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WHITE PAPER

WP8010: Determination of Multiple Quality Attributes of Antibody-Oligonucleotide Conjugate with SEC-MALS and AEX-MALS

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Summary

Multi-angle light scattering coupled to liquid chromatography (LC-MALS) is a powerful tool for analyzing antibody-oligonucleotide conjugates (AOCs), providing a robust and straightforward approach to obtaining multiple quality attributes. In this white paper, we demonstrate the successful application of LC-MALS with both size-exclusion chromatography (SEC) and anion exchange chromatography (AEX) to quantify the oligonucleotide-antibody ratio (OAR) and overall payload concentration of two AOC samples. Through a single LC-MALS experiment, we identify the complex molecular species present in the samples, including aggregates, fragments, and different OAR populations.

Introduction

Therapeutic oligonucleotides conjugated to antibodies represent a fast-growing modality for immunotherapies and oncology [1,2]. However, much like their antibody-drug conjugate (ADC) cousin, AOCs are complex in their payload heterogeneity and linker chemistry[3]. With different conjugation chemistry conditions, the resulting drug products can vary in stoichiometries and, ultimately, drug safety and efficacy. Thus, there is an urgent need to measure multiple CQAs for these complex therapeutic formulations.

Currently, no efficient workflow exists to characterize AOCs systematically and comprehensively. For instance, analytical size-exclusion chromatography (SEC) with UV detection, while routinely used for aggregation and fragmentation screening, lacks information on the composition of the peaks and may miss key differences in payload distribution among samples. This makes SEC-UV alone inadequate for complex samples such as heterogenous AOC formulations. Similarly, anion exchange chromatography (AEX) is commonly used to separate potential OAR variations. AEX, however, often cannot resolve aggregates or fragments and may not fully resolve different OAR variants, limiting the technique's use in purification analysis.

Mass spectrometry (MS) is another common technique used to quantify OAR heterogeneity, though its employment for AOC samples has proven challenging. Within the conjugate, negative charges at the phosphate backbone of the pendant oligonucleotide eclipse the charge contribution by the antibody, leading to a significantly negative overall charge profile. While typical mass spectrometry techniques for protein analysis employ ionization in the positive mode, negatively charged oligonucleotides are nearly unobservable under anything but negative ion mode, complicating the study of a hybrid protein-nucleic acid modality like AOCs. Furthermore, subjecting such samples to even mild MS ionization conditions could obscure biophysical details like aggregation and sample polydispersity. Beyond these challenges, MS also demands careful sample preparation that can affect irreversible and reversible aggregation events, potentially inducing precipitation and structural modifications.

Evaluating the nature of AOC samples under native and high salt conditions requires a gentler, more versatile, and more robust analytical technique. Here, we demonstrate an easy-to-implement LC-MALS method for analyzing multiple CQAs of AOCs using common aqueous mobile phases for SEC and AEX. The combination of SEC or AEX with MALS and UV detection at two wavelengths quantifies AOC payload heterogeneity, identifies the oligo-antibody ratio and quantify the payload content.

Materials and Methods

AOC samples, oligonucleotide controls, and bare antibody were kindly provided by Takeda Pharmaceutical Company. Each sample consisted of the same human IgG1 isotype control monoclonal antibody (mAb) with MCC (maleimidomethyl cyclohexane-1-carboxylate) linker functionalized luciferase-targeting siRNA (siLuc-MCC, theoretical molar mass = 14.443 kDa, MCC theoretical molar mass = 236.27 g/mol). Sample 1 primarily contained molecules with a single oligonucleotide conjugated to each antibody (OAR1). Sample 2 primarily contained molecules with two oligonucleotides conjugated to each mAb (OAR2). The human IgG1 isotype control mAb molar mass is verified with MS by Takeda.

SEC and AEX were performed using an ACQUITY™ Premier UPLC™ with an eλ PDA detector (Waters Corporation), microDAWN™ MALS detector (Wyatt Technology, LLC), and microOptilab™ dRI detector (Wyatt). UV data were collected at 280 nm and 260 nm with 1.2 nm bandwidth. Data were collected and analyzed using ASTRA™ software (Wyatt). The autosampler was kept at 6 °C, and columns were kept at 25 °C throughout the experiments.

