Meet Aunty – the Queen of protein stability

Introduction

Aunty is the fastest, highest throughput protein stability platform on the planet (Figure 1). Other stability methods each have their drawbacks: some are slow, others don't give the full stability picture, and some use way too much sample. Aunty blasts through those issues to crank out actionable results at breakneck speeds. Full-spectrum fluorescence in Aunty gives you the freedom to use intrinsic or dye-based fluorescence, whichever strikes your fancy, to determine the melting temperatures of your sample. Static and dynamic light scattering (SLS & DLS) run in parallel to each other to spot aggregates with superior sensitivity before, during, or after heating. In Aunty, fluorescence, SLS and DLS work together to get the whole story on thermal stability.

Minimizing sample consumption and maximizing throughput are part of the main goals of biologics characterization workflows. Aunty reads all 96 samples every minute of your experiment and only needs 8 µL of each sample. The SBS-format plates of 96 quartz wells are automation-friendly and give impeccable optical performance. Plus, quartz glass is chemically compatible with almost anything. Sealing the wells after loading keeps samples in and contaminants out. Sample concentrations from 25 µg/mL to 300 mg/mL, depending on the sample, can run on the same plate and in the same experiment. Temperature control (15–95 °C) combined with sealed samples give unprecedented data resolution without sacrificing flexibility. Ramp samples from 0.1–10 °C/minute or hold a single temperature for hours, or even days, to fast-forward long term stability testing. Fluorescence, SLS, and DLS can run together or separately on one sample or many, so experiments can be tailor-fit to your needs. Once the experiment finishes, overlay graphs, compare results, and use the information to draw the right conclusions on stability

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

of constructs, modifications, conjugations, conditions, or formulations.

This app note shows how to use Aunty's full-spectrum fluorescence, show-stopping SLS, ISO-compliant DLS, and temperature control to determine key stability measurements on proteins and viral vectors.

1. Thermal ramp (T_m & T_{agg})

Heating up proteins usually makes them unfold, rearranging their amino acids. As this happens, the fluorescence behavior of their tryptophan and tyrosine residues also tends to change. Tracking the changes in the intrinsic fluorescence of a protein allows you to monitor a protein's conformational stability and determine a melting temperature (T_m). You can use this to rank order the stability of different formulations, constructs, mutants, or even conjugates.

Proteins tend to aggregate due to thermal stress. Aggregation often happens alongside unfolding and the 2 processes are in many cases, though not always, linked. SLS is a super sensitive method of detecting aggregation ASAP during a

thermal ramp. Simultaneously monitoring protein unfolding and aggregation is key to effective thermal stability screens, which accelerate developability testing and reduce the time it takes to bring novel, life-saving therapies to the clinic.

Aunty's plate-based stability screening is the greatest of all time. It covers all the bases of protein unfolding and aggregation, reading 96 samples every minute of a thermal ramp experiment and only using 8 µL of each sample with sensitivity to protein concentrations down to 25 µg/mL. Trastuzumab in PBS at 1 mg/mL and 25 µg/mL had similar T_ms, 71.1 and 70.3 °C, respectively (Figure 2). The temperature of the onset of aggregation, or T_{agg}, at 25 µg/mL was lower than the T_{agg} at 1 mg/mL (76 versus 79.1 °C). Total SLS intensity was higher in the 1 mg/mL sample, though, indicating that although the mAb was more resistant to the onset of aggregation at the higher concentration, it tended to form larger or more numerous aggregates in that case.

