Uncover the secrets of your ADC with Stunner and Uncle

Introduction

Antibody-drug conjugates (ADCs) consist of 3 chief components: a monoclonal antibody (mAb), a linker, and a small molecule drug. The resulting combinations are potent but characterizing such a complex molecule brings in challenges that aren't present in small molecules or mAbs individually. Monitoring antibody concentration, the drug-antibody ratio (DAR), and watching for aggregates are all critical during the early stages of ADC development, but there are other concerns, too. Adding the small molecule can change the surface properties of the precursor mAb, often making it more hydrophobic. Choice of drug, linker, and attachment site all affect this change in surface properties. These changes also impact the ADC's developability profile, particularly its thermal stability. This, in turn, often creates the need to optimize the ADC's formulation.

Determining the DAR of ADCs and looking for aggregates typically involve a combination of HPLC and detection techniques, like size-exclusion chromatography and mass spectrometry. However, these methods often take too long, require too much sample, or need too much optimization to work for every single candidate ADC. Stunner (Figure 1A) uses UV/Vis absorbance on just 2 µL of sample to determine the concentration and DAR of up to 96 samples at-a-time.[1,2]

User-stored reference spectra help Stunner's Unmix algorithms deconvolute the UV/Vis absorbance spectrum of an ADC into its component parts. From these deconvoluted spectra, Stunner can quantify the total amount of protein and drug payload present and use these to calculate the DAR. Simultaneously, Stunner detects aggregates by looking at the size and size distribution of the ADC sample with dynamic light scattering (DLS).

A

B

Figure 1: Stunner (**A**) is the only plate-based system that pulls together UV/Vis and DLS data on the same 2 µL sample. Uncle (**B**) is the original all-in-one biologics stability platform.

Developability assessments of ADCs include characterization of their biophysical properties, to eventually understand their thermal stability in a range of conditions. Evaluating these properties early on identifies potential problems, for example high aggregation propensity, before

they become more serious. It also gives a head-start on formulation screening. However, these advantages depend on high-throughput methods with low sample consumption since ADC material is precious and not readily available in early-phase research and discovery.

Uncle fills that niche as the original all-in-one platform for protein stability with 3 detection methods: full-spectrum fluorescence, static light scattering (SLS), and DLS to fully profile ADC thermal stability from 9 µL of sample (Figure 1B). Temperature control (15–95 °C) and sealed sample holders supply greater flexibility in how that profiling can be performed. Multiple parameters, including fluorescence, aggregation, size, polydispersity, and thermal unfolding, can be assessed from the same sample volume in just one experiment, allowing you to obtain orthogonal and complimentary information. Uncle can measure up to 48 samples at a time, enabling quick access to a wide range of results when characterizing mAbs and ADCs with high throughput.

In this study, we conjugated a mAb with 3 different payload/linker combinations to model ADCs. Concentration, DAR, and ADC quality were assessed on Stunner and a set of criteria were established to identify promising ADC candidates. A subset of 3 candidates was then used for formulation screening and thermal stress testing on Uncle. The results show how to use Stunner and Uncle to screen ADCs, determe developability profiles, and optimize formulations.

Methods

Monoclonal antibody conjugation and ADC quantification

Fluorescein isothiocyanate (FITC, Thermo Fisher 46425), Alexa Fluor™ 350 (AF350) NHS ester (Thermo Fisher A10168), and AF350 C_5 maleimide (Thermo Fisher A30505) were reacted at the indicated dye-to-protein molar ratios with 10 mg/mL monoclonal antibody in 50 mM borate buffer, pH 8.5, 100 mM bicarbonate buffer, pH 8.3, or 1X phosphate-buffered saline (PBS), respectively. 100 µg of tris-(2-carboxyethyl) phosphine (TCEP, Thermo Fisher T2556) were added to the maleimide reaction as a reducing agent. The reaction mixes were incubated for 1 hour at room temperature, protected from light.