For SEC, separation was performed using an ACQUITY Premier protein SEC column, 1.7 µm, 250 Å, 4.6 mm x 150 mm (Waters). The mobile phase was Dulbecco's PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 6.7) flowing at 0.35 mL/min. To analyze molar mass and OAR, 3 µL of each AOC sample was injected. Unconjugated mAb (injected at 3 µL), siLuc-MCC (injected at 1 µL), and free siLuc (injected at 0.15 µL) were used to establish the UV extinction coefficients for use in both the SEC-MALS and AEX-MALS analyses.

For AEX, separation was performed using a Gen-Pak™ FAX column, 2.5 µm, 4.6 mm x 100 mm (Waters). Mobile phase A was 25 mM Tris pH 7.5, and mobile phase B was 25 mM Tris pH 7.5 with 1 M NaCl. For elution, a linear gradient from 75% A and 25% B to 30% A and 70% B was applied over 20 min. The flow rate was 0.75 mL/min. For AEX-MALS analyses, 10 µL of each AOC were injected.

Results and Discussion

mAb and siLuc-MCC system suitability controls

To enable accurate conjugate analysis for the AOC samples, we first quantified the molar mass, heterogeneity, and extinction coefficients of the mAb and siRNA by SEC-MALS. UV extinction coefficients of the antibody and siRNA can come from computer predictions. However, these predicted values sometimes can carry up to ± 10% error compared to experimental values[4]. To enable more accurate analysis, we can measure the experimental UV extinction coefficients of the mAb and siRNA directly from chromatography, as shown in Table 1.

Table 1. Experimental UV Extinction Coefficients determined via online SEC-MALS

	ε_{280} (mL mg^{-1} cm^{-1})	ε_{260} (mL mg^{-1} cm^{-1})
Human IgG1 isotype control mAb	1.546 ± 0.000	0.847 ± 0.000
siLuc-MCC	10.805 ± 0.015	21.795 ± 0.090

All values are average and standard deviation of three injections.

The human IgG1 isotype control mAb predominantly contained protein monomer with a molar mass of 145.8 ± 0.1 kDa determined by MALS (Figure 1). This is in excellent agreement with intact mass spectrometry (145.496 kDa). The mAb contains mainly monomer, with minimal dimer.

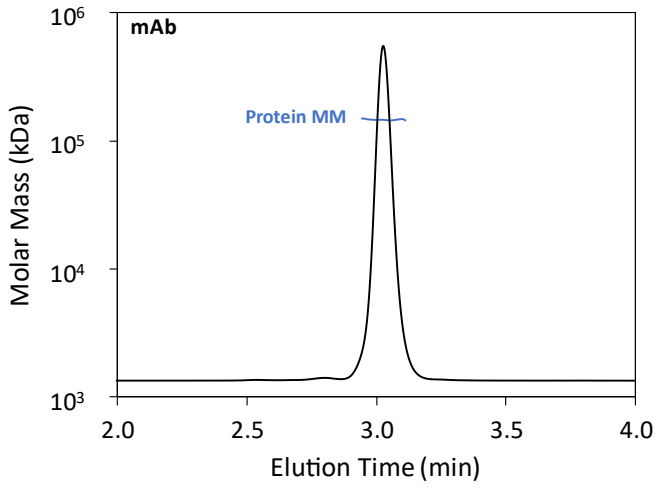

Figure 1. SEC-MALS of human IgG1 isotype control mAb. UV 280 nm chromatogram is shown with the molar mass overlaid.

Interestingly, the free siLuc RNA contains only the monomer, but SEC-MALS showed that the MCC-linker

functionalized siLuc-MCC RNA contains a distinct dimer (Figure 2). The presence of the siLuc-MCC dimer could be a quality consideration for the siRNA. The measured molar mass for the siLuc is 14.0 ± 0.1 kDa, whereas the molar mass for the siLuc-MCC monomer is 14.2 ± 0.3 kDa, and for the dimer is 28.3 ± 0.4 kDa, matching the expected theoretical molar mass of 14.4 kDa for the siLuc-MCC monomer.