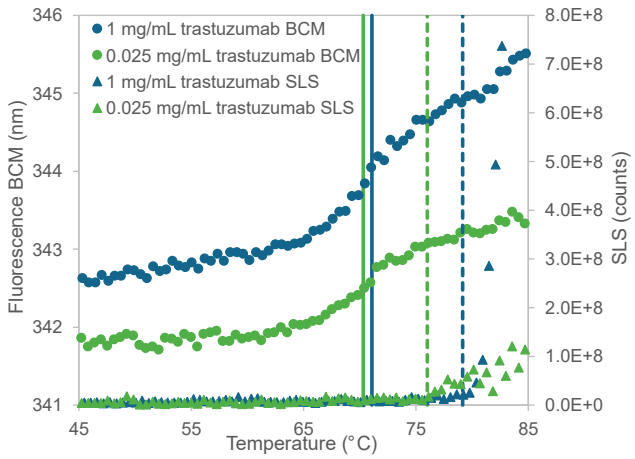


Figure 2: Trastuzumab was prepared to concentrations of 1 mg/mL (blue) and 25 µg/mL (green) in PBS and heated from 25 to 95 °C using Aunty's T_m & T_{agg} app with fluorescence (circles, left y-axis) and SLS (triangles, right y-axis) measurements taken every 30 seconds. T_ms (solid lines) and T_{agg}s (dashed lines) are the average of duplicates.

2. Sizing & polydispersity

It's always important to make sure your biologics and other therapeutics are the correct size. Small particles might indicate contamination, degradation or impurities, while large particles could be aggregates. Some sizing methods can't handle the entire size range of particles that might come your way. Most soluble proteins are 1–20 nm in diameter, viruses 20–100, and nanoparticles can be almost any size. Aunty's DLS is a fast, sensitive, and reliable way to look for aggregated protein or to tell if your gene therapy is the correct size. Best of all, it's versatile enough to cover sizes from 0.3–1000 nm.

3 NIST polystyrene bead standards were determined to have average diameters of 22, 102, and 512 nm. These were all within the manufacturer's specified size ranges of 21–25, 90–104, and 503–517 nm, respectively (Figure 3).

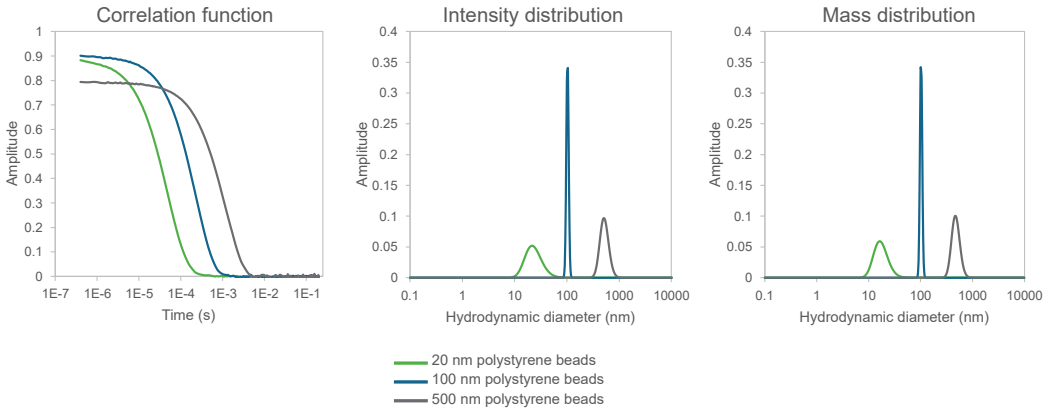


Figure 3: Correlation functions (left), intensity distributions (middle) and mass distributions (right) of 20 (green), 100 (blue), and 500 (grey) nm polystyrene beads diluted 1:100, 1:500, and 1:1000 measured using Aunty's Sizing & polydispersity app at 25 °C with 5 acquisitions of 4 seconds, each. Curves are representative of triplicates.

The PDI of all samples was ≤0.1, indicating they were monodisperse preparations.

