

# Conquer ADC stability characterization with Aunty

## Introduction

Conjugating potent cytotoxic payloads to monoclonal antibodies results in powerful anti-cancer therapeutics, but the resulting antibody-drug conjugates (ADCs) have unique stability challenges.<sup>1</sup> Attaching a hydrophobic small molecule drug to a hydrophilic amino acid, like cysteine or lysine, tends to reduce stability relative to the monoclonal antibody (mAb) alone, as the added hydrophobic interactions make the ADC more likely to unfold and aggregate. Identifying excipients which reduce these interactions and stabilize ADCs is one of the key steps in formulation optimization.

Formulation screens tend to use methods and tools that take a lot of sample, a lot of time, or are difficult to automate. Those methods are usually one-trick ponies, too, only giving a single type of data. That means you need to use many different assays to get the info you need to make the decision to move an ADC formulation to the next stage of development – or leave it behind.

Aunty lets you interrogate ADC conformational and colloidal stability in up to 96 formulations at a time with a combination of static light scattering (SLS), dynamic light scattering (DLS), and full spectrum fluorescence. The one-of-a-kind quartz Aunty plate only needs 8  $\mu$ L of sample per well, maximizing throughput and data output while minimizing sample consumption. Aunty can ramp the temperature from 15–95 °C at rates from 0.1–10 °C/minute or hold a temperature for days at a time. With SBS-format plates and an API to control experimental setup and operation, Aunty's entire process is automation-ready. Regardless of how you design your ADCs or formulations, Aunty is ready to churn out actionable results with unmatched flexibility.

This app note shows how Aunty's full-spectrum fluorescence, SLS, DLS, and temperature control work together to determine key conformational

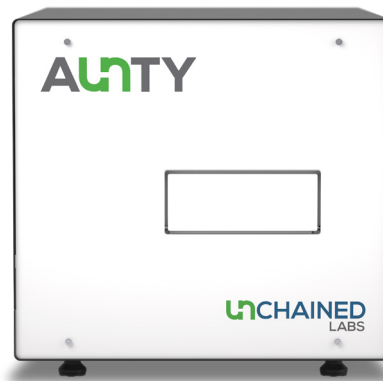


Figure 1: Aunty is the world's only flexible, automation-friendly, fit-for-purpose, plate-based thermal stability platform.

and colloidal stability parameters during a formulation excipient screen of ADCs and mAbs.

## Methods

Herceptin, Kanjinti, Kadcyla, and Enhertu (Evidentis GmbH, Potsdam, Germany) were exchanged into 10 mM histidine buffer, pH 6, using Big Tuna's default parameters for protein solutions from 0.5–50 mg/mL. Initial and final protein concentrations, size, and PDI were checked in quadruplicate on Stunner with buffer blanks. All 4 proteins were then diluted to 10 mg/mL and either 80 mg/mL sucrose or 0.9% NaCl was added to the formulations.

Dilution series of the 4 protein samples in 2 formulations were prepared (1, 2, 4, 8 and 10 mg/mL) and 8  $\mu$ L of each dilution were loaded in triplicate into Aunty plates. The 'colloidal stability' app was used with buffer blanks to determine  $B_{22}$  and  $k_D$  at 25 °C using 5x4 seconds DLS acquisitions and auto-attenuation. The same samples were then heated from 25 to 95 °C at a rate of 1 °C/minute with Aunty's 'T<sub>m</sub> & T<sub>agg</sub> with sizing' application, using automatic excitation settings throughout. Aunty Analysis was used to determine T<sub>m</sub> from the change in the barycentric mean (BCM) of the protein's intrinsic fluorescence

in the range 310–370 nm.  $T_{agg}$  was determined from the increase in SLS intensity and  $T_{size}$  from the increase in z-average diameter.

