Application Note

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Antibody-Drug Conjugate (ADC) Analysis with the Agilent ProteoAnalyzer System

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Abstract

This application note demonstrates the use of the Agilent ProteoAnalyzer system for antibody-drug conjugate (ADC) analysis by capillary electrophoresis with sodium dodecyl sulfate (CE-SDS). The high resolution delivered by the Agilent ProteoAnalyzer system enables precise sizing and assessment of heterogeneity of these samples. Representative figures and quantitative data from cysteine- and lysine-conjugated ADCs confirm the accuracy and reproducibility of the system for ADC characterization and quality control.

Introduction

Antibody-drug conjugates (ADCs) are targeted biopharmaceuticals composed of a monoclonal antibody (mAb) connected to a drug through a stable linker. Factors such as drug-to-antibody ratio (DAR), conjugation strategy, and linker chemistry significantly influence ADC therapeutic efficacy, safety, and biophysical properties. Advanced analytical techniques like capillary electrophoresis with sodium dodecyl sulfate (CE-SDS), hydrophobic interaction chromatography (HIC), and mass spectrometry are essential for characterizing ADC critical quality attributes (CQAs) such as heterogeneity, purity, DAR, and payload location.

CE-SDS is used in ADC analysis to determine molecular weight, size heterogeneity, and purity. The technology can provide high resolution for closely related size variants, making it ideal for monitoring antibody fragments dissociable by SDS. The electrophoretic profile depends on the drug conjugation sites, such as cysteine or lysine residues (Figure 1). Additionally, different linker chemistries can impact the antibody structure, and therefore the separation profile, by forming nondisulfide covalent crosslinks between light chains and heavy chains during the ADC manufacturing process.[1,2]

The Agilent ProteoAnalyzer system automates CE-SDS analysis of both reduced and nonreduced proteins and enables efficient workflows by separating up to 12 samples in parallel.[3] While accurate sizing of antibody samples can be difficult, the system allows for the use of custom size calibration ladders to correct for migration differences caused by secondary structures. Previous work has shown that the NIST mAb and its associated antibody fragments can be used as a ladder when analyzing mAbs[4] and is used here for sizing nonreduced ADCs. This application note demonstrates the capabilities of the ProteoAnalyzer system for comprehensive ADC analysis, with examples for both cysteine- and lysine-conjugated ADCs.

Experimental

Commercially available ADC samples and corresponding naked mAb standards were obtained and prepared for analysis with the Agilent ProteoAnalyzer system. Samples include: SiLu SigmaMAb Universal Antibody Standard Human (Sigma, p/n MSQC4-1MG), SigmaMAb Antibody Drug Conjugate (ADC) Mimic (Sigma, p/n MSQC8-0.5MG), Trastuzumab, Trastuzumab deruxtecan (MedChem Express, p/n HY-138298A), and Trastuzumab emtansine (T-DM1) (MedChem Express, p/n HY-P9921). Samples were reconstituted to 10 mg/mL and further diluted to 1.5 mg/mL in nuclease free water. Sample concentration was confirmed by NanoDrop (settings: Protein A280; curve type: other E1%; extinction coefficient: 14.3).

Each sample was then prepared under both reducing and nonreducing conditions according to the Agilent Protein Broad Range P240 kit (p/n 5191-6640) manual.[5] The samples were covalently labeled by incubating with the supplied reagents at 70 °C for 10 minutes).[6] The reduced and nonreduced antibodies were analyzed across multiple capillaries of a ProteoAnalyzer system with the ProteoAnalyzer Broad Range Kit lower marker (LM) only method. For nonreduced conditions, the sample injection was decreased to 7 kV, 6 seconds for optimal results.[6] Sizing analysis of the nonreduced samples was performed using the NIST mAb as the ladder.[4]