3. Thermal ramp (T_m & T_{agg} including sizing)

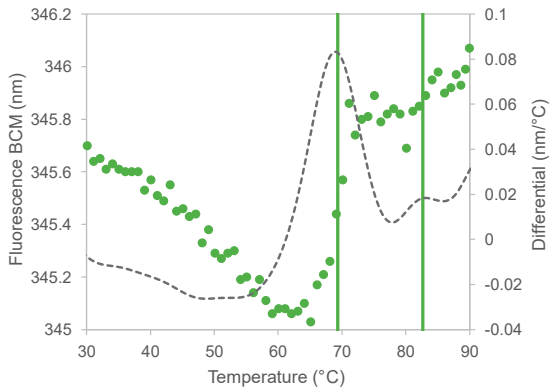
When you take Aunty's fluorescence and SLS and add DLS during a thermal ramp, across a 96-well plate, you make Aunty THE stability platform. Accuracy and reproducibility are no sweat, and Aunty's unprecedented resolution gives you all the information you need to see the full stability picture. No matter how much or how little info you have on a protein, Aunty can show what you need to know and more.

The NISTmAb protein is reference material intended to evaluate the performance of methods for characterizing antibodies. Aunty determined 2 T_ms from 1 mg/mL NISTmAb in 12.5 mM L-histidine, 12.5 mM histidine-HCl, pH 6.0 at 69.7 and 83.5 °C (**Figure 4A**). These results are within 0.5 °C of the published differential scanning calorimetry (DSC) T_ms of 69.2 and 83.1 °C.[1] While most proteins aggregate during a thermal ramp, NISTmAb's SLS intensity showed no significant increase, suggesting no aggregation in this condition. Aunty's side-by-side collection of SLS and DLS lets us confirm this result using an orthogonal technique (**Figure 4B**). The antibody increased in hydrodynamic size by only about 5 nm, with the increase starting at approximately 80 °C. As protein aggregates tend to be in the size range of 100s of nanometers, it's likely that the reported size increase, which coincides closely with T_m2, is due to protein unfolding and not its aggregation. Accurately combining multiple detection methods on 96 samples makes Aunty the fastest, highest throughput stability platform on the planet.

4. Thermal ramp (T_m & T_{agg} with reporter dye)

Some proteins don't have fluorescent residues – or are in an environment that quenches their fluorescence. In these cases, you can use a reporter dye, like SYPRO Orange, to assess stability. Aunty's flexible full-spectrum

A



	T_m1 (°C)	T_m2 (°C)
Aunty	69.7 ± 0.8	83.5 ± 1.2
DSC	69.2 ± 0.07	83.1 ± 0.14

B

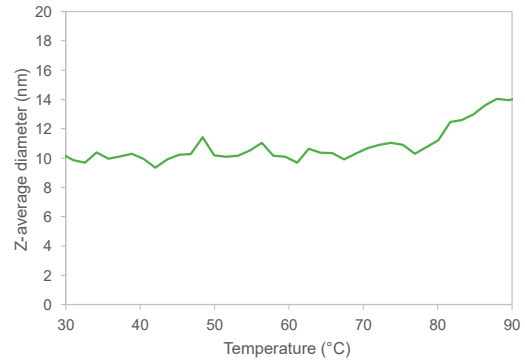


Figure 4: NISTmAb was diluted to 1 mg/mL in 12.5 mM L-histidine, 12.5 mM histidine-HCl, pH 6.0 and run using Aunty's T_m & T_{agg} including sizing app with default settings. T_ms (**A, vertical lines**) were determined from changes in fluorescence BCM (left y-axis, green dots) based on peaks in the first-order differential (right y-axis, dashed line). Inset table shows average Aunty and published DSC T_ms plus or minus the standard deviation. Z-average diameters (**B**) of the protein during the thermal ramp were determined alongside fluorescence.

fluorescence with excitation LEDs at 280 and 470 nm can handle stability testing of all these kinds of molecules with a variety of dyes. SLS and DLS are compatible with either intrinsic or dye-based fluorescence assays, so you'll get aggregation information whichever way you go. Aunty lets you tailor your assays to suit your molecule, rather than forcing them to fit with your instrumentation.