The antibodies and ADCs were also diluted to 2 mg/mL in the 2 formulations with 5X SYPRO Orange and loaded in triplicate in Aunty plates. Kadcykla was additionally prepared with 1X and 10X SYPRO Orange. All of the samples were heated from 25 to 95 °C at a rate of 1 °C/minute with Aunty's 'T<sub>m</sub> & T<sub>agg</sub> with reporter dye' application, using automatic settings. Aunty Analysis was used to determine T<sub>m</sub> from the increase in SYPRO Orange fluorescence (area under the emission spectrum from 525–640 nm) and T<sub>agg</sub> from the increase in SLS intensity.

## Results

Propensity to aggregate is a key attribute in any formulation study and the diffusion interaction parameter ( $k_D$ ) is an effective method of identifying samples with poor developability profiles.<sup>2</sup> Aunty's 'colloidal stability' app uses DLS to determine the diffusion coefficients of a dilution series of ADCs or proteins and then calculates the  $k_D$  from the linear fit. Negative values of  $k_D$  indicate attractive analyte interactions and high aggregation propensity, positive values indicate repulsive self-interaction and low propensity to aggregate.

All 4 of the molecules had a positive slope to their diffusion coefficients with increasing concentration when formulated in 10 mM histidine, pH 6, with 80 mg/mL sucrose (Figure 2). Faster diffusion at higher concentration indicates repulsive intermolecular forces and consequently lower propensity to aggregate. When formulated in the same buffer but with 0.9% NaCl, all 4 had negative slopes and showed signs of attractive interactions. Kadcykla and Enhertu, both trastuzumab-based ADCs, had stronger attractive interactions when formulated with NaCl than Herceptin or Kanjinti, which are trastuzumab and a trastuzumab biosimilar, respectively.

As with  $k_D$ , positive values of the second virial coefficient ( $B_{22}$ ) indicate repulsive intermolecular forces and negative  $B_{22}$  numbers indicate

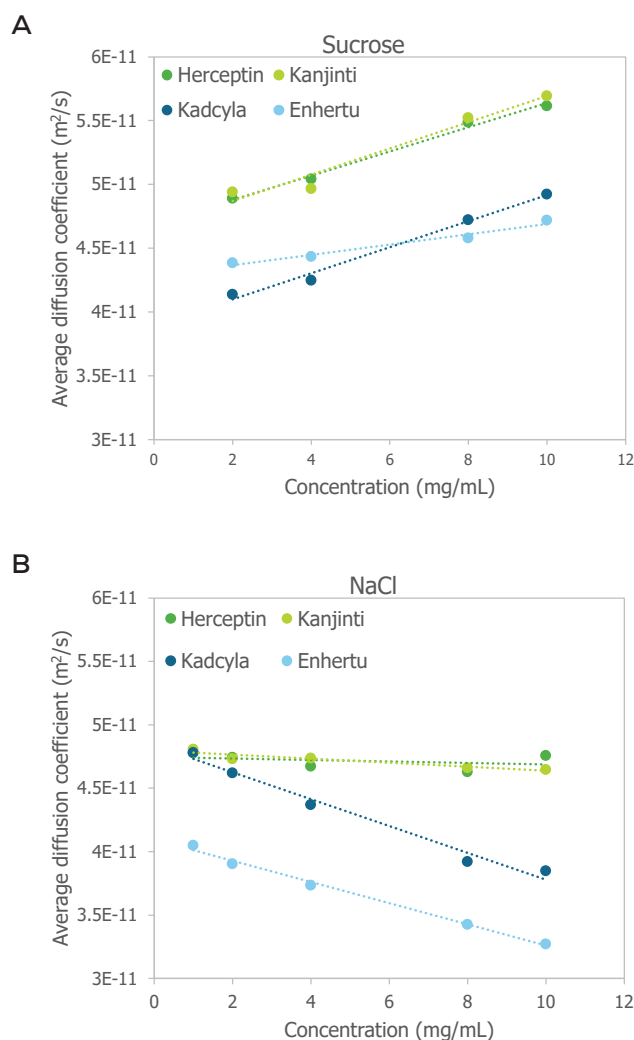


Figure 2: Average diffusion coefficient of triplicates versus concentration of Herceptin, Kanjinti, Kadcykla and Enhertu in 10 mM histidine, pH 6, with either 80 mg/mL sucrose (A) or 0.9% NaCl (B) with linear regression (dotted lines).

