Decoding ADC spectra for DAR measurements at warp speed with Stunner

Introduction

Characterizing ADCs by UV/Vis absorbance is the fastest and simplest approach for determining antibody concentration and drug-antibody ratio (DAR) analysis. This type of measurement is ideal when you need a method that can be standardized across multiple labs, or when screening large numbers of samples during discovery and process development. However, there are several major potholes that can get in the way of analysis when you're working with samples where you don't already know every detail about the antibody, drug, or linker spectra.

Traditional UV/Vis determination of DAR requires prior knowledge of drug-linker absorbance spectra and accurate extinction coefficients before your method is fully ready-to-go. Many ADCs are also challenging to use in the classic two-wavelength approach because their drug and antibody absorbance spectra are too similar, making both method development and analytical readouts more difficult.

Stunner comes fully equipped with all the brains, speed, and flexibility needed for analysis of DAR for every kind of ADC, conjugated fAb, or other protein-conjugate (Figure 1). Using advanced spectral-unmixing algorithms, Stunner can deconvolute overlapping ADC spectra to extract both the protein and drug-linker contributions– even when no prior spectral information is available. This approach makes analysis possible even for highly overlapping spectra or when there is no prior drug-linker spectrum knowledge.

For a more classic analysis approach, Stunner's two-wavelength application (ADC Dual Wvl) empowers simple two-wavelength DAR analysis. Stunner can also be taught drug-linker spectra and use them for full-spectrum DAR analysis in the ADC Quant application, providing improved robustness and reduced noise compared to relying only on two wavelengths.

Figure 1: Stunner is the only plate-based dynamic light scattering & UV/Vis tool built for biologics.

Because aggregation is a common challenge in ADC development, each UV/Vis-based ADC application on Stunner can be paired with dynamic light scattering (DLS) to screen for particle size and detect aggregates in the same measurement.

This application note demonstrates how Stunner simplifies ADC characterization by delivering rapid, precise measurements of antibody concentration, DAR, size and aggregation – from just 2 µL, in less than a minute per sample, and on an automation-friendly, 96-well plate-based format.

Materials & Methods

Trastuzumab (HY-P9907), trastuzumab emtansine (HY-P9921), trastuzumab deruxtecan (HY-138298A), trastuzumab vedotin (HY-164992), naratuximab (HY-P99374), naratuximab emtansine (HY-132260), gemtuzumab (HY-P9997), gemtuzumab ozogamicin (HY-109539) were all obtained from MedChemExpress.

Stunner (P/N: 900-2003), Stunner plates (P/N: 701-2125) and Stunner Client and Analysis v10.1 were used for this work (Figure 2). The ADC Quant, ADC Dual Wvl, and ADC Decode applications were used for experimental setup and analysis.

Results

Back to basics

The most basic approach to quantifying antibody concentration, drug-linker concentration, and DAR also relies on knowing the most about the UV/Vis absorbance behavior of your two molecule types. If the molar extinction coefficient is known in units (L/mol)/cm for both the antibody and the drug at two wavelengths, then DAR and both concentration values can be determined by solving a system of equations.[1]

The Dual Wvl application on Stunner simplifies this analysis by automatically performing all calculations, either by using manually entered molar extinction coefficients, or by applying the values you've saved as Analytes in the Stunner software.

Figure 3 shows the consistency of the ADC Dual Wvl application when measuring antibody concentration, drug concentration, and DAR values for three trastuzumab-derived ADCs: trastuzumab emtansine, trastuzumab deruxtecan, and trastuzumab vedotin. Molar extinction coefficients derived from literature were applied when available.[2-5] Samples were diluted across a 1:2 dilution series spanning an eightfold concentration range starting from antibody concentrations of 4.8, 5.0, and 2.9 mg/mL, respectively. The ratio of measured drug and mAb concentrations spanned a range of 1.89-2.17 across all samples and each dilution step. The DAR values over all dilutions were stable and spanned the range of 0.98-1.04 going from dilution to dilution. The stability of these ratios demonstrates excellent linearity and precision across the range of measurements.

However, determining the correct molar extinction coefficient values can be difficult, especially when analyzing large sample sets or working with molecules that are not fully characterized. Extinction coefficients determined in organic solvents may not translate directly to aqueous conditions, and significant spectral overlap between the drug and antibody can make determining molar extinction coefficients impractical. In these situations, a more flexible and intelligent analytical approach is needed.