After incubation, Unagi was used to buffer exchange the reaction mixes into PBS and to remove payload-related impurities. Protein concentrations, drug-antibody ratio (DAR), size, and polydispersity index (PDI) were checked in quadruplicate on Stunner. DLS acquisition settings of 5 acquisitions at 1 second each were used with the software's automatic outlier exclusion.

ADC thermal stability and formulation excipient screening

Spike-ins of Tween 80, trehalose, and arginine were added to aliquots of each ADC and the mAb to final concentrations of 0.01%, 80 mg/mL and 10 mg/mL, respectively. Protein concentrations were set to 2 mg/mL. Each sample was loaded into cuvettes of a Uni (an array of sixteen 9 µL quartz cuvettes, sealed by silicone gaskets) in triplicate. Protein unfolding and aggregation experiments were performed with the T_m & T_{agg} application on Uncle. Samples were ramped in Uncle from 15–95 °C at a rate of 0.3 °C/minute and excited at 266 and 473 nm, while simultaneously monitoring fluorescence emission and SLS. Uncle Analysis software determined the T_m from the barycentric mean (BCM) of the fluorescence intensity curves from 300–380 nm and the T_{agg} from the intensity of light scattered at 266 nm.

Results

Identifying the optimal reaction conditions and combination of drug, linker, and mAb means doing a lot of high-throughput screening on a number of conditions. It also means determining the thresholds or target values for a number of different parameters, like DAR and % recovery. Lastly, it means checking to make sure that the resulting ADC is of high quality and does not contain aggregates. In some cases, these criteria are linked and thus using a multi-parameter

assay tool like Stunner can accelerate the process, plus allow new insights to come to light.

In order to determine the DAR of an ADC, Stunner simultaneously determines the concentrations of antibody and drug in solution. Increasing the molar reaction ratio of AF350 NHS ester to mAb increased the DAR of the resulting ADC (Figure 2). When using 2:1 or 10:1 molar ratios, the DAR approximately equaled the molar ratio. However, this linear relationship weakened as the molar ratio increased, with the 20:1 and 40:1 experiments resulting in DARs of 15 and 30.3, respectively. Since the linker in this experiment preferentially reacts with lysine residues in the mAb, it's likely that the DAR would reach a plateau based on the kinetics and the number of available reactive sites.

Using the antibody concentration we can also determine the % recovery for the conjugation and purification steps of all 4 different molar ratios. With the exception of the 40:1 reaction, all of the recoveries were >80%, which we considered acceptable for this application.

As part of the Unmix deconvolution, Stunner also determines a turbidity correction value for the background, based on the UV/Vis spectra, and reports it as an A330 value. Turbidity was near-zero in the 2:1 and 10:1 reactions, but higher in the 20:1 and 40:1 reactions. Higher turbidity generally indicates the formation of large particles, which can be protein, ADC, or dye-related aggregates. This may explain lower recovery in the 40:1 reaction: some of the ADC was most likely lost due to the formation of large aggregates that precipitated.

Conjugating a dye or (in real life) a drug changes the biophysical properties of a mAb, including its hydrodynamic diameter. In the case of our AF350 NHS ester conjugates, increasing the DAR also increased the hydrodynamic diameter of the ADC, relative to the parental mAb (Figure 3). The 2.1 DAR ADC had a diameter that was close to the unreacted mAb while the other ADCs were larger. The size increase trended in the same direction as increasing DAR – more conjugated dye results in a larger ADC.

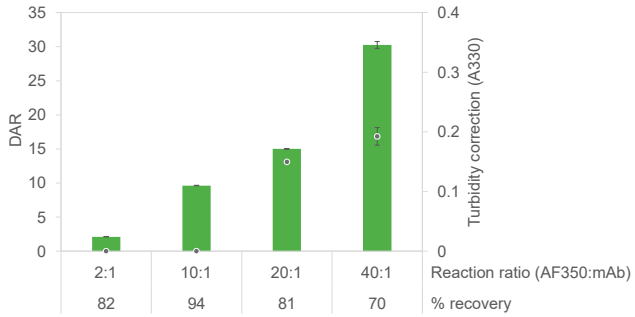

Figure 2: Drug-antibody ratio (DAR) of mAb labeled with AlexaFluor™ 350 NHS ester reacted at 2:1, 10:1, 20:1 and 40:1 dye-to-protein molar ratios after purification (green bars, left y-axis) with Stunner's turbidity assessment (grey dots, right y-axis). Percent recovery of the conjugated mAbs is also shown. Error bars are 1 standard deviation of quadruplicate measurements.