attractive self-interactions. While their interpretations are similar,  $B_{22}$  is based on SLS, not DLS. Aunty measures both  $k_D$  and  $B_{22}$  in a single application. Herceptin, Kanjinti, and Kadcykla all had relatively high, positive  $k_D$ s with sucrose while Enhertu had the smallest  $k_D$  (Table 1). All 4 molecules had negative  $k_D$ s with NaCl, but only Enhertu had both a negative  $k_D$  and a negative  $B_{22}$  in this formulation. Thus, sucrose tended to have a stabilizing effect on all of the molecules, with this stabilization being weakest for Enhertu. NaCl also had the strongest destabilizing effect on Enhertu among the analytes tested. Both ADCs had lower values for  $k_D$  and  $B_{22}$  than the mAbs, indicating they are more prone to aggregation, especially at high concentrations.

Sample	Excipient	$k_D$	$B_{22}$
Herceptin	Sucrose	21.6	1.93E-04
Herceptin	NaCl	-3.3	1.20E-05
Kanjinti	Sucrose	14.2	2.13E-04
Kanjinti	NaCl	-3.3	6.54E-06
Kadcyla	Sucrose	12.1	1.64E-04
Kadcyla	NaCl	-21.9	1.89E-04
Enhertu	Sucrose	4.6	1.78E-04
Enhertu	NaCl	-20.4	-2.72E-05

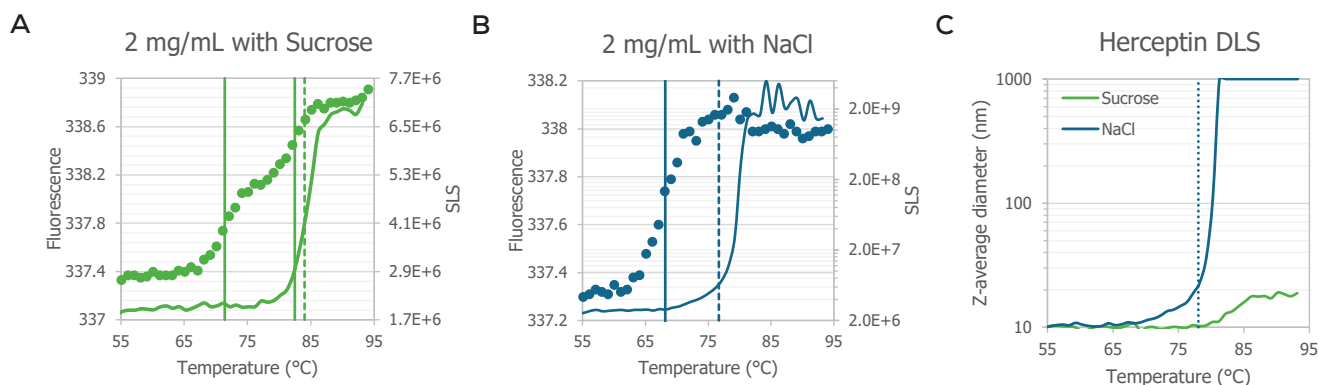
**Table 1:** Diffusion interaction parameter ( $k_D$ ) and second virial coefficient ( $B_{22}$ ) of Herceptin, Kanjinti, Kadcyla and Enhertu 10 mM histidine, pH 6, with either 0.9% NaCl or 80 mg/mL sucrose.

