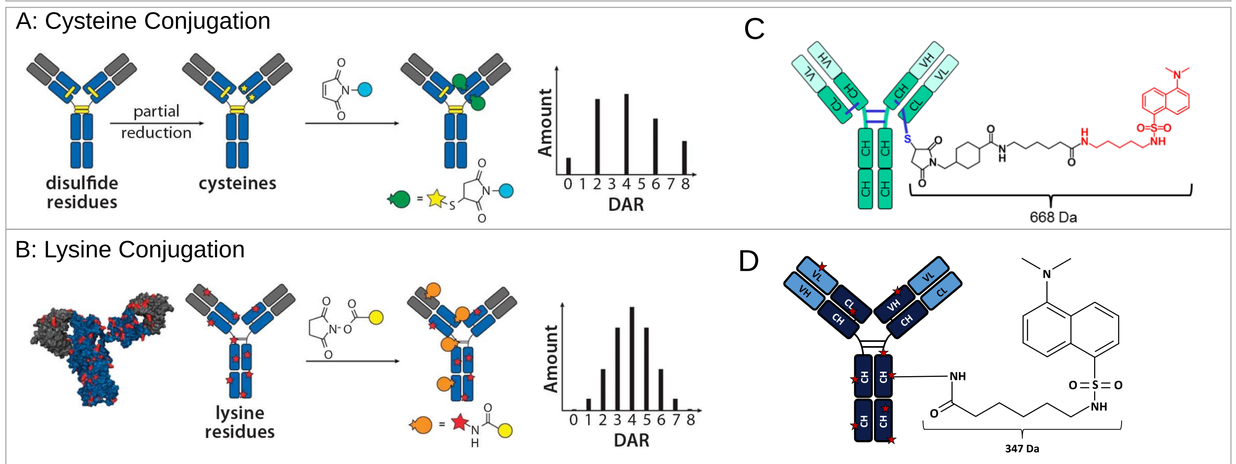

Fig. 1. A and B: Schematic of cysteine and lysine conjugates and their expected DAR profiles (3) C: Schematic of cystein-conjugated SigmaMAb ADC Mimic (4). D: Schematic of the home-made lysine-conjugated ADC mimic.

I. Workflow: High-Throughput Characterization using SEC-HPLC-MS

DAR Analysis with Native SEC-MS

Cysteine-conjugated ADC

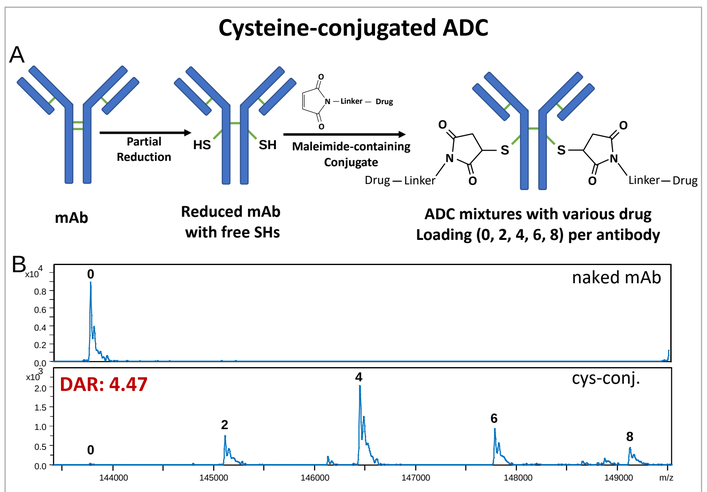

Fig. 2. A: Schematic of cysteine-conjugation reaction B: Native SEC-MS spectrum of SigmaMAb ADC mimic with a schematic of the possible conjugation sites. The DAR is the weighted sum of annotated peaks‘ intensities.

Lysine-conjugated ADC

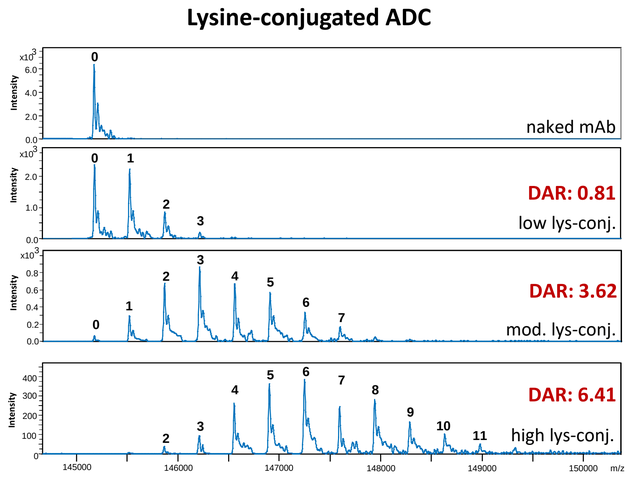

Fig. 3. Native SEC-MS spectrum of home-made lysine-conjugated ADC mimic with increasing drug load. The DAR is the weighted sum of annotated peaks‘ intensities