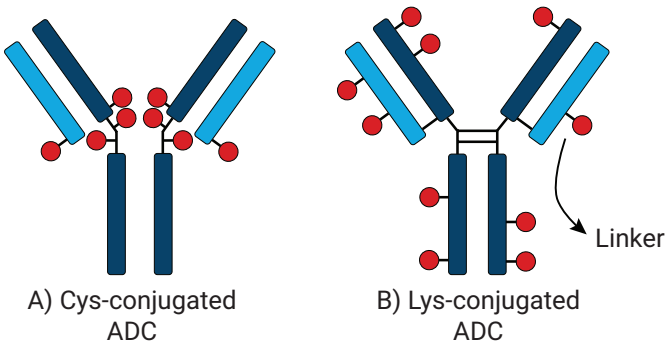

Figure 1. Schematic representation of (A) cysteine- and (B) lysine-conjugated antibody-drug conjugates (ADCs). Red circles indicate the potential drug conjugation sites.

Results and discussion

Reduced CE-SDS analysis of cysteine-conjugated ADCs

CE-SDS analysis of cysteine-conjugated ADCs under reduced conditions is expected to show a light chain (LC) and heavy chain (HC). In addition to these peaks, partial separation of the LC without a payload (L0) and an LC with one payload (L1) may also be observed. Electrophoretic separation of the L0 and L1 depends on the size and properties of the payloads and antibody.[7,8]

Analysis of the SigmaMAb ADC Mimic and its naked counterpart, the SigmaMAb Antibody Standard, was performed using the ProteoAnalyzer under reduced conditions. The standard is an IgG1 monoclonal antibody, with an expected profile of two major peaks, indicative of the LC and HC (Figure 2A). The ADC Mimic is the SigmaMAb Standard linked to dansyl fluorophores by way of an LC-SMCC crosslinker. The electrophoretic profile of the ADC shows the expected LC and HC, with separation of the LC into two peaks, consistent of an L0 and L1 payload (Figure 2B).

Another example of a cysteine-conjugated ADC is Trastuzumab deruxtecan, consisting of the mAb Trastuzumab covalently linked to the topoisomerase I inhibitor deruxtecan. The Trastuzumab mAb and the Trastuzumab deruxtecan ADC showed similar profiles, with prominent peaks for both the LC and HC, and a small NGHC, as shown in Figure 2C–D. While the ProteoAnalyzer could not resolve the differences between the conjugated and unconjugated forms, separation of the LC, NGHC, and HC peaks still enables calculations of CQAs such as percent glycosylation and percent thioether.

Sizing and concentration analysis of triplicate replicates of both the mAbs and the cysteine-conjugated ADC showed highly reproducible results. All samples had a sizing precision of less than 2.5% CV and quantification precision of less than 10% CV (Table 1).