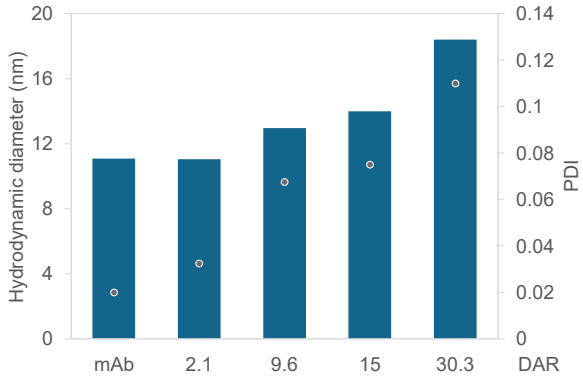

Figure 3: Hydrodynamic diameter (blue bars, left y-axis) and PDI (grey dots, right y-axis) of purified mAb labeled with AlexaFluor™ 350 NHS ester from Figure 2.

ADCs with aggregates or other protein- or drug-related impurities have higher PDIs than well-behaved, monodisperse ADCs. As a rule-of-thumb, proteins and ADCs with PDIs ≤0.1 are monodisperse while a PDI >0.2 is indicative of protein aggregation. The AF350 NHS ester ADCs had PDIs <0.1 except the 30.3 DAR sample, which had a PDI of 0.11. Taken together, the higher PDI, observed low recovery, and high turbidity all indicate this 'drug' load on the mAb did not result in a soluble or stable ADC.

Testing on Stunner found that the 2.1 DAR ADC had a hydrodynamic diameter and PDI very close to the unreacted mAb, indicating the ADC preparation is at least as homogeneous as that of the parent mAb and it is unlikely aggregation occurred during the conjugation reaction or purification steps.

ADCs that appear suitable for further consideration immediately following conjugation can become problematic later. DLS can often spot issues before they become larger problems. The initial intensity and mass distributions of the AF350 ester ADC with a DAR of 15 showed single, well-defined peaks at 12 nm, which is a size typical of monodisperse antibodies (Figure 4). However, after 1 week of storage at 4 °C the intensity distributions showed that large aggregates had appeared. The mass distributions show the aggregates were only a small fraction of the overall mass, probably about 1% of the total amount of ADC, but aggregates have a potential to increase over time. The DAR 30.3 sample was even more severely aggregated (data not shown).

Stunner allows for the measurement of DAR, turbidity, diameter, and PDI in a single experiment. When combined, this information gives useful metrics and actionable results for selecting and refining reaction conditions for mAb conjugation. Based on the above pilot experiments, we identified 4 criteria for choosing reaction conditions to yield samples appropriate for further analysis:

1. DAR between 2–4.
2. Hydrodynamic diameter close to 12 nm
3. PDI ≤0.1
4. Recovery >80%

We identified molar conjugation ratios for 3 conjugate payloads that met or exceeded our criteria and took the resulting ADCs into further formulation and thermal stability testing on Uncle (Table 1).