Aggregation at room temperature is an important piece of an antibody's or ADC's stability story, but it's far from the only part. Unfolding and aggregation behavior at elevated temperatures give vital clues to how well a therapeutic will perform, its manufacturability, and its developability.<sup>3</sup> Since protein unfolding and aggregation are typically linked, tracking both events simultaneously in the same sample is a must. In a thermal ramp experiment, Aunty captures the changes in an antibody's intrinsic fluorescence and increases in amount of light it scatters, as well as changes in its hydrodynamic diameter, to monitor conformational and colloidal stability in each formulation side-by-side.

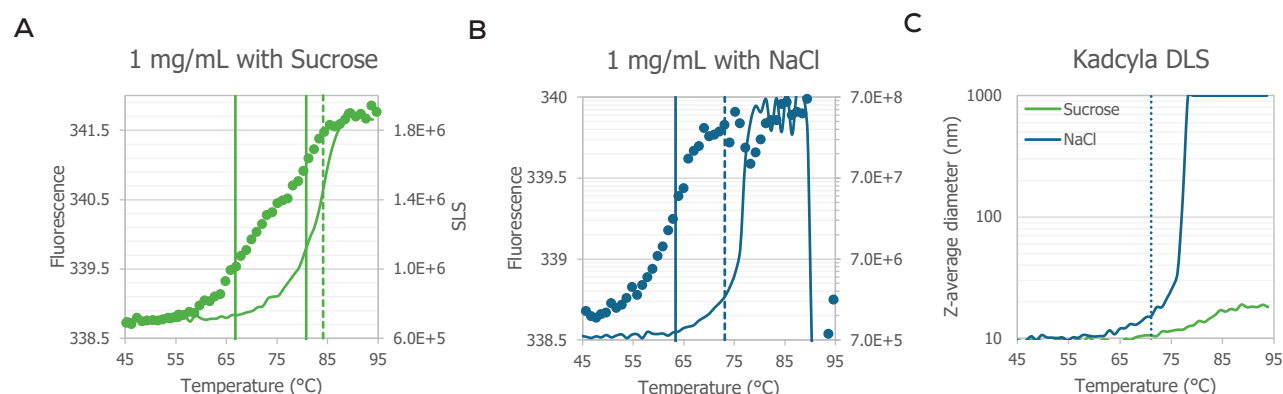
Most mAbs show a distinct red shift in their intrinsic fluorescence emission during unfolding with melting temperatures at the inflection points.<sup>4</sup> Herceptin in 10 mM histidine with sucrose had 2  $T_m$ s at 71.4 and 82.5 °C and a  $T_{agg}$  at 84 °C (**Figure 3A**). Increases in the SLS intensity of a mAb often indicate the formation of aggregates (by default, the  $T_{agg}$  in Aunty is defined as the temperature at which the SLS intensity is twice that of the baseline value). Replacing sucrose with NaCl as an excipient destabilized Herceptin. This formulation showed a  $T_m$  of 68.2 °C and  $T_{agg}$  of 76.7 °C (**Figure 3B**).

Not only did Herceptin unfold and aggregate earlier in the presence of NaCl, but the aggregates were also, on average, larger than with sucrose. From a baseline of 10 nm in both formulations, the z-average diameter reached a maximum of ~18 nm in sucrose beyond 87.2 °C, while that size was already reached at about 77 °C in NaCl (**Figure 3C**). The maximum z-average in NaCl was larger than Aunty's DLS maximum detectable size of 1000 nm.

Similar methods can be used to determine the conformational and colloidal stability of an ADC and its mAb component. Using the same analysis parameters and application on Aunty, Kadcyla with sucrose had  $T_m$ s at 66.7 and 80.8 °C and a  $T_{agg}$  at 84.1 °C (**Figure 4A**). As with Herceptin, replacing sucrose with NaCl destabilized Kadcyla. In 10 mM histidine with 0.9% NaCl, Kadcyla had a  $T_m$  of 63.4 °C and a  $T_{agg}$  of 73.1 °C (**Figure 4B**).