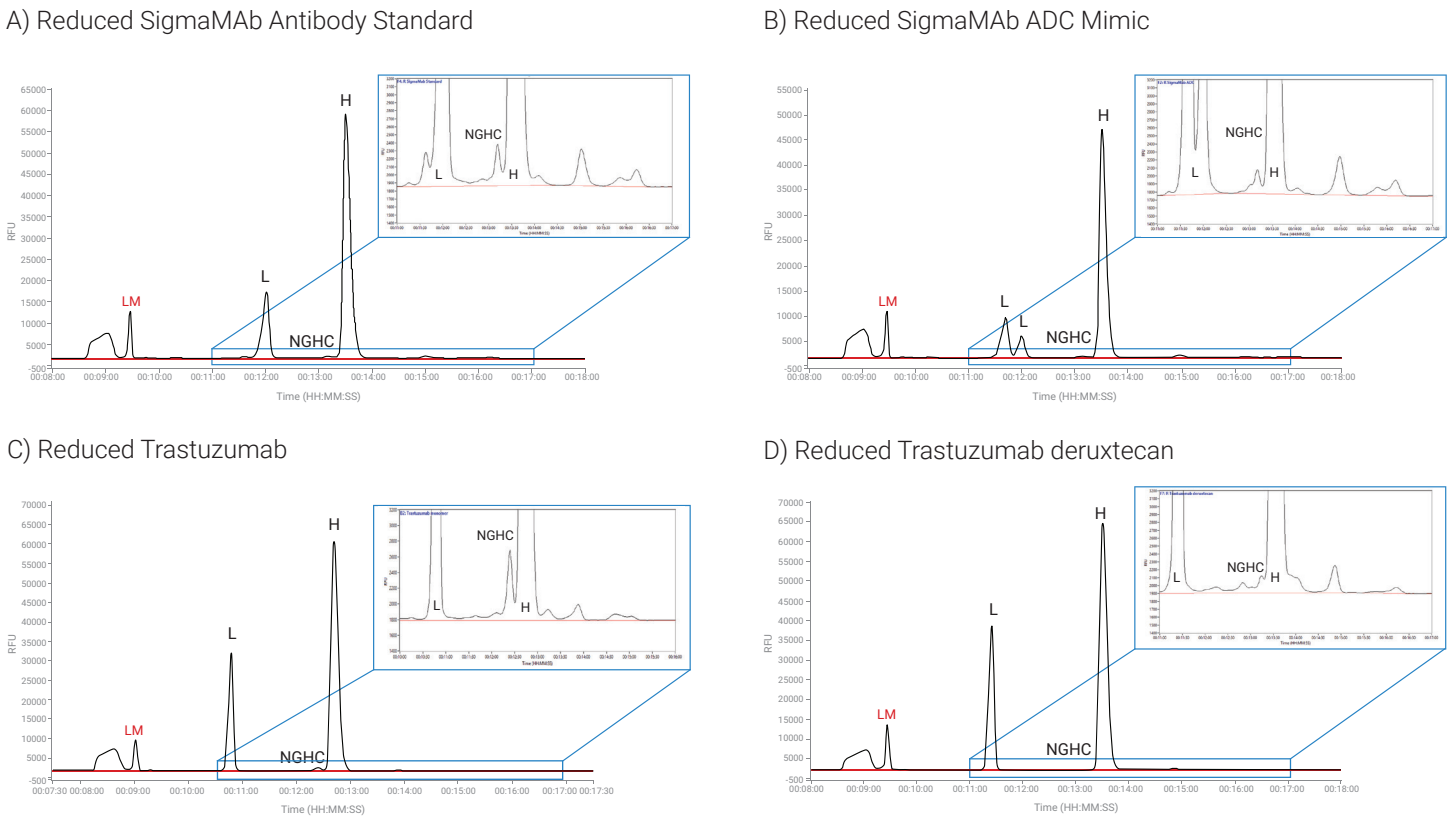

Figure 2. Reduced analysis of (A) SigmaMAb Antibody Standard, (B) SigmaMAb ADC Mimic, (C) Trastuzumab, and (D) Trastuzumab deruxtecan using the Agilent ProteoAnalyzer system and the Agilent Protein Broad Range P240 kit.

Table 1. Average sizing and concentration of (A) SigmaMAb Antibody Standard, (B) SigmaMAb ADC Mimic, (C) Trastuzumab, and (D) Trastuzumab deruxtecan under reduced conditions using the Agilent ProteoAnalyzer system and the Agilent Protein Broad Range P240 kit (n=3).

			Size (kDa)		Concentration (ng/µL)	
	mAb chains	Appx. size	Average	%CV	Average	%CV
R SigmaMAb Standard	L	25	36.87	0.31	239.65	1.54
	NGHC		56.97	0.51	7.40	9.25
	H	50	62.60	0.55	830.24	0.99
R SigmaMAb ADC	L	25	32.80	0.61	190.98	0.41
	L	25	37.77	0.40	96.09	0.90
	NGHC		58.63	0.43	6.92	1.93
	H	50	65.77	0.84	846.18	1.44
R Trastuzumab	L	25	25.80	1.69	523.95	0.28
	NGHC		54.17	2.32	18.44	1.37
	H	50	60.03	2.11	1267.31	2.77
	L	25	27.05	0.26	431.20	0.58
R Trastuzumab deruxtecan	NGHC		57.90	0.49	2.59	7.84
	H	50	62.25	0.34	869.34	0.85