DAR Analysis with Reduced SEC-MS

Reduced lysine-conjugated ADC

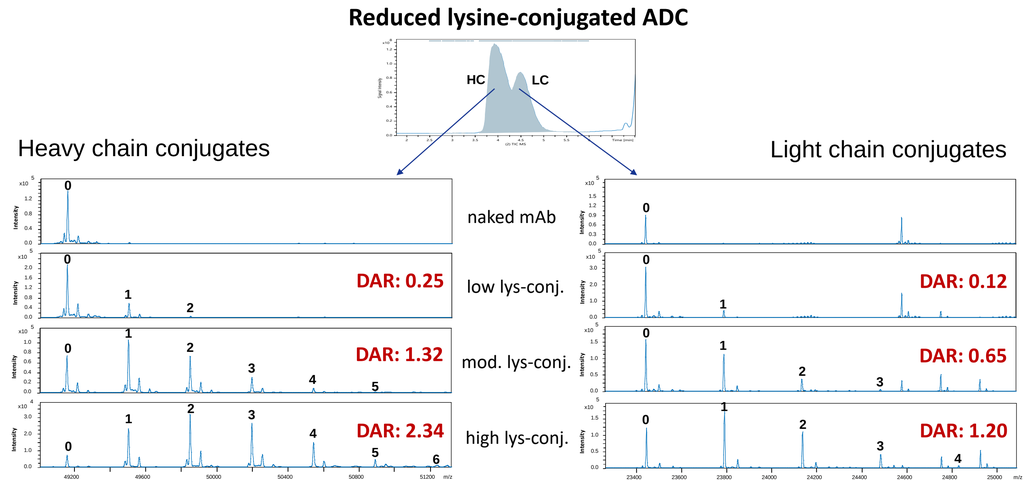

Fig. 4. Reduced SEC-MS spectrum of heavy and light chain of home-made lysine-conjugated ADC mimic with increasing drug load. The DAR is the weighted sum of annotated peaks‘ intensities