**Figure 3:** Barycentric mean of the intrinsic protein fluorescence 310–370 nm (left y-axes, circles) and static light scattering intensity (right y-axes, solid line) of 2 mg/mL **Herceptin** in 10 mM histidine, pH 6, with 80 mg/mL sucrose (**A**) or 0.9% NaCl (**B**) with  $T_m$ s (solid vertical lines) and  $T_{agg}$ s (dashed vertical lines). Hydrodynamic diameter (**C**) of the same samples with  $T_{size}$  (dotted vertical line). Curves are representative of triplicates.



**Figure 4:** Barycentric mean of the intrinsic protein fluorescence 310–370 nm (left y-axes, circles) and static light scattering intensity (right y-axes, solid line) of 1 mg/mL **Kadcylyl** in 10 mM histidine, pH 6 with 80 mg/mL sucrose (**A**) or 0.9% NaCl (**B**) with  $T_m$ s (solid vertical lines) and  $T_{agg}$ s (dashed vertical lines). Hydrodynamic diameter (**C**) of the same samples with  $T_{size}$  (dotted vertical line). Curves are representative of triplicates.

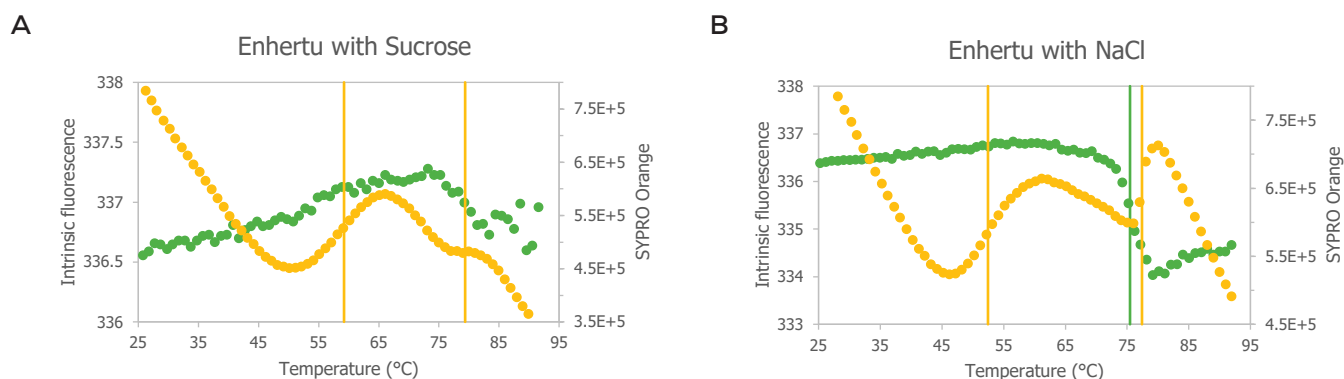
Once the aggregates became too large or started precipitating, the SLS intensity became noisy and decreased.  $T_{size}$  of Kadcylyl with NaCl, based on the increase in DLS z-average diameter, was 71.1 °C (**Figure 4C**). Kadcylyl aggregates also tended to be larger in NaCl than in sucrose with maximum hydrodynamic diameters of >1000 nm and 18 nm, respectively.

Many of the small molecule drugs used to make ADCs fluoresce under ultraviolet light in the same wavelengths as the tryptophan and tyrosine residues of the mAb. Enhertu is one such ADC. This crosstalk makes it difficult or even impossible to determine the  $T_m$  by BCM or two-wavelength analysis. Aunty's full-spectrum fluorescence detection and second excitation LED at 470 nm lets you use fluorescent indicator dyes, like SYPRO

Orange, to identify the  $T_m$  of those difficult-to-analyze samples.

The intrinsic fluorescence of Enhertu with sucrose changed during heating but could not be analyzed to identify  $T_m$ s (**Figure 5A**). However, SYPRO Orange analysis showed it has 2  $T_m$ s at 59.2 and 79.3 °C. Enhertu's intrinsic fluorescence underwent a blue shift during heating with NaCl and Aunty was able to identify a  $T_m$  at 75.5 °C (**Figure 5B**). This was quite close to  $T_{m2}$  by SYPRO Orange, 77.4 °C. Enhertu's  $T_{m1}$  with NaCl was 6.8 °C lower than with sucrose. Aunty's full spectrum fluorescence detection makes it easy to design and customize assays to suit anyone's current or future protein stability needs.