Nonreduced CE-SDS analysis of cysteine-conjugated ADCs

Under nonreducing CE-SDS conditions, cysteine-conjugated ADCs show characteristic fragments such as the LC and HC, as well as heavy-light (HL), heavy-heavy (HH), and heavy-heavy-light (HHL) chains. The ADC dissociates in the presence of SDS, based on the number and location of drugs linked to the antibody. The presence of the drug prevents the interchain disulfide bond from reforming.[9] An illustration of the potential fragments that can be expected with cysteine-conjugated IgG1 ADCs is shown in Figure 3. ADC samples were analyzed on the ProteoAnalyzer system to demonstrate the ability of the system to assess cysteine-conjugated ADC samples. Representative examples of the electrophoretic profiles of the SigmaMAb and Trastuzumab ADCs under nonreduced conditions are shown in Figure 4.

Drug to Antibody Ratio	Position of Payload		Fragments formed under NR conditions					
0	DAR0		mAb					
2	$DAR2_f$			HHL				L
	$DAR2_h$		mAb					
4	$DAR4_{ff}$				HH			2L
	$DAR4_{fh}$			HHL				L
	$DAR4_{hh}$					2HL		
6	$DAR6_{ffh}$				HH			2L
	$DAR6_{fhh}$					HL	H	L
8	DAR8						2H	2L

Figure 3. Expected fragments of positional isomers from cysteine-conjugated IgG1 ADCs.

The SigmaMAb Standard shows four prominent peaks. When sized with the NIST mAb as ladder, the most prominent peak had an average size of 156 kDa, close to the expected molecular weight of the intact mAb (HHLL) at approximately 150 kDa. The other three peaks displayed average sizes of 35, 103, and 131 kDa, representative of the L, HH, and HHL species, respectively (Figure 4A, Table 2). The SigmaMAb ADC Mimic displays multiple peaks with sizes consistent with the L, H, HL, HH, HHL, and HHLL species (Figure 4B). There are three distinct peaks within the area of the L chain, likely representative of the different payload locations. The average size and concentration of each peak is shown in Table 2.

The Trastuzumab and the Trastuzumab deruxtecan ADC are shown in Figure 4C–D. The monomeric Trastuzumab sample shows a large peak at approximately 150 kDa, indicative of the intact mAb, or the HHLL. Smaller peaks with sizes similar to the L, H, HL, HH, and HHL chains are also evident. Comparison of the size of these peaks to those in the Trastuzumab deruxtecan provides confidence in the peak assignment of the sample. Upon nonreduced analysis, the ADC shows two large peaks at 22 and 56 kDa, consistent with an increased amount of an L and an H chain. The three smaller peaks to the right of the electropherogram had average sizes of 73, 100, and 126 kDa, indicative of the HL, HH, and HHL fragments. Together, these examples demonstrate that cysteine conjugated Ig1 ADCs can be successfully analyzed using the ProteoAnalyzer system.