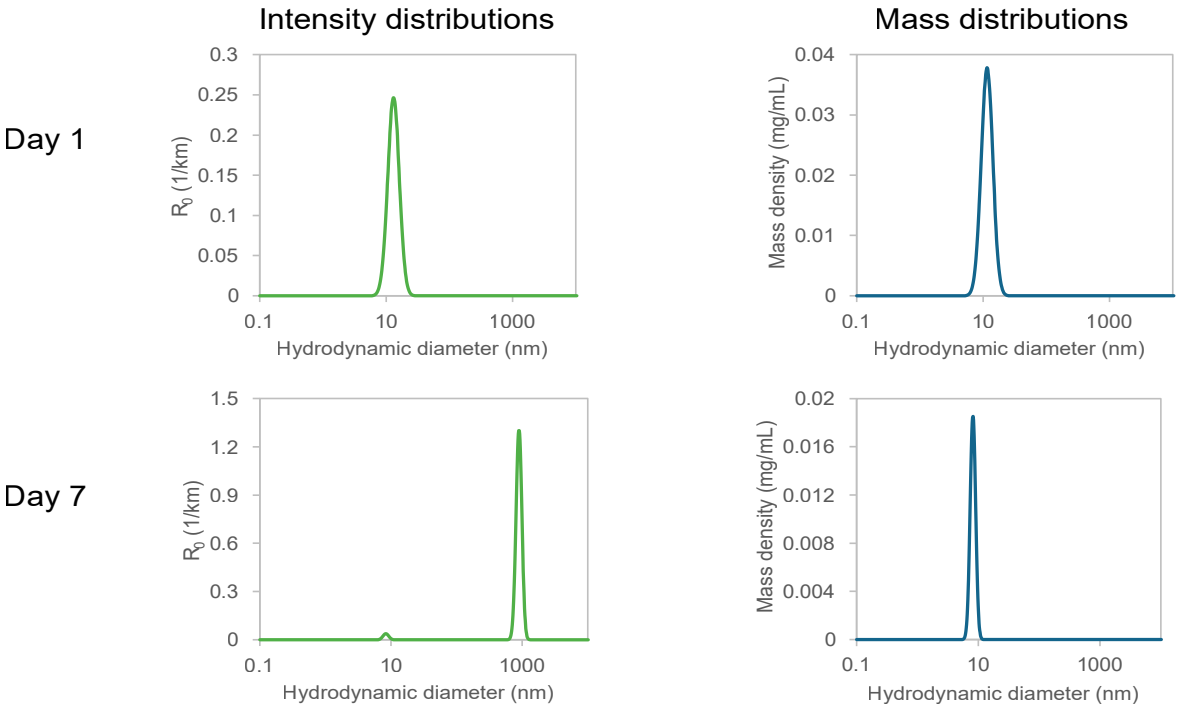

Figure 4: Intensity distributions (green, left) and mass distributions (blue, right) of mAb labeled with AlexaFluor™ 350 NHS ester with a DAR of 15 (see Figures 2 & 3) immediately after reaction/purification (top) and after 7 days of storage at 4 °C (bottom).

Label	Molar ratio (Dye: mAb)	DAR	Hydrodynamic diameter (nm)	PDI	Recovery
FITC	10:1	3.7	13.2	0.1	86%
AF350 NHS Ester	2:1	2.1	11.1	0.03	82%
AF350 C_5 maleimide	10:1	2.1	11.4	0.1	93%

Table 1: Dye-to-protein labelling molar ratio, resulting DAR, hydrodynamic diameter, PDI, turbidity and % recovery of mAb labeled with FITC, AlexaFluor™ 350 NHS ester and AlexaFluor™ 350 C_5 maleimide, selected for additional thermostability testing.

Thermal stability screening is a key method for high-throughput developability testing of ADCs, mAbs, and formulations.[3,4] Intrinsic fluorescence can track the melting of proteins and mAbs, but ADCs can add an extra wrinkle. Many drugs used in ADCs also fluoresce with UV excitation and the resulting drug emission spectra can interfere with analysis. The mAb component of ADCs typically emits fluorescence from 300–450 nm (Figure 5). As an ADC unfolds/melts, the peak of emission tends to shift to longer wavelengths ('red shift'), and to decrease. However, in case of the analytes used here, the AF350 conjugated to the mAb emits light in the 400–550 nm range when excited by a UV laser, overlapping slightly with the protein's intrinsic fluorescence emission.