II. Workflow: Comprehensive Characterization using LC-MS/MS

Determination of Conjugation Sites

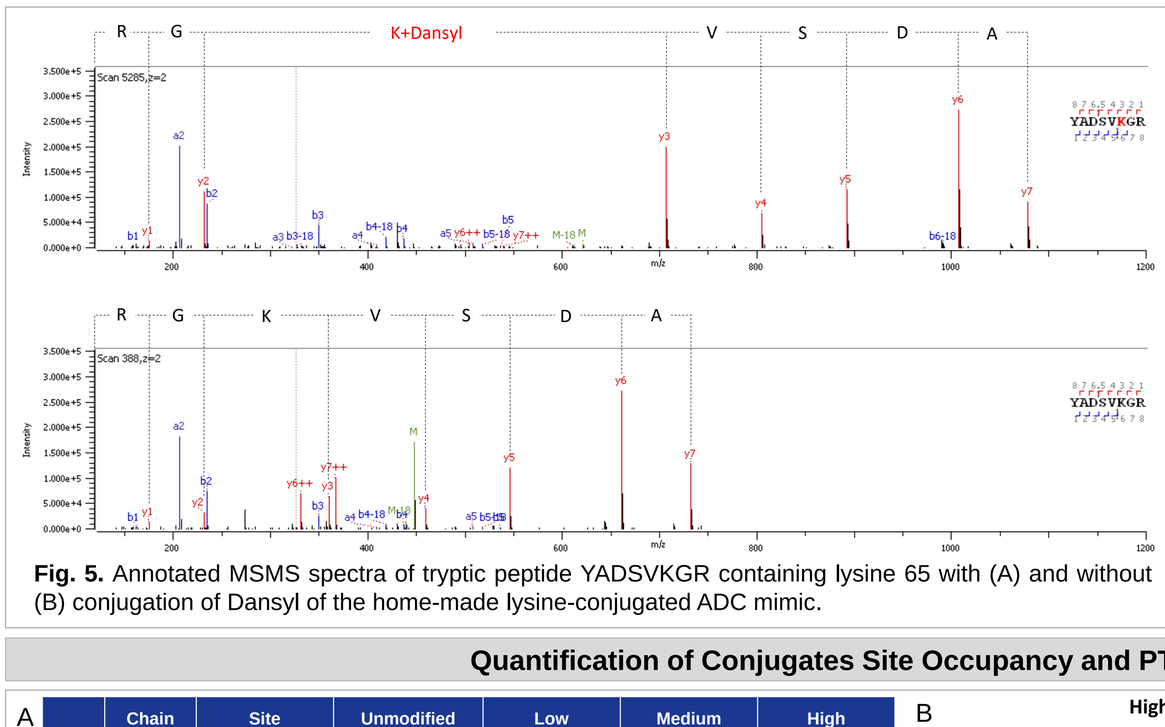

Fig. 5. Annotated MSMS spectra of tryptic peptide YADSVKGR containing lysine 65 with (A) and without (B) conjugation of Dansyl of the home-made lysine-conjugated ADC mimic.

Validate	Protein alias name	Start AA	End AA	Var. Pos. Protein	z	Sequence	Mod. Summary	Calc. m/z
True-positive	HC	20	38	30	3	R.LSCAASGFNIkDTYIHWVR.Q	K11(Dansyl-X/346.1351)	862.0874
True-positive	HC	39	50	43	2	R.QAPGkGLEWVAR.I	K5(Dansyl-X/346.1351)	829.4296
True-positive	HC	60	67	65	2	R.YADSVkGR.F	K6(Dansyl-X/346.1351)	621.3028
True-positive	HC	68	87	76	3	R.FTISADTSkNTAYLQMNSLR.A	K9(Dansyl-X/346.1351)	869.7576
True-positive	HC	125	150	136	3	K.GPSVFPLAPSSkSTSGGTAALGCLVK.D	K12(Dansyl-X/346.1351)	945.8188
True-positive	HC	209	216	213	1	K.PSNTkVDK.K	K5(Dansyl-X/346.1351)	1234.6136
True-positive	HC	214	221	216	3	K.VDkKVEPK.S	K3(Dansyl-X/346.1351)	430.2372
True-positive	HC	226	251	249	4	K.THTCPPCPAPELLGGPSVFLFPPkPK.D	K24(Dansyl-X/346.1351)	798.4036
True-positive	HC	259	291	277	5	R.TPEVTCVVVDVSHEDPEVkFNWYVDGVEVHNAK.T	K19(Dansyl-X/346.1351)	829.5952
True-positive	HC	278	293	291	3	K.FNWYVDGVEVHNAkTK.P	K14(Dansyl-X/346.1351)	751.6981
True-positive	HC	292	295	293	2	K.TkPR.E	K2(Dansyl-X/346.1351)	424.2284
True-positive	HC	305	325	320	3	R.VVSVLTVLHQDWLNGkEYKCK.V	K16(Dansyl-X/346.1351)	954.8276
True-positive	HC	305	325	323	3	R.VVSVLTVLHQDWLNGKEYkCK.V	K19(Dansyl-X/346.1351)	954.8276
True-positive	HC	321	325	323	1	K.EYkCK.V	K3(Dansyl-X/346.1351)	1073.4795
True-positive	HC	324	329	325	2	K.CkVSNK.A	K2(Dansyl-X/346.1351)	541.2621
True-positive	HC	326	337	329	3	K.VSNkALPAPIEK.T	K4(Dansyl-X/346.1351)	538.2971
True-positive	HC	330	341	337	3	K.ALPAPIEkTISK.A	K8(Dansyl-X/346.1351)	538.6372
True-positive	HC	338	343	341	2	K.TISkAK.G	K4(Dansyl-X/346.1351)	497.2755

Fig. 6. Excerpt of the result table of identified conjugation sites of the heavy chain of the home-made lysine-conjugated ADC mimic using Byologic® software (Protein Metrics Inc.).