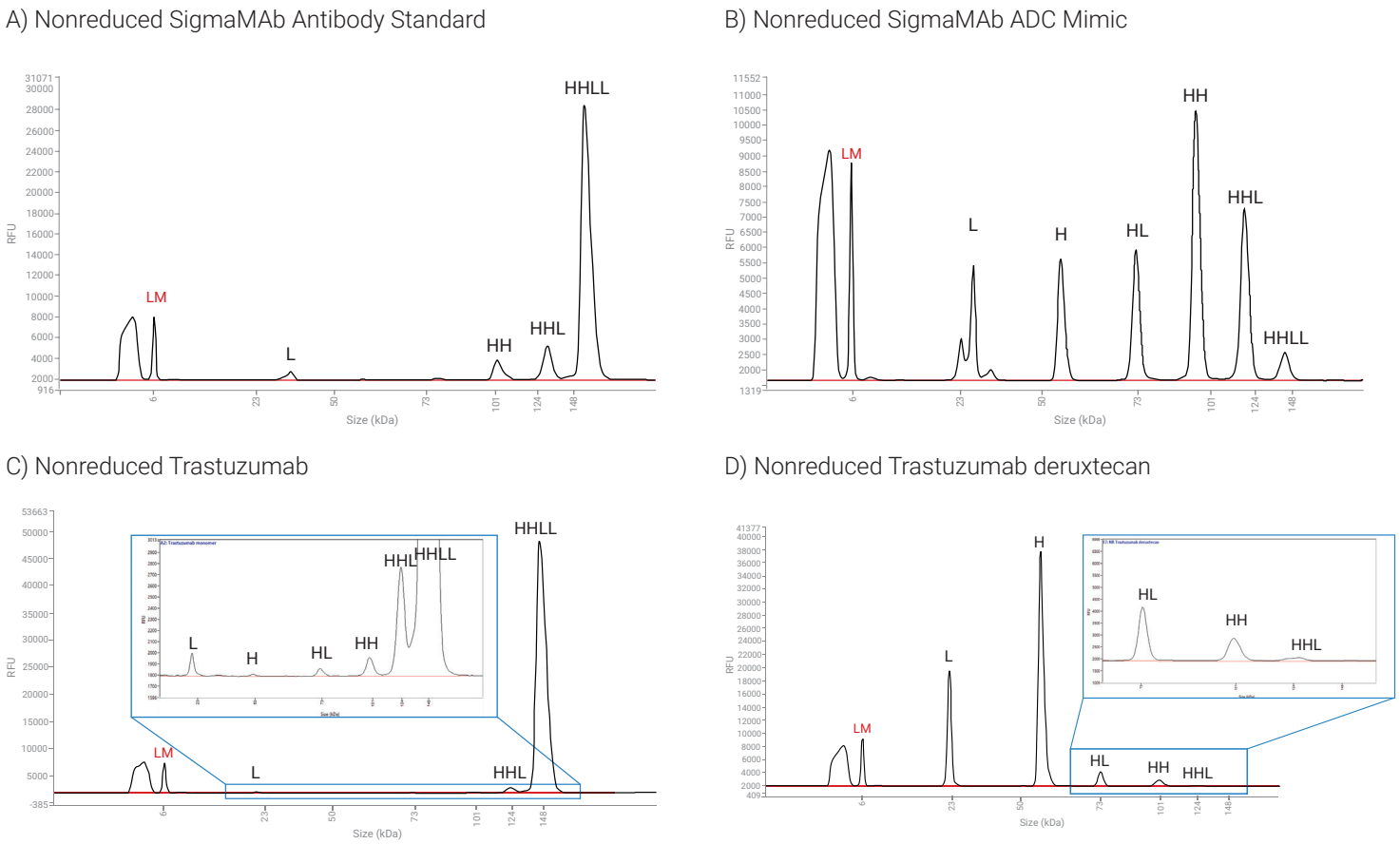

Figure 4. Nonreduced analysis of (A) SigmaMAb Antibody Standard, (B) SigmaMAb ADC Mimic, (C) Trastuzumab, and (D) Trastuzumab deruxtecan using the Agilent ProteoAnalyzer system and the Agilent Protein Broad Range P240 kit with NIST mAb as the ladder.

Table 2. Average sizing and concentration of (A) SigmaMAb Antibody Standard, (B) SigmaMAb ADC Mimic, (C) Trastuzumab, and (D) Trastuzumab deruxtecan under nonreduced conditions using the Agilent ProteoAnalyzer system and the Agilent Protein Broad Range P240 kit with NIST mAb as the ladder (n=3).

			Size (kDa)		Concentration (ng/µL)	
	mAb chains	**Appx. size**	**Average**	**%CV**	**Average**	**%CV**
NR SigmaMAb Standard	L	25	35.43	1.55	29.79	3.38
	HH	100	103.03	3.23	60.92	3.68
	HHL	125	131.60	3.23	94.53	2.16
	HHL (intact)	150	156.77	3.06	822.93	0.56
NR SigmaMAb ADC	L	25	24.73	2.07	35.05	3.44
			28.63	1.72	79.74	2.03
			34.63	2.05	13.51	5.97
	H	50	56.53	1.67	104.77	0.89
	HL	75	76.10	2.94	105.56	0.46
	HH	100	100.20	3.34	213.74	0.26
	HHL	125	127.67	4.27	136.89	0.31
	HHLL (intact)	150	154.77	3.84	25.37	5.77
NR Trastuzumab	L	25	21.77	0.53	6.46	0.68
	H	50	48.80	0.35	0.83	9.47
	HL	75	72.13	0.21	2.34	6.45
	HH	100	98.93	0.21	6.06	4.95
	HHL	125	122.50	0.67	34.38	1.89
	HHLL (intact)	150	144.20	0.42	1641.45	0.55
NR Trastuzumab deruxtecan	L	25	22.37	0.52	280.83	0.97
	H	50	56.53	0.51	662.96	1.94
	HL	75	73.30	1.21	41.45	2.36
	HH	100	100.07	1.01	22.56	2.26
	HHL	125	126.03	1.55	4.37	4.60