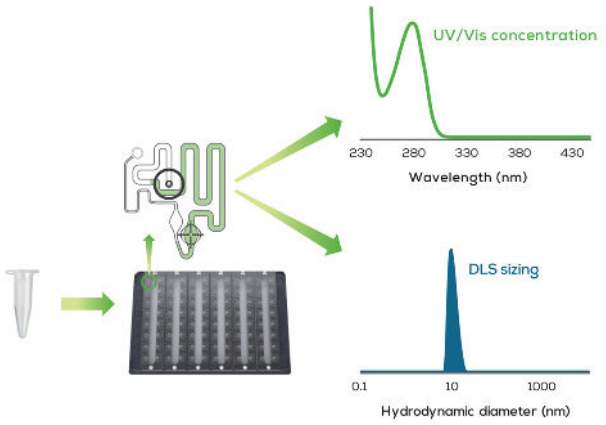

Figure 2: Microfluidic circuits in the automation-compatible, 96-well Stunner plate make it possible to collect UV/Vis concentration, size and PDI measurements from 2 µL samples.

Decoding ADC spectra

When molar extinction coefficients and spectra are unknown, Stunner's ADC Decode applications are available to read ADC absorbance and use powerful Unmix algorithms to apply the best fit of the underlying protein spectrum and visualize the underlying drug-linker absorbance spectrum.

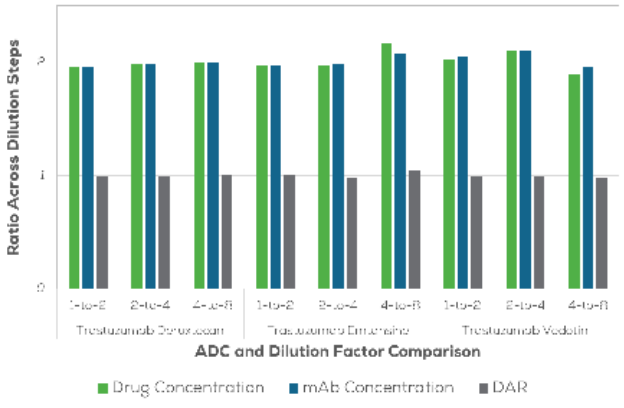

Figure 3: Across a 1:2 dilution series antibody concentration and drug concentration consistently decreased by a factor of 2, while DAR remained constant. Samples were measured by the ADC Dual Wvl application.

When an ADC is measured by UV/Vis absorbance using the ADC Decode application, Stunner will use a protein spectrum from Stunner's analyte library (typically an antibody) for analysis. However, when the antibody concentration of an ADC sample is unknown, there is a range of possible values bounded by the ADC's overall absorbance as the maximum and zero as the minimum (Figure 4). Stunner's ADC Decode application uses absorbance features related to tryptophan in the 280-300 nm region to determine the optimal fit and optimal antibody concentration.

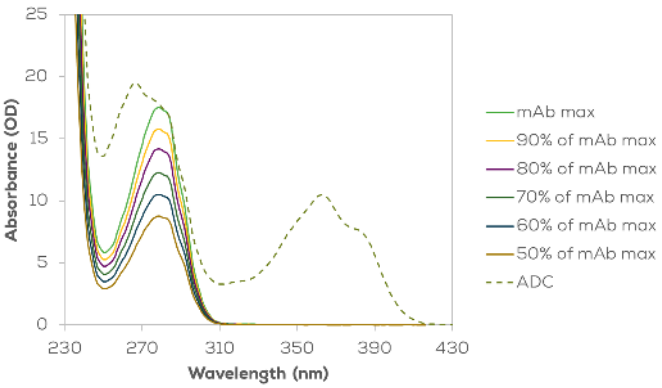

Figure 4: The range of possible protein (mAb) spectra that could be extracted from the absorbance spectrum of an ADC (dashed line, trastuzumab deruxtecan). Each line represents a different possible protein concentration and would correspond with different possible complementary drug-linker spectra (not shown).

Based on the measured ADC spectrum, the algorithm in ADC Decode is able to optimize for a best-fit solution and determine the concentration of the antibody and the resulting drug-linker absorption spectra. Examples of decoded spectra for trastuzumab emtansine, trastuzumab deruxtecan, and trastuzumab vedotin are shown in Figure 5. When analyzed in quadruplicate the variability of the drug absorbance detected had coefficients of variation (CVs) of 0.6%, 0.5%, and 0.6%, respectively. The measured DAR values of each had CVs of 0.5%, 0.2%, and 0.5%, respectively.