The UV extinction coefficients and measured molar masses determined by SEC-MALS were then used for the analysis of the complete AOC by SEC-MALS and AEX-MALS.

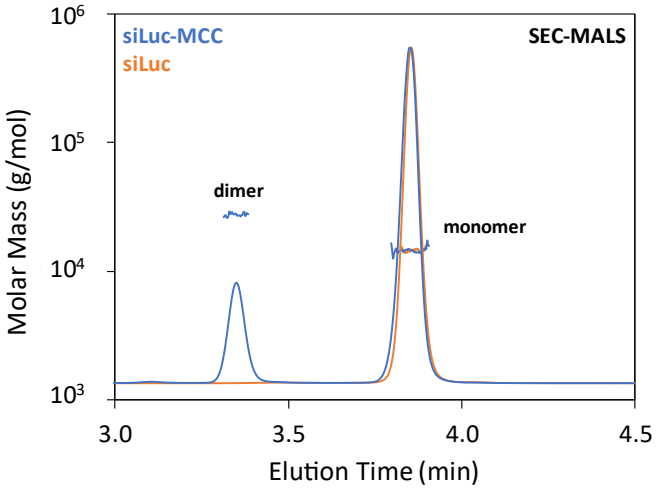

Figure 2. SEC-MALS of siLuc and siLuc-MCC. UV 260 nm chromatogram is shown with the molar mass overlaid.

Sample 1: UV-UV-MALS reveals unexpected species by SEC and AEX

Combining LC separation with MALS detection and UV absorbance at 280 nm and 260 nm enabled direct measurement of the composition of the eluting species, thus providing insight into the OAR and heterogeneity of each peak. SEC and AEX revealed that Sample 1 consisted of >90% w/w monomer with OAR1, as expected. However, each technique revealed slightly different minor species in the sample.

SEC-MALS confirmed that the main drug species is indeed OAR1. SEC-MALS also showed that this AOC sample contains an OAR1 dimer and a low abundance HMW aggregates (<2% w/w). Despite the heterogeneous nature of the HMW aggregates, the OAR for the HMW is also approximately 1.

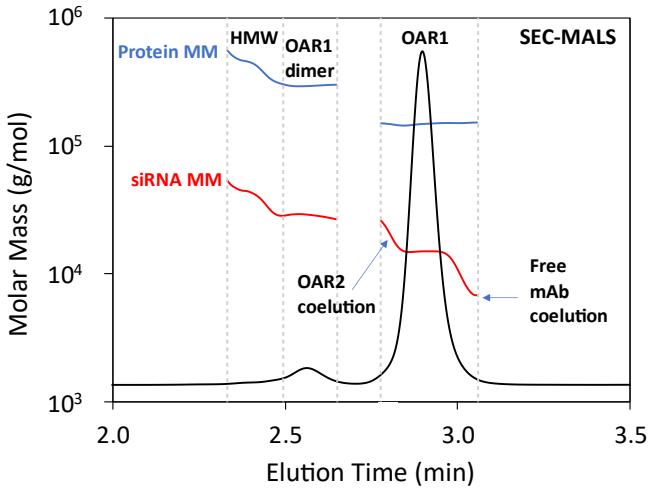

Figure 3. SEC-MALS of AOC Sample 1. The UV 280 nm chromatogram is shown with the molar mass overlaid. Peak boundaries are indicated by the vertical dashed lines.

The SEC-MALS data also suggest a small amount of unconjugated antibody co-elutes with the main monomer. This is evidenced by a decrease in the measured siRNA molar mass on the trailing edge of the monomer peak in combination with a constant (monomer) molar mass for the mAb, suggesting a decrease in the siRNA:mAb stoichiometry. Since the free mAb does not present itself as a distinct chromatogram feature, the presence of a separate species would not be detected with SEC-UV alone. Similarly, SEC-MALS data suggest a small amount of OAR2 co-elutes with the main monomer, evidenced by the increase in siRNA molar mass and constant mAb molar mass on the leading edge of the monomer peak.