Once you have the protein melting points and aggregation temperatures, it's simple to



**Figure 5:** Barycentric mean of the intrinsic protein fluorescence 310–370 nm (left y-axes, green circles) and SYPRO Orange fluorescence (right y-axes, yellow circles) of 2 mg/mL **Enhertu** in 10 mM histidine, pH 6, with 80 mg/mL sucrose (**A**) or 0.9% NaCl (**B**) with  $T_m$ s (solid vertical lines). Curves are representative of triplicates.

determine which formulations, mAbs, or ADCs are most stable. Just compare the  $T_m$ s and  $T_{agg}$ s – the samples with the highest values are the winners. Herceptin and Kanjinti had similar  $T_{m1}$ s and  $T_{agg}$ s in the tested formulations (Figure 6).

Kadcyla had a lower  $T_{m1}$  and  $T_{agg}$  in both formulations and Enhertu had lower  $T_{agg}$ s. (Enhertu was not amenable to  $T_m$  analysis by intrinsic fluorescence, and it was in consequence excluded from this comparison.) Sucrose stabilized all the tested mAbs and ADCs over NaCl.

SYPRO Orange  $T_m$ s were generally lower than those determined by intrinsic fluorescence (Figure 7A). Similar  $T_m$ s were identified for Kadcyla with 1X, 5X, and 10X dye concentrations (data not shown). It's therefore unlikely that the dye was having a concentration-dependent impact on the stability of the mAbs or ADCs. SYPRO Orange increases in fluorescence when it binds to hydrophobic patches in the protein that are exposed during unfolding. Aunty determines  $T_m$ s from this signal increase. As, in contrast, intrinsic fluorescence  $T_m$ s are determined by exposure of tryptophan and tyrosine residues to the aqueous buffer, the two methods to determine  $T_m$ s (intrinsic fluorescence and reporter dye fluorescence) are distinct and shouldn't be compared directly to each other. The relative stability rankings of the samples by the methods, however, are usually comparable. Herceptin and Kanjinti  $T_m$ s by SYPRO Orange were approximately equal to each other in presence of both NaCl and sucrose, while Kadcyla's  $T_m$ s were lower. These are the same

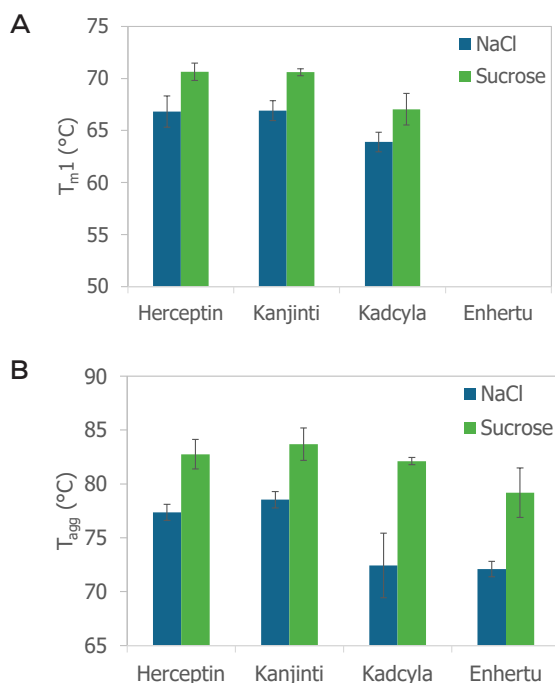


Figure 6: Intrinsic fluorescence  $T_{m1}$  (A) and  $T_{agg}$  (B) of 2 mg/mL Herceptin, Kanjinti, Kadcyla, and Enhertu in 10 mM histidine, pH 6, with either 0.9% NaCl or 80 mg/mL sucrose by Aunty's 'T<sub>m</sub> & T<sub>agg</sub> including sizing' app. Measurements were done in triplicate; error bars are 1 standard deviation.

trends seen in their intrinsic fluorescence  $T_m$ s. Enhertu in NaCl had the lowest  $T_{m1}$  by SYPRO Orange. Enhertu was generally the least stable of the tested molecules, but sucrose stabilized it, similarly to the effect on the other biologics tested.