Since molar extinction coefficients may not always be available for early-stage compounds, Stunner's ADC applications are equipped to enable relative comparisons based

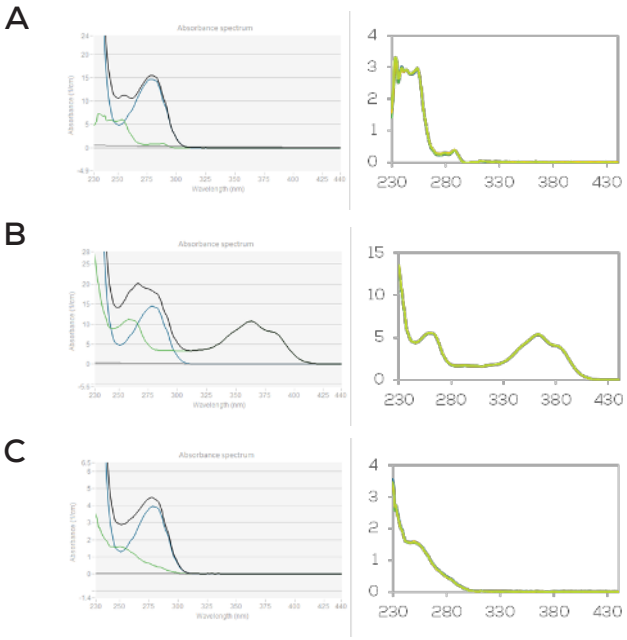

Figure 5: The resulting spectra (left) when ADC Decode is used to analyze trastuzumab emtansine (**A**), trastuzumab deruxtecan (**B**), and trastuzumab vedotin (**C**). The accompanying graphs (right) show the precise overlay of the spectra of the drug component across all triplicates.

solely on absorbance information. For the above example, trastuzumab emtansine had a DAR of 3.6 when measured by hydrophobic interaction chromatography and a DAR of 3.3 when measured by Stunner – however, if molar extinction coefficient values were unavailable, the ratio of drug spectra to protein spectra absorbances ($A_{252\ nm}$ / $A_{280\ nm}$ = 0.48) could be used to compare process steps, conjugation conditions, or batches.

The 1:2 dilution series of the three trastuzumab-derived ADC samples described previously were analyzed side-by-side by the ADC Decode and the ADC Dual Wvl applications (Figure 6). Antibody concentration measurements of unconjugated trastuzumab had a CV of 0.2% (not shown) while trastuzumab emtansine, trastuzumab deruxtecan, and trastuzumab vedotin had CVs of 0.4%, 0.3%, and 0.4%, respectively. DAR measurements were also precise, with CVs ranging from 0.5-1.4% for the highest concentration samples measured and 0.8-4.3% for the lowest concentrations.

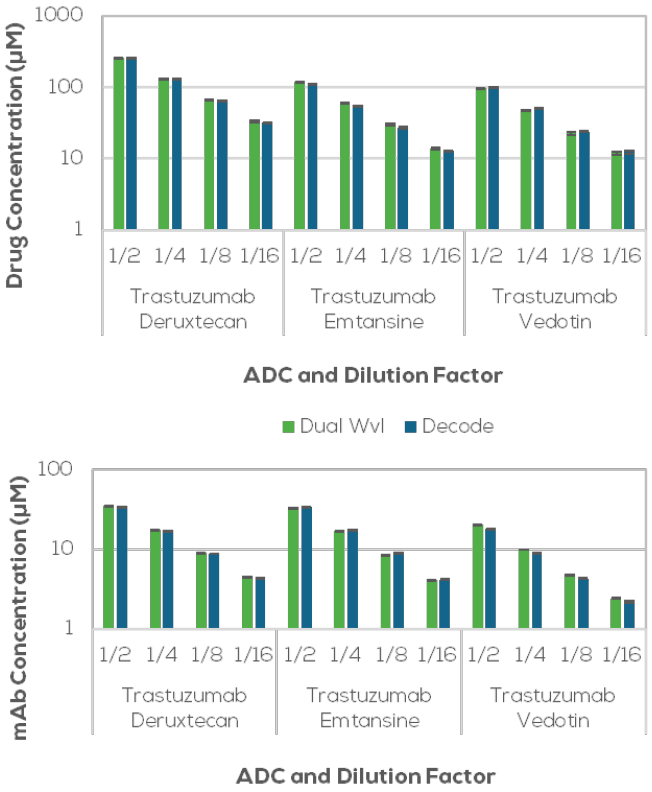

Figure 6: Across a 1:2 dilution series antibody concentration and drug concentration decreased linearly when measured by both the ADC Decode and the ADC Dual Wvl applications for three trastuzumab-derived ADCs. Note that the y-axes are log scale for improved visibility at low concentrations.

Using the ADC Decode + Sizing application, stock concentrations of the three trastuzumab-derived ADCs were evaluated for size and aggregation by DLS (Figure 7). Trastuzumab emtansine measured 11.8 ± 0.4 nm, trastuzumab deruxtecan 14.3 ± 0.5 nm, and trastuzumab vedotin 10.2 ± 0.8 nm. The reproducibility and overlap of the mass distributions indicate that the bulk of the sample mass is consistent in size. However, the larger size of trastuzumab deruxtecan could indicate aggregation and should be further investigated. This kind of analysis enables rapid aggregation screening and helps identify process or formulation conditions that cause aggregation.