Quantification of Conjugates Site Occupancy and PTMs

A

	Chain	Site	Unmodified	Low	Medium	High
Conjugation	HC	K30	0.0%	0.0%	0.6%	1.2%
		K43	0.0%	1.0%	5.9%	8.5%
		K65	0.0%	6.4%	29.5%	38.0%
		K76	0.0%	1.1%	4.3%	6.1%
		K249	0.0%	2.2%	15.2%	22.3%
		K251	0.0%	0.7%	4.3%	6.3%
		K277	0.0%	0.4%	1.0%	1.1%
		K293	0.0%	2.7%	8.6%	10.4%
		K320	0.0%	0.3%	1.5%	1.6%
		K323	0.0%	1.8%	4.8%	4.6%
		K325	0.0%	1.7%	3.9%	4.1%
		K329	0.0%	0.9%	4.6%	5.1%
		K417	0.0%	0.0%	4.8%	9.1%
		K442	0.0%	0.0%	2.3%	3.7%
	LC	K42	0.0%	4.8%	19.6%	24.5%
		K107	0.0%	0.4%	3.5%	5.6%
		K169	0.0%	0.5%	4.0%	6.0%
		K183	0.0%	2.7%	10.2%	18.1%
		K188	0.0%	1.6%	8.3%	15.5%
		K190	0.0%	3.4%	6.6%	12.5%
		K207	0.0%	0.5%	3.5%	7.0%
Oxidation	HC	M83	0.8%	0.5%	0.4%	0.5%
		M255	3.0%	2.6%	3.0%	3.8%
		M361	1.6%	1.3%	2.2%	2.2%
		M431	1.3%	1.1%	1.9%	2.4%
Deamidation	HC	N55	1.4%	1.3%	1.4%	1.4%
		N300	6.2%	5.9%	5.9%	5.9%
		N364	0.8%	0.8%	1.0%	1.0%
		N387/N392/N393	0.2%	0.3%	0.3%	0.4%
	LC	N30	7.6%	10.6%	10.4%	9.0%

B

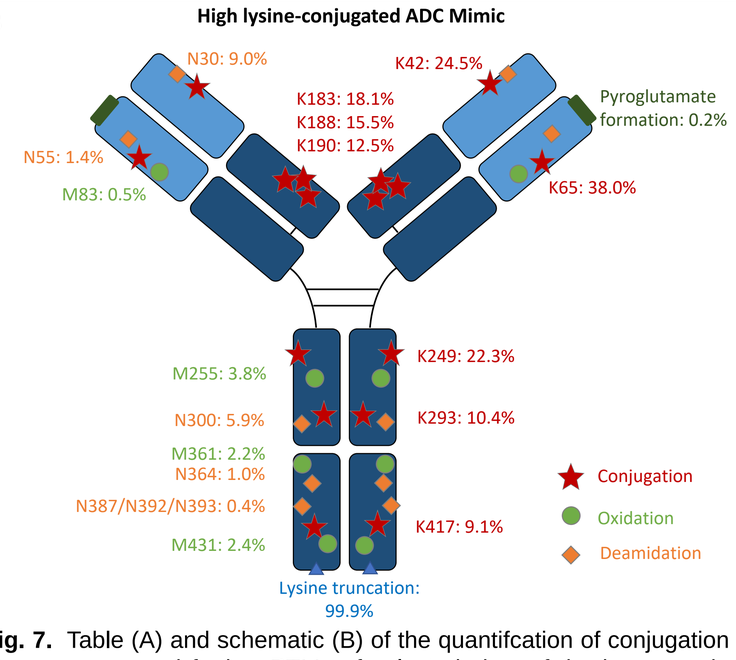

Fig. 7. Table (A) and schematic (B) of the quantifcation of conjugation site occupany and further PTMs of selected sites of the home-made lysine-conjugated ADC mimic samples.

Further Possible Analyses

- Amino acid sequence and sequence variants
- Terminal truncations, signal peptide residue
- N-Terminal pyro-Glu, C-Terminal Lys and Amidation
- Deamidation, Oxidation, Glycation
- N-Glycosylation profile and Disulfide linkages

Conclusion

Using both workflow, we are able to (I) analyze lysine- and cysteine-conjugated ADCs with high-throughput SEC-MS methods under native and reduced conditions for exact DAR determination and (II) determine the precise site of the conjugation, quantify the conjugation site occupancy and quantify further posttranslational modifications. Using this approach, we show that the coupling reaction conditions lead to a conjugation site occupancy of up to 38% of distinct lysine residues without influencing the deamidation (*e.g.* GFYPSDIAVEWESNGQPENNYK: 0.2-0.4%) and oxidation level (*e.g.* DTLMISR: 2-4%).

These analytic workflows are highly suitable for both high-throughput and comprehensive characterization of ADCs. Therefore, this approach can be used in the development process of ADCs for comparison of production lots, stability studies and forced degradation analyses for quality control and assurance.