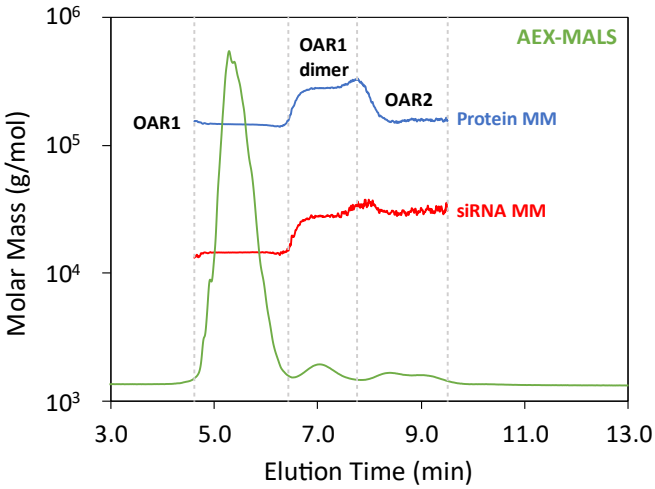

Figure 4. AEX-MALS chromatogram of AOC Sample 1. UV 280 nm chromatogram is shown with the molar mass overlaid. Peak boundaries are indicated by the vertical dashed lines.

Table 2. SEC-MALS and AEX-MALS results of Sample 1. Species are defined as shown in Figure 3 and Figure 4.

	SEC-MALS [1]				AEX-MALS [2]			
	Mass Fraction (%)	mAb M_w (kDa)	siRNA M_w (kDa)	OAR	Mass Fraction (%)	mAb M_w (kDa)	siRNA M_w (kDa)	OAR
OAR1[3]	93.4 ± 0	148.6 ± 0.1	14.9 ± 0	1.02 ± 0.00	90.8 ± 0.1	146.4 ± 0.3	14.5 ± 0.1	1.01 ± 0.00
OAR2	ND[4]	--	--	--	3.9 ± 0.1	169.8 ± 4.0	29.4 ± 1.6	1.75 ± 0.06
OAR1 dimer	5.1 ± 0.1	296.2 ± 1.2	29 ± 0.1	0.99 ± 0.00	5.4 ± 0.1	274.7 ± 0.9	26.8 ± 0.6	0.99 ± 0.02
HMW	1.5 ± 0.1	384 ± 3.2	36.8 ± 0.4	0.97 ± 0.00	ND[4]	--	--	--
Average OAR				1.02 ± 0.00				1.03 ± 0.01

[1] All values are the average and standard deviation of three injections with a total injected mass of 38.3 µg.
[2] All values are the average and standard deviation of three injections with a total injected mass of 127.6 µg.
[3] Including coeluting free mAbs.
[4] ND: not determined; this species was not resolved under this separation method.

AEX-MALS confirmed the presence of OAR1 monomer and dimer with similar abundance as determined by SEC-MALS. AEX-MALS successfully separated the small amount of OAR2 species (Figure 4), which coeluted with the main peak as observed by SEC-MALS. This OAR2 species makes up only ~ 4% of the total mass. It is important to note that without the molar mass information provided by MALS, AEX-UV alone could not distinguish aggregates and OAR variants, especially since the elution order is unpredictable.

Both SEC-MALS and AEX-MALS showed that the conjugation chemistry is specific in making the correct OAR, although the process may have introduced aggregation.

Whether it is the OAR1 dimer or the HMW aggregates, these non-covalently bound species are generally difficult to resolve with mass spectrometry, especially under native conditions. Here, SEC-MALS and AEX-MALS provided an easy-to-use and robust method for studying their presence and composition. The key results are tabulated in Table 2. Due to the coelution of some species, the weight average molar mass (M_w) is slightly different than the theoretical values. However, the OAR number is consistent with expectations.