Uncle has full-spectrum fluorescence detection, so you can see if the drug and antibody emission spectra overlap. It also has tools to tailor the analysis to find the appropriate method for a specific sample. This flexibility also allows Uncle to use thermal stability dyes like CPM or Sypro Orange and nucleic acid dyes like Sybr Gold for a wide range of targets including ADCs, viral vectors, LNPs, and membrane proteins. In this set of ADCs, analysis focused on the BCM in the range 300–380 nm, to omit interference by the dyes conjugated.

High T_ms and T_{agg}s generally indicate a better developability profile than lower values for mAbs and ADCs, but also for formulations.[4] Some common excipients, like Tween 80, trehalose, and arginine, are added for a variety of reasons.[5] Tween 80 is a surfactant and detergent that reduces surface adsorption and protein denaturation at air-water interfaces.

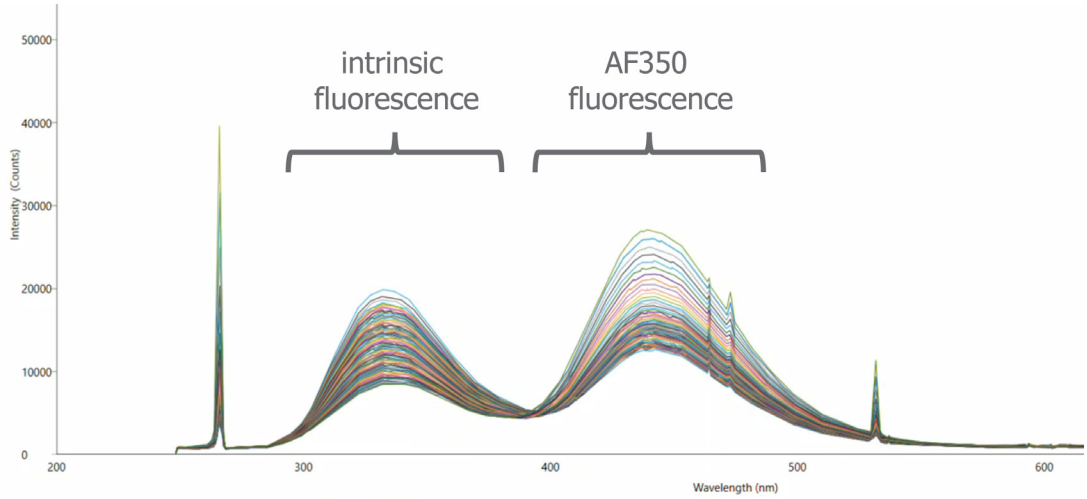

Figure 5: Emission spectra of AlexaFluor™ 350-labeled mAb excited by 266 and 473 nm lasers in Uncle during a thermal ramp from 15–95 °C. The fluorescence emissions of the protein itself and the dye are clearly visible as distinct peaks centered at ~330 and 440 nm, respectively.

Trehalose is a cryo-protectant and arginine is often used to reduce viscosity of high concentration mAb therapies. All 3 excipients also impact the melting and aggregation of mAbs and ADCs exposed to thermal stress. These impacts are not universal; they may vary depending on the sequence and structure of the mAb, but also the location and identity of a drug or linker.

Rank-ordering the formulations based on their T_m and T_{agg}, with PBS serving as the "default," shows which excipients have the largest impact on conformational and colloidal stability of the mAb (Figure 6). The inclusion of trehalose (yellow) had the largest positive impact on the T_m and T_{agg} of the mAb. Arginine, a chaotrope, decreased T_m and increased T_{agg}. The increase of T_{agg} with arginine (purple) was smaller than the increase with trehalose, relative to PBS alone. Tween 80 (blue) had no impact on T_m or T_{agg}. It's also worth noting that the protein started unfolding much earlier than it started aggregating, or in other words: $T_m << T_{agg}$ for all samples tested.