The T_m by intrinsic fluorescence and T_{agg} of 3 mg/mL bovine IgG in PBS were 71.3 and 70.8 °C, respectively (**Figure 5A**). By adding 20X SYPRO Orange™, we could use the increase in the dye's

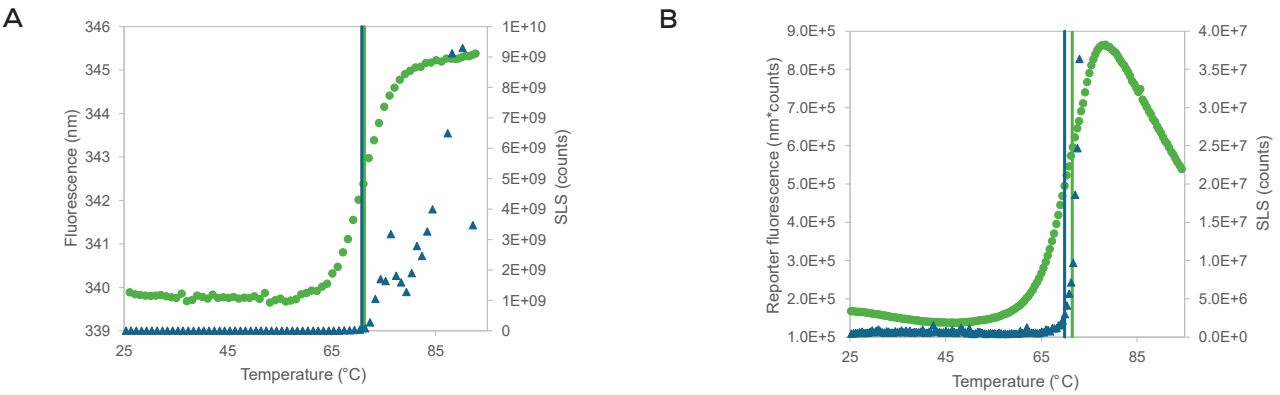


Figure 5: Bovine IgG was prepared to a concentration of 3 mg/mL in PBS and measured with Aunty's T_m & T_{agg} app with the UV LED only (**A**) or spiked with 20X SYPRO Orange and measured using the T_m & T_{agg} with reporter dye app and the blue LED only (**B**). Intrinsic or SYPRO Orange fluorescence (green dots, left y-axes) were used to determine T_ms (green vertical lines). SLS (blue triangles, right y-axes) was used to determine T_{agg}s (blue vertical lines).

fluorescence intensity when it bound to exposed hydrophobic patches to determine a T_m of 71.5 °C (Figure 5B). Under these conditions, the T_{agg} of the bovine IgG was 69.9 °C.

5. Capsid stability

Viral vectors experience just as many stresses during processing as other biologics, but they can fail in completely unique ways. Protein unfolding and aggregation are certainly issues for viral vectors, but so is the loss of the nucleic acid payload by genome ejection. Functional assays can take days to detect these issues, but Aunty's Capsid stability app can do it in under an hour. By monitoring the increase in SYBR Gold fluorescence, Aunty can tell when viral genomes are ejected from the capsid.[6] With sensitivity down to 5e11 vg/mL, you can get stability insights earlier than ever.

AAV9 in PBS had a genome ejection T_m at 60.6 °C, based on the increase in SYBR Gold fluorescence, and a T_{agg} at 76.3 °C by SLS (Figure 6). The T_m indicates the vector lost its nucleic acid payload earlier than the overall disruption of the capsid proteins, which likely coincided with the T_{agg}. Aunty monitored both fluorescence and SLS simultaneously and automatically calculated the T_m and T_{agg} values.

