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The Use of a GroEL-BLI Biosensor to Rapidly Assess Pre-Aggregate Populations for Antibody Solutions Exhibiting Different Stability Profiles

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Running Title: GroEL-BLI biosensor assay to detect preaggregate species in antibody solutions

Keywords: Bio-layer interferometry, GroEL, monoclonal antibody, bispecific antibody, protein aggregation, stability, formulation.

Abstract

An automated method using biotinylated GroEL-streptavidin biosensors with Bio-Layer Interferometry (GroEL-BLI) was evaluated to detect the formation of transiently formed, pre-aggregate species in various pharmaceutically relevant monoclonal antibody (mAb) samples. The relative aggregation propensity of various IgG1 and IgG4 mAbs was rank-ordered using the GroEL-BLI biosensor method, and the least stable IgG4 mAb was subjected to different stresses including elevated temperatures, acidic pH, and addition of guanidine-HCl. The GroEL-BLI biosensor detects mAb pre-aggregate formation mostly prior to, or sometimes concomitantly with, observing soluble aggregates and subvisible particles using size exclusion chromatography (SEC) and microflow imaging (MFI), respectively. A relatively unstable bispecific antibody (Bis-