Creating an ADC changes the properties of the precursor mAb's surface, often making it more hydrophobic. This also impacts the thermal stability profile. In the case of conjugation with FITC or the AF350 NHS ester, a hydrophilic lysine residue became more hydrophobic. With the maleimide reaction, disulfide bonds were reduced with TCEP to free cysteines which then reacted with the hydrophobic drug. Uncle lets you use the same tools to characterize the thermal stability of mAbs and ADCs, to make comparisons across different drugs and linkers easy.

Conjugation of the mAb with FITC (blue) slightly decreased T_{agg} but had no impact on T_m (Figure 7A). However, adding Alexa Fluor™ 350 NHS ester (yellow) increased T_m by 0.7 °C and T_{agg} by 0.6 °C. Even though both of these conjugation reagents target amines, their linkers and structures are different and they had opposite effects on the stability of the protein. The breaking of disulfide bonds and addition of Alexa Fluor™ 350 to free cystines with a maleimide reaction (purple) slightly decreased T_m by 0.3 °C but decreased T_{agg} by 3.6 °C. This was the biggest change in behaviors due to conjugation, by a large margin.

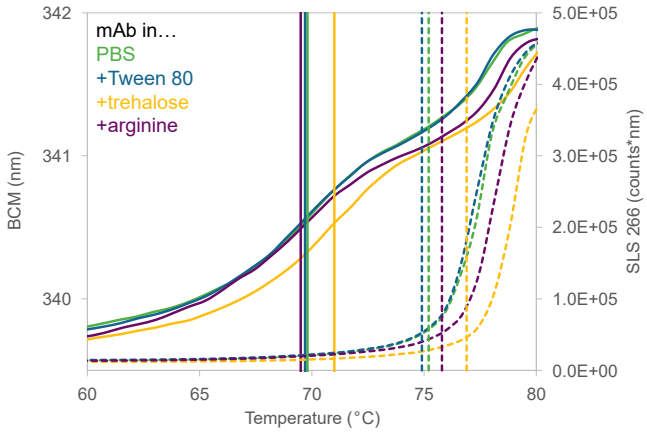

Figure 6: Melting (solid curves, left y-axis) and aggregation (dashed curves, right y-axis) of 2 mg/mL mAb in PBS (green) and with Tween 80 (blue), trehalose (yellow) or arginine (purple) spiked-in. T_m (solid vertical lines) and T_{agg} (dashed vertical lines) values for each formulation are also depicted. Curves are representative of triplicates, T_ms and T_{agg}s are averages of the same triplicates.

The choice of linker, drug, and amino acid target all impact the thermal stability of an ADC. Their behaviors aren't always predictable based on related compounds. However, just like with mAbs, ADCs with less-than-desirable thermal stability profiles can often be ameliorated by choosing the proper formulation and excipients. Adding trehalose to the formulations stabilized all ADCs, just as it did for the parent mAb (Figure 7B). The maleimide conjugate showed the largest improvement in thermal stability, going from a T_{agg} of 71.6 °C in PBS to 76.2 °C with trehalose, an improvement of nearly 5 °C.

The other excipients tested, arginine and Tween 80, had various impacts on the thermal stability of the ADCs, but trehalose had the largest stabilizing effect. The complete results are shown in Table 2. Even though arginine tended to decrease T_ms and increase T_{agg}s, it improved both parameters when added to the ADC containing AF350 maleimide.

The results also show the reproducibility of Uncle's T_m and T_{agg} measurements. The standard deviations of all the values were less than 1 °C, which showcases Uncle as a reliable and robust method of determining thermal stability.