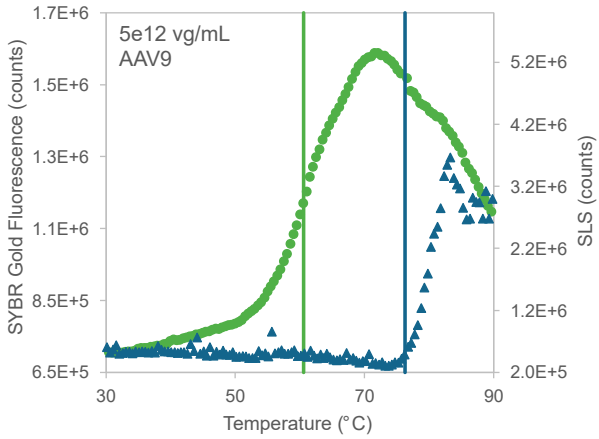


Figure 6: AAV9 was diluted to 5e12 vg/mL in PBS with 20x SYBR Gold and heated from 25–95 °C using Aunty's Capsid stability app with default settings. Increases in SYBR Gold fluorescence (green dots) and SLS (blue triangles) were monitored during heating and the T_m (green vertical line) and T_{agg} (blue vertical line) were assigned at the fluorescence transition and onset of SLS increase, respectively.

6. Colloidal stability (k_D, B_{22}, G_{22})

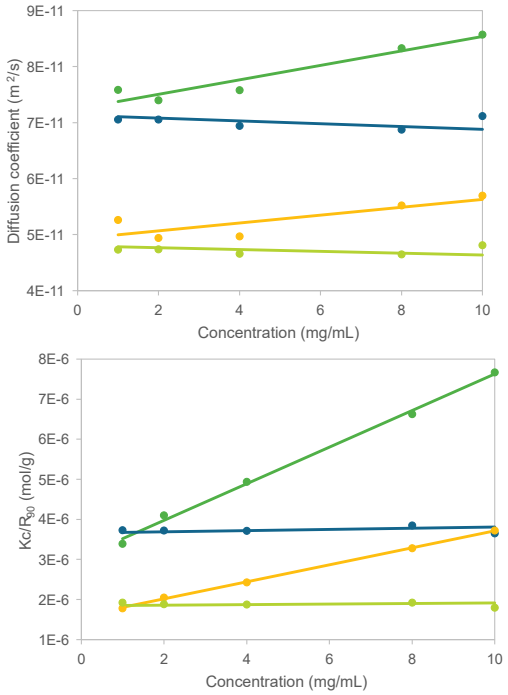
Identifying factors that reduce or prevent aggregation is one of the goals of developability and formulation screening for biologics. Choice of buffer, addition of excipients, or introducing mutations to the analyte all impact a therapeutic's colloidal stability. Using Aunty's Colloidal stability app puts its superior light scattering to work, screening if a protein 'likes' a given formulation or if aggregation is on the horizon by measuring the

three colloidal stability parameters k_D, B_{22}, and G_{22} all in one go.

Attractive intermolecular forces make proteins diffuse more slowly as their concentration increases; repulsive forces make them diffuse faster. Protein molecules that repel each other are less likely to aggregate and, therefore, have better colloidal stability.[2] The diffusion interaction parameter k_D expresses this relationship. A positive k_D indicates repulsive intermolecular forces and low aggregation propensity, negative k_D values indicate the opposite. The second virial coefficient B_{22} is a another measure of protein self-associations in solution.[3] It's determined from SLS data – a linear regression of static light scattering versus concentration (known as a Debye plot). Just like with k_D, positive values of B_{22} indicate repulsion and negative values indicate attraction. The main difference lies in how they're measured: B_{22} is based on static light scattering and k_D on DLS.

G_{22}, the Kirkwood-Buff integral, is a more rigorous way to measure the net self-interaction parameter of protein molecules especially at high concentrations, by accounting for protein-solvent and protein-solute interactions in your sample.[4] Typically, k_D and B_{22} are most appropriate for concentrations ≤20 mg/mL. Higher than that and G_{22} becomes more useful. Unlike in the cases of B_{22} and k_D, in G_{22} a negative value indicates net repulsive forces between protein molecules, while a positive G_{22} indicates possible self-association. Also, unlike k_D and B_{22}, a discrete G_{22} value is defined for each concentration within the dilution series.