Sample 2: AEX-MALS confirms suspected OAR1 contaminant observed by SEC-MALS

Both SEC and AEX agree that Sample 2 consisted of a product with overall OAR2, as designed. Like Sample 1, dimer and high-molecular-weight species were evident by SEC-MALS, which were somewhat captured by AEX-MALS. Unlike Sample 1, SEC-MALS suggests monomer species with undesired OAR, which were then confirmed by AEX-MALS.

SEC-MALS revealed the existence of an OAR2 dimer, consisting of two antibodies and four siRNA molecules, along with HMW aggregates with an average OAR close to 2 (Figure 5 left, and Table 2). Additionally, a small amount of OAR1 species coeluted with the main OAR2 species in SEC. Despite only achieving partial separation with SEC, combined UV-UV-MALS detection can still identify the complex nature of the sample by measuring the molar mass of the mAb and siRNA throughout the chromatogram.

Like Sample 1, the conjugation chemistry for Sample 2 is mostly specific. By AEX, the OAR1 contaminant fully resolved from the OAR2 main drug species (Figure 5, right), and we can quantify that it comprises ~ 7.5 % w/w of the sample. However, compared to Sample 1, this chemistry seemed to have generated more dimer and aggregates as a percentage of the total mass. The presence of the OAR1 species may result from unconjugated mAb or may indicate that the conjugation chemistry is reversible[5]. Future LC-MALS studies with stress conditions could reveal more about the reversibility, stability, and degradation of AOC samples.

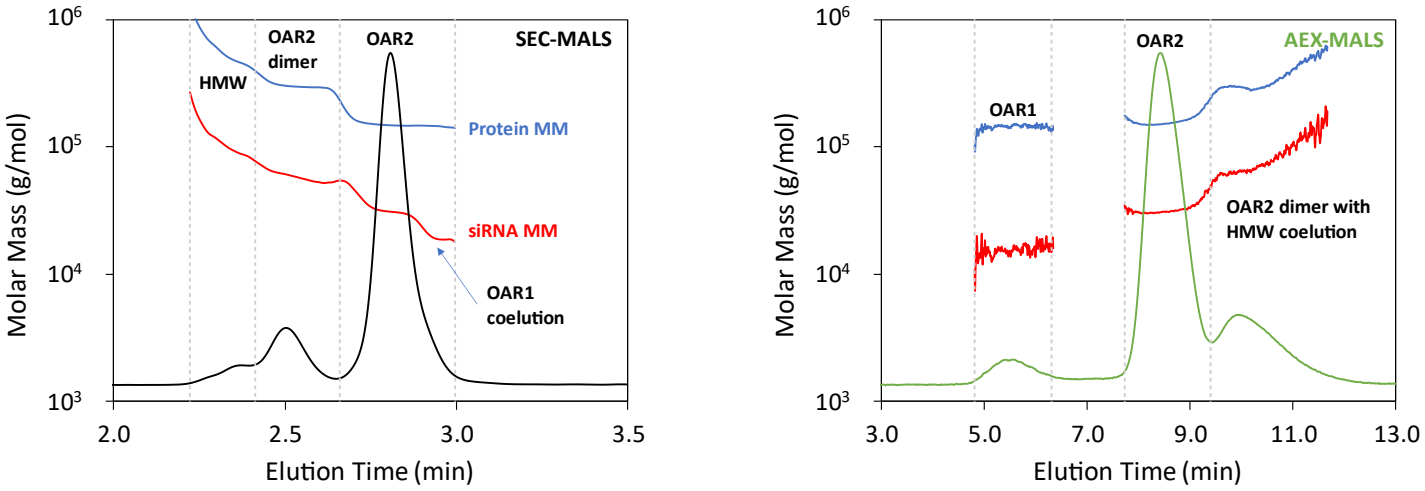

Figure 5. Left: SEC-MALS of AOC Sample 2. The UV 280 nm chromatogram is shown with the molar mass overlaid. Peak boundaries are indicated by the vertical dashed lines. Right: AEX-MALS of AOC Sample 2. The UV 280 nm chromatogram is shown with the molar mass overlaid. Peak boundaries are indicated by the vertical dashed lines.