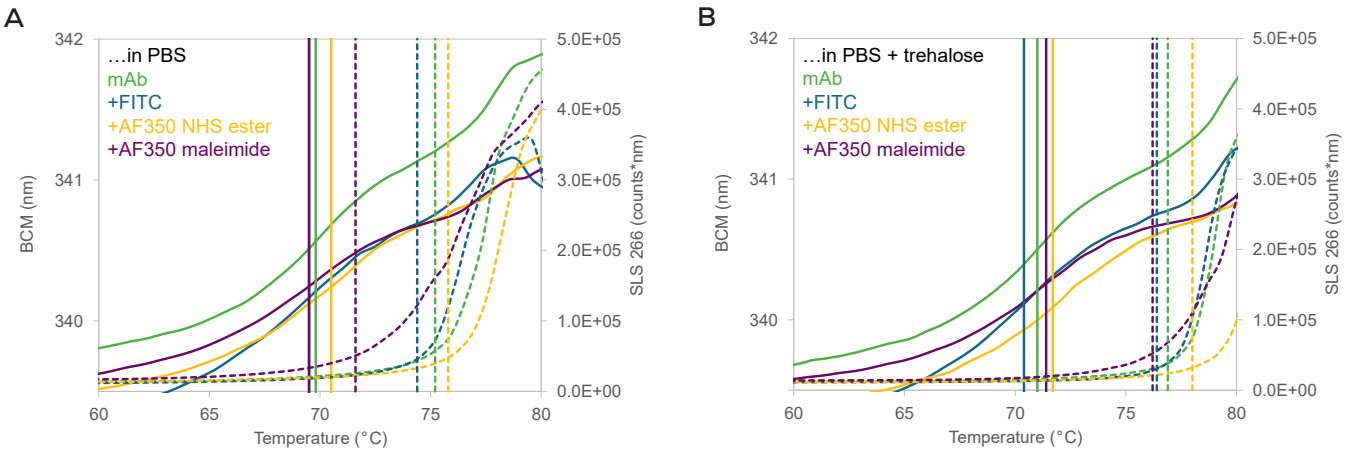

Figure 7: Melting (solid curves, left y-axis) and aggregation (dashed curve, right y-axis) of 2 mg/mL unconjugated (green), FITC-labeled (blue), AlexaFluor™ 350 NHS ester-labeled (yellow) and AlexaFluor™ 350 C_5 maleimide-labeled (purple) mAb in PBS (A) or PBS with trehalose (B). T_ms (solid vertical lines) and T_{agg}s (dashed vertical lines) for each species are also depicted. Curves are representative of triplicates, T_ms and T_{agg}s are averages of the same triplicates.

Conclusion

ADCs aren't just antibodies. They also include 2 small molecules, the drug and linker, which makes characterizing ADCs that much more complex. While many of the tools for examining mAbs can be used for ADCs, you have to bring specific knowledge to the table to make good decisions regarding process and developability.

Stunner and Uncle bring that knowledge with high-throughput, low volume assessments of DAR, concentration, aggregation, and thermal stability. With their help it's easy to identify problematic conjugation reactions, aggregation-prone samples, and optimize formulations to bring a safe, effective ADC to market as fast as possible.

Sample		PBS	+trehalose	+arginine	+Tween 80
mAb	T_m	69.8 ±0.1	71.0 ±0.1	69.5 ±0.1	69.7 ±0.0
	T_{agg}	75.2 ±0.3	76.9 ±0.2	75.8 ±0.3	74.9 ±0.1
+ FITC	T_m	69.5 ± 0.1	70.4 ± 0.1	69.3 ± 0.2	69.4 ± 0.3
(DAR 3.7)	T_{agg}	74.4 ± 0.3	76.4 ± 0.1	75.0 ± 0.2	74.2 ± 0.4
+ AF350 NHS ester	T_m	70.5 ± 0.1	71.7 ± 0.7	69.6 ± 0.1	70.6 ± 0.2
(DAR 2.1)	T_{agg}	75.8 ± 0.2	78.0 ± 0.9	75.5 ± 0.2	75.8 ± 0.2
+ AF350 maleimide	T_m	69.5 ± 0.1	71.5 ± 0.2	70.1 ± 0.2	70.0 ± 0.2
(DAR 2.1)	T_{agg}	71.6 ± 0.2	76.2 ± 0.4	74.4 ± 0.2	71.5 ± 0.9

Table 2: Melting (T_m) and aggregation (T_{agg}) temperatures of all analytes and formulations in this study. Values are depicted as the mean ± 1 standard deviation of triplicate measurements.

References

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