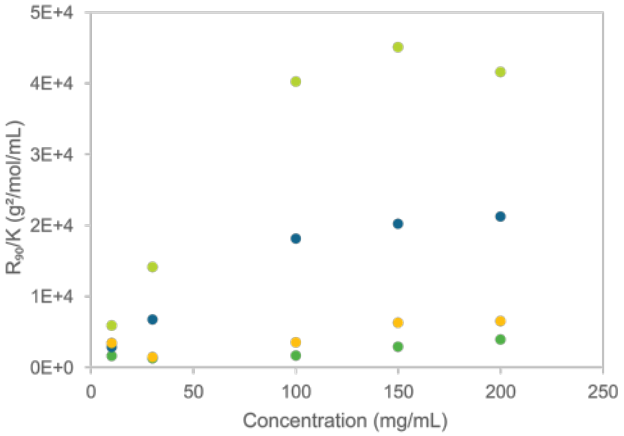
Aunty's Colloidal stability app calculates all 3 parameters for your analyte. Just pipette in a dilution series (ideally with 5 concentrations in triplicate), and you're good to go. You'll always get k_D, but if you include a buffer blank, you'll get B_{22}. If you tell Aunty the molecular weight of your analyte, you'll get G_{22}, too. It's the quickest way to figure out a protein's aggregation propensity in a given formulation, or to figure out if there's a risk when you go from lower concentrations to higher concentrations, for example when re-formulating a therapeutic from intravenous administration to subcutaneous injections.



	k_D (mL/g)	B_{22} (mol*mL/g²)
Trastuzumab with sucrose, 42°C	17.8	2.3e-4
Trastuzumab with NaCl, 42°C	-3.5	7.3e-6
Trastuzumab with sucrose, 25°C	14.2	1.1e-4
Trastuzumab with NaCl, 25°C	-3.3	3.3e-6

Figure 7: Dilution series of 1, 2, 4, 8, and 10 mg/mL trastuzumab were prepared in 10 mM histidine, pH 6 with either 80 mg/mL sucrose or 0.9% NaCl. The samples were measured in triplicate at either 25 or 42 °C with Aunty's Colloidal stability app, using default settings, to determine k_D and B_{22} based on the linear regression of the diffusion coefficients (top graph) or the Debye plot (bottom graph). The points on the graph are the average of triplicates and the table shows the results.

Trastuzumb in 10 mM histidine, pH 6 with 80 mg/mL sucrose had relatively large, positive values of k_D while adding 0.9% NaCl instead made it have negative values at 25 and 42 °C (Figure 7). While B_{22}s for all the tested conditions were positive, the values with sucrose were ~30 times higher than NaCl, indicating the repulsive forces with sucrose were much stronger. This suggests that trastuzumab has better colloidal stability in histidine with sucrose than with NaCl.



mAb1	Concentration mg/mL				
	10	30	100	150	200
with sucrose, 42°C	92.2	3.9	-0.5	-0.2	-0.1
with NaCl, 42°C	24.1	-5.2	-4.4	-5.0	-4.7
with sucrose, 25°C	189.7	-1.5	-1.3	-0.6	-0.7
with NaCl, 25°C	6.8	-24.1	-11.8	-12.4	-12.4

Figure 8: Dilution series of 10, 30, 100, 150, and 200 mg/mL mAb1 were prepared in 10 mM histidine, pH 6 with either 80 mg/mL sucrose or 0.9% NaCl. The samples were measured in triplicate at either 25 or 42 °C with Aunty's Colloidal stability app, using default settings, to determine G_{22} based on the deviation of R_{90}/K from linearity. The points on the graph are the average of triplicates and the table shows the results.

While the addition of sucrose to trastuzumab in a histidine buffer stabilized it, sugars are not always the best stabilizers. The R_{90}/K graph from G_{22} analysis of another monoclonal antibody mAb1 with NaCl at higher protein concentrations is trending to a plateau, indicating net repulsive forces in presence of salt (Figure 8). The trend towards lower G_{22} values in presence of NaCl (compared with sucrose) shows the same. This indicates that at high concentrations, for example over 50 mg/mL, NaCl is a more stabilizing excipient of mAb1 in 10 mM histidine, pH 6.