Lysine-conjugated ADC analysis

Lysine-conjugated ADCs present different analytical considerations compared to their cysteine-conjugated counterparts. In nonreduced CE-SDS analysis, lysine-conjugated ADCs retain their cysteine disulfide bonds, resulting in electrophoretic profiles that closely resemble those of naked mAbs. This similarity allows for straightforward assessment of monomeric purity and overall molecular integrity.

A representative example is Trastuzumab emtansine (T-DM1), an ADC generated by linking the anti-tubulin drug DM1 to lysine residues of Trastuzumab by way of an SMCC linker. The manufacturing process involves first attaching the linker to form the intermediate TmAb-MCC, followed by conjugation of DM1 to the linker. The intermediate can form nondisulfide covalent linkages between HCs and LCs, which can be detected by CE-SDS. The drug load distribution for T-DM1 per antibody ranges from 0 to 8, with an average drug-to-antibody ratio (DAR) of approximately 3.5.

CE-SDS analysis of Trastuzumab emtansine under nonreducing conditions with the ProteoAnalyzer displays a major peak at ~150 kDa, consistent with the intact mAb and similar to the naked Trastuzumab control (Figure 5). Additionally, the electropherogram profile did not show significant fragmentation. Analysis of the monomeric structure allows for reliable monomeric purity CQA assessment of the ADC using the ProteoAnalyzer.

Under reduced conditions, CE-SDS analysis shows the expected LC, HC, as well as higher molecular weight species (Figure 6). These higher molecular weight fragments, not observed in the naked Trastuzumab control, were sized at approximately 75, 100, and 125 kDa using an imported nonreduced NIST mAb as ladder (Table 3). Their presence suggests the formation of nondisulfide covalent linkages between HC and LC during manufacturing as previously described.[2]

It is important to note that for lysine conjugated ADCs, the analytical sensitivity of the ProteoAnalyzer system may be affected by the DAR. If all lysine residues are conjugated to payloads, detection of the ADC may be compromised due to a lack of available lysines for dye binding.