Table 3. MALS results of Sample 2. Species are defined as in Figure 5.

	SEC-MALS [1]				AEX-MALS [2]			
	Mass Fraction (%)	mAb M_w (kDa)	siRNA M_w (kDa)	OAR	Mass Fraction (%)	mAb M_w (kDa)	siRNA M_w (kDa)	OAR
OAR1	ND[4]	--	--	--	7.5 ± 0.2	147.5 ± 8.6	15.6 ± 1.5	1.07 ± 0.04
OAR2[3]	79.2 ± 0.1	150.1 ± 0.3	30.0 ± 0.1	2.03 ± 0.01	67.8 ± 0.4	156.9 ± 0.4	31.6 ± 0.1	2.04 ± 0.00
OAR2 dimer	15.8 ± 0.0	310.0 ± 1.0	59.9 ± 0.7	1.96 ± 0.02	22.1 ± 0.2	322.7 ± 3.3	73.7 ± 2.1	2.31 ± 0.05
HMW	5.0 ± 0.1	529.5 ± 5.2	99.0 ± 2.3	1.89 ± 0.04	ND[4]	--	--	--
Average OAR				1.99 ± 0.02				2.07 ± 0.03

[1] All values are the average and standard deviation of three injections with a total injected mass of 8.01 µg.
[2] All values are the average and standard deviation of three injections with a total injected mass of 26.7 µg.
[3] Including coeluting OAR1 species.
[4] ND: not determined; this species was not resolved under this separation method.

Conclusions

Separation combined with multiple detector – including MALS, UV at multiple wavelengths, and differential refractive index – greatly expands the capability of traditional liquid chromatography-based analyses for antibody-oligonucleotide conjugates. SEC provides size-based separation yielding key information about AOC aggregates, fragments, and unconjugated nucleic acid, but SEC-UV alone may miss key features and differences in payload distribution. Similarly, AEX utilizes charged-based separation to provide better resolution of pay-load variants, but AEX-UV alone may misidentify the eluting peaks. By combining UV260, UV280, MALS, and dRI detection with either separation technique, the molar mass and composition of the eluting species are measured simultaneously throughout the entire chromatogram, enabling accurate payload quantitation for antibody-oligonucleotide conjugates. Based on the results reported in this white paper, we conclude that LC-MALS provides users with a platform technology to measure multiple critical quality attributes in a single rapid, robust method. The LC-MALS method is applicable throughout AOC product and process development, including research, formulation, and QA/QC. This translates to a simplified system setup, saving both time and resources and reducing errors with a unified single workflow.

References

1. Mullard, A. Antibody–oligonucleotide conjugates enter the clinic. *Nature Reviews Drug Discovery* **21**, 6–8 (2021). *https://doi.org/10.1038/d41573-021-00213-5*
2. Dovgan, I., Koniev, O., Kolodych, S. & Wagner, A. Antibody–Oligonucleotide Conjugates as Therapeutic, Imaging, and Detection Agents. *Bioconjugate Chem.* **30**, 2483–2501 (2019). *https://doi.org/10.1021/acs.bioconjchem.9b00306*
3. Dugal-Tessier, J., Thirumalairajan, S. & Jain, N. Antibody-Oligonucleotide Conjugates: A Twist to Antibody-Drug Conjugates. *J Clin Med* **10**, 838 (2021). *https://doi.org/10.3390%2Fjcm10040838*
4. Batabyal, D., Lord, H., Ahlstrom, B. & Wikström, M. Determination of the experimental extinction coefficient of therapeutic proteins using the Edelhoch method. *Biologicals* **71**, 42–47 (2021). *https://doi.org/10.1016/j.biologicals.2021.03.003*
5. Szijj, P. A., Bahou, C. & Chudasama, V. Minireview: Addressing the retro-Michael instability of maleimide bioconjugates. *Drug Discov Today Technol* **30**, 27–34 (2018). *https://doi.org/10.1016/j.ddtec.2018.07.002*

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