7. Isothermal stability

Long-term stability testing is often a bottleneck for biologics. Traditional methods are clunky and clumsy, and you'd rather put this testing off – while looking into long term stability early in development leaves you more room to react to potential issues. Aunty helps keep the process moving with sealed plates that can hold samples in the instrument at temperature for hours or days. With the Out-of-the-box apps, you can leave the sample in an incubator and check their fluorescence, SLS, and DLS when you need to, without tying up the instrument for the entire duration of the experiment. After all, the analytical methods should be the simplest part of stability testing.

Arginine is usually added to protein formulations to reduce viscosity. However, it has chaotropic properties – it limits aggregation but drives protein unfolding.[5]

Bovine IgG in the presence of arginine had a more rapid change in its fluorescence when held at 62 °C than the protein without arginine (Figure 9A). In these same samples, increases in SLS and z-average diameter were slower with arginine (Figure 9B). These results show arginine

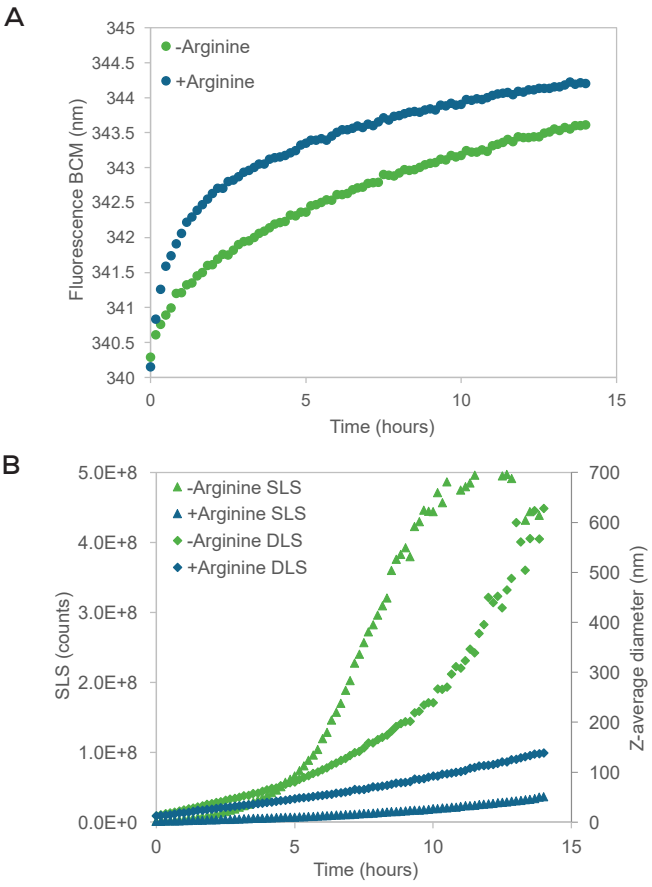


Figure 9: 1 mg/mL bovine IgG was prepared in PBS with either 0 (green) or 500 mM (blue) arginine. Aunty's Isothermal application was used with default settings to hold the samples at 62 °C for 14 hours while monitoring unfolding (**A**) via fluorescence or aggregation (**B**) by SLS (triangles) and DLS (diamonds).

promoted protein unfolding but inhibited aggregation. Aunty's sealed samples and multiple detection modes makes it obvious when and how a protein starts to misbehave on any time scale.

Conclusion

Aunty combines full-spectrum fluorescence, SLS, and DLS with finely tuned thermal control and an all quartz 96-well plate to make the Queen of high-throughput stability characterization. Blast through stability screens which use 8 µL of each sample and check 96 samples in sealed wells every minute of your experiment. Flexible assays track melting, aggregation, colloidal stability, size, and polydispersity of proteins. With viral vectors, Aunty can also keep tabs on genome ejection. That way you can make the right decisions to optimize the stability of constructs, formulations, conjugations, or modifications of your biologic.

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