$T_{agg}$ s for the ADCs and mAbs with SYPRO Orange recapitulate the trends shown in the intrinsic fluorescence experiments – the ADCs are less stable than the mAbs and sucrose stabilized

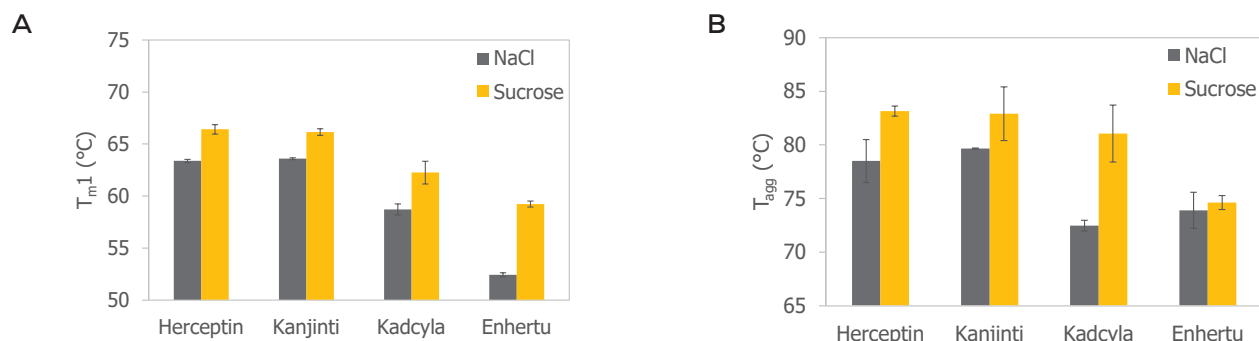


Figure 7:  $T_m$  with SYPRO Orange (A) and  $T_{agg}$  (B) of 2 mg/mL Herceptin, Kanjinti, Kadcyla, and Enhertu in 10 mM histidine, pH 6, with either 0.9% NaCl or 80 mg/mL sucrose and 5X SYPRO Orange by Aunty's 'T<sub>m</sub> & T<sub>agg</sub> with reporter dye' app. Measurements were done in triplicate; error bars are 1 standard deviation.

most of them, relative to NaCl (Figure 7B). Adding SYPRO Orange appears to have had little effect on the aggregation behavior of most of the formulations. The only striking difference is that, unlike in the other experiment, the  $T_{agg}$ s of Enhertu in the different formulations are approximately equal. However, the final SLS intensity of Enhertu with NaCl was much higher than with sucrose (not shown), indicating that the ADC formed larger aggregates with NaCl than in presence of sucrose.

## Conclusion

ADCs are powerful anti-cancer therapeutics and have the potential to address a wide variety of other diseases. However, they have their own unique challenges when it comes to stability and stability characterization. This creates a need for flexible tools, like Aunty, which can address multiple stability concerns and make it easier to adapt formulations for ADCs. Using Aunty, we showed that the trastuzumab-based ADCs Kadcyla and Enhertu were less stable than the trastuzumab therapeutics Herceptin and Kanjinti, based on their intrinsic and dye-based data:  $T_m$ ,  $T_{agg}$ ,  $k_D$ , and  $B_{22}$ . However, a histidine formulation with sucrose stabilized all of them, relative to one containing NaCl. Aunty is the true high-throughput stability platform with SLS, DLS and fluorescence measurements on a full 96-well plate every minute of an experiment.

## References

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