ADC Quant

The final ADC application available on Stunner is the ADC Quant application, called ADC + Sizing when DLS is included. The ADC Quant application is able to take antibody and drug spectra saved to Stunner's database from

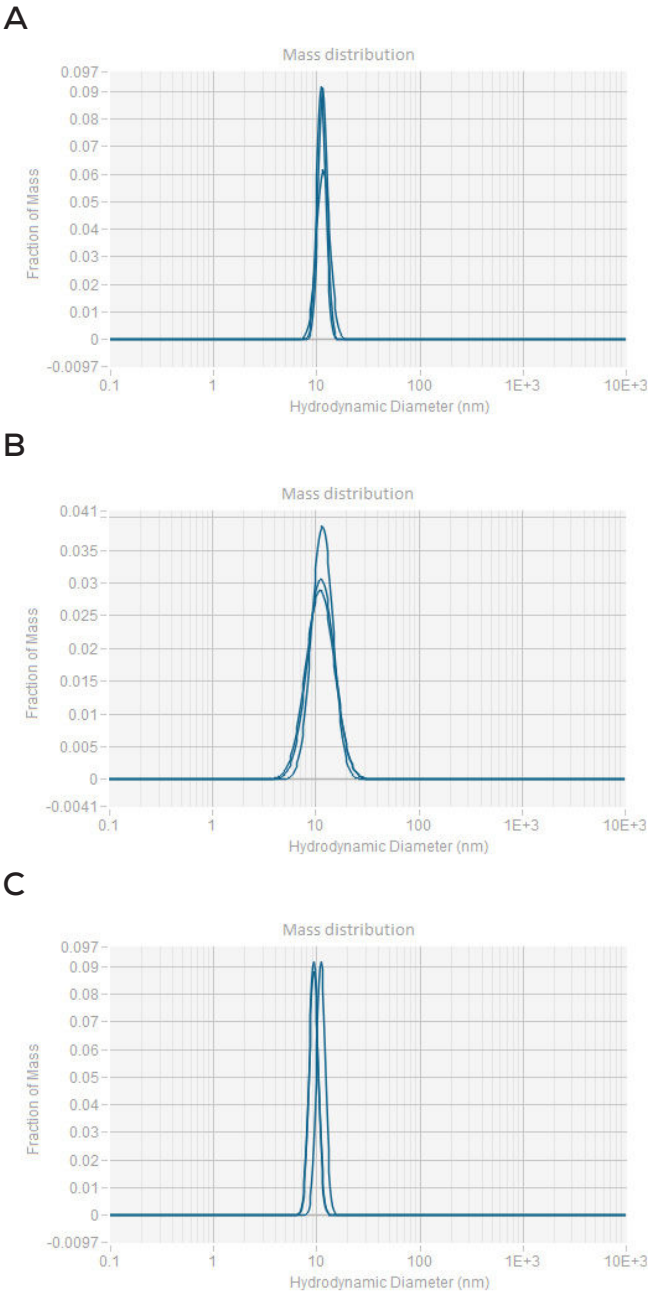

Figure 7: Mass distributions from a DLS analysis of trastuzumab emtansine (**A**), trastuzumab deruxtecan (**B**), and trastuzumab vedotin (**C**), each measured in triplicate using the ADC Decode + Sizing application.

prior experiments and apply them to ADC measurements to accurately deconvolute spectra and determine antibody and drug spectra. When working with ADCs, the ADC Quant applications have the advantage of using the full drug and antibody spectra to evaluate concentration, which is generally recommended as an advantage over the two-wavelength methodology.

Conclusion

Stunner pushes the limit for what is possible in ADC characterization by bringing together robust UV/Vis analytics with sensitive DLS sizing in a single assay done on only 2 µL of sample. Whether untangling overlapping spectra with ADC Decode, applying known spectra data with ADC Dual Wvl or ADC Quant, or screening for aggregation by adding DLS, Stunner provides accuracy and precision throughout the ADC development pipeline. By using minimal sample volume, ultra-simple workflows, and rapid data collection Stunner enables a completely new look at ADCs characterization.

References

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5. Nakatsuji, M.; et al. Human Lipocalin-Type Prostaglandin D Synthase–Based Drug Delivery System for Poorly Water-Soluble Anti-Cancer Drug SN-38. PLOS ONE 2015, 10(11), e0142206. *Note that extinction coefficient determined in DMSO at 380 nm and applied to peak in aqueous at 363 nm.

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