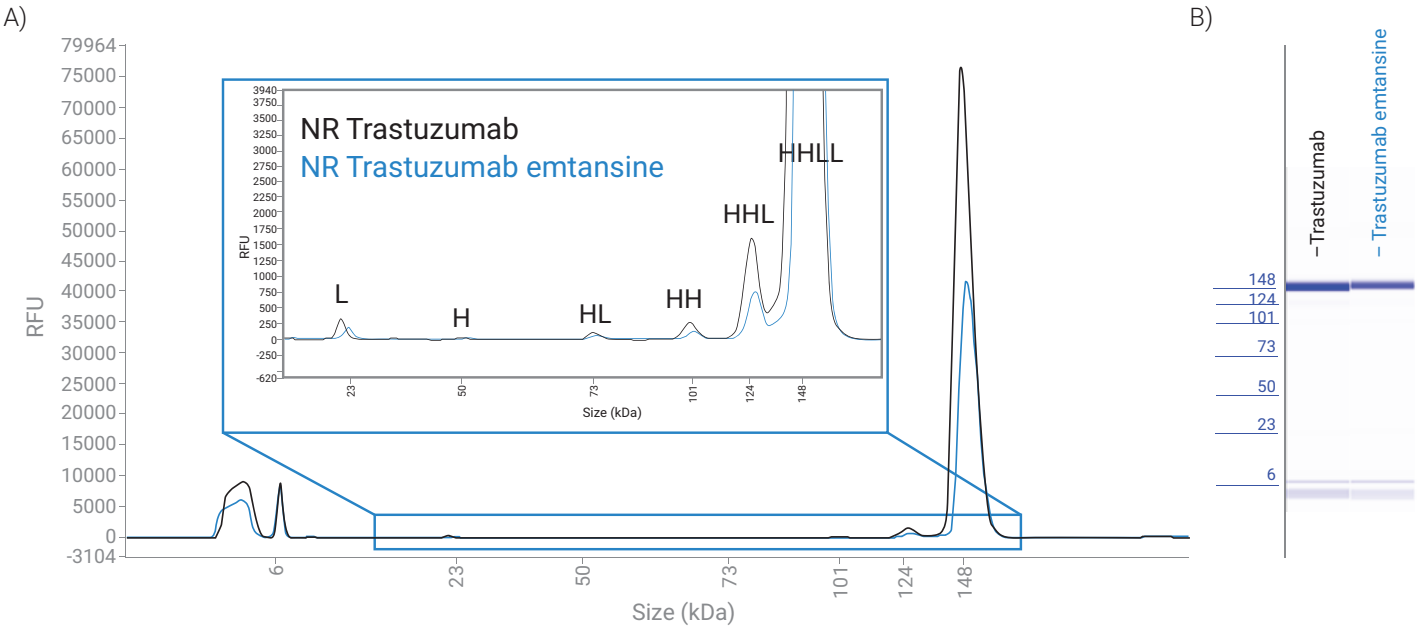

Figure 5. (A) Electropherogram overlay and (B) gel image of nonreduced CE-SDS analysis of Trastuzumab control (black trace) and Trastuzumab emtansine (blue trace) using the Agilent ProteoAnalyzer system and the Agilent Protein Broad Range P240 kit with NIST mAb as the ladder.

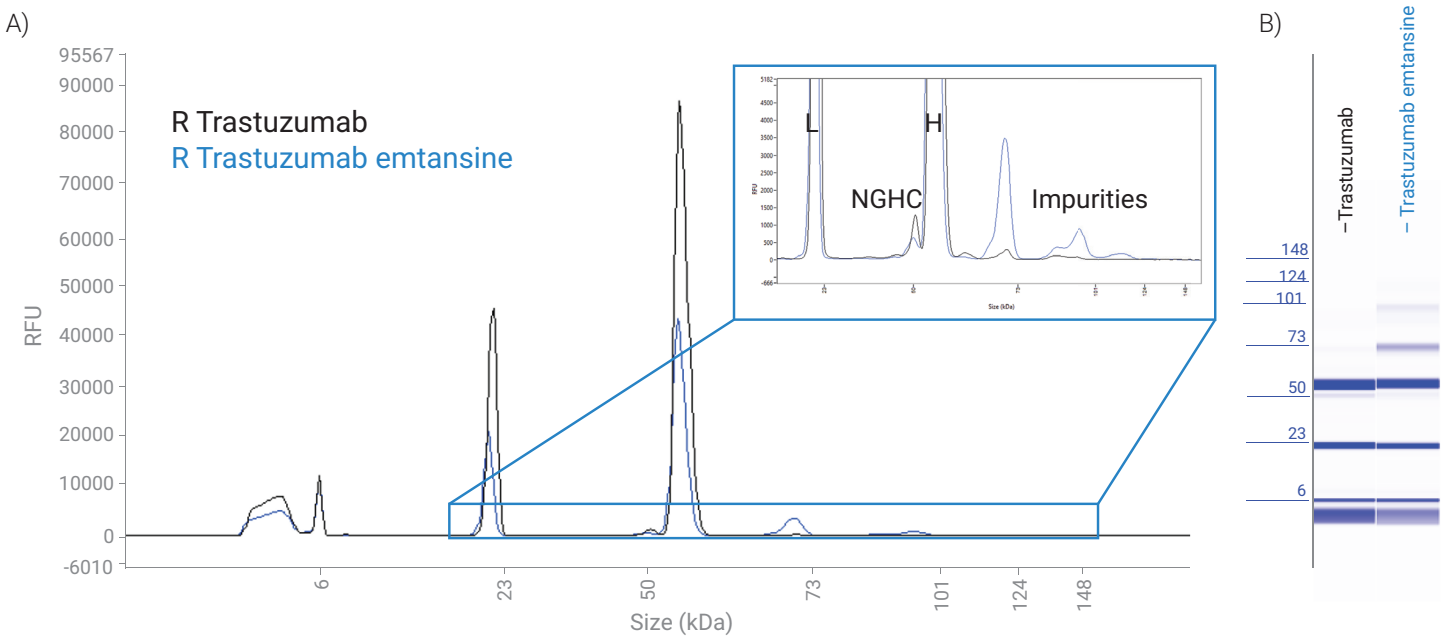

Figure 6. (A) Electropherogram overlay and (B) gel image of reduced CE-SDS analysis of Trastuzumab control (black trace) and Trastuzumab emtansine (blue trace) using the Agilent ProteoAnalyzer system and the Agilent Protein Broad Range P240 kit with NIST mAb as the ladder.

Table 3. Average sizing and concentration of Trastuzumab emtansine under nonreduced and reduced conditions using the Agilent ProteoAnalyzer system and the Agilent Protein Broad Range P240 kit with NIST mAb as the ladder (n=3).

			Size (kDa)		Concentration (ng/µL)	
	mAb chains	**Appx. size**	**Average**	**%CV**	**Average**	**%CV**
NR Trastuzumab emtansine	L	25	22.30	1.35	3.79	5.69
	H	50	50.70	1.71	0.45	5.24
	HL	75	72.63	0.65	1.41	3.43
	HH	100	100.03	0.74	2.78	1.42
	HHL	125	123.83	1.02	17.03	2.06
	HHLL (intact)	150	146.97	0.89	965.88	1.44
	Impurity		225.70	0.94	12.69	2.64
R Trastuzumab emtansine	L	25	21.50	0.47	267.45	0.26
	NGHC		51.33	0.22	12.70	1.68
	H	50	55.13	0.21	652.78	0.71
	HL	75	71.03	0.16	69.48	0.44
	Impurities		88.60	0.20	7.49	3.10
	Impurities		97.43	0.41	21.04	3.88
	Impurities		115.27	0.70	5.29	1.99

Conclusions

The Agilent ProteoAnalyzer system provides detailed characterization of cysteine-conjugated ADCs by CE-SDS analysis. Under reduced conditions, the electropherograms display distinct peaks for the LC, HC, and, in some cases, partially separated LC species with and without payloads (L0 and L1). These results highlight the heterogeneity introduced by drug conjugation and demonstrate the system's precision in sizing and quantification.

Nonreduced CE-SDS analysis further distinguishes characteristic fragments such as LC, HC, HL, HH, and HHL chains, providing insight into the positional isomers. This data can be combined with HIC for full characterization of the average DAR and payload location. The presence and relative abundance of these fragments are consistent with expected molecular weights and conformations, confirming the system's capability to resolve complex ADC mixtures. Together, these results illustrate that the ProteoAnalyzer system offers robust and reproducible analysis of cysteine-conjugated ADCs, supporting both purity assessment and structural characterization.

CE-SDS analysis of lysine-conjugated ADCs such as Trastuzumab emtansine demonstrates that nonreduced profiles closely resemble those of unconjugated mAbs, allowing for monomeric purity assessment. Reduced profiles reveal unique crosslinked species, providing insight into the effects of conjugation chemistry on antibody structure. For complete characterization, CE-SDS should be complemented with additional analytical methods, such as HIC.[10]

Overall, this application note demonstrates that the ProteoAnalyzer system provides detailed, reproducible, and insightful analysis for both cysteine- and lysine-conjugated ADCs. The ability of the system to resolve, size, and quantify fragments supports both purity assessment and structural characterization, making it a valuable tool for ADC development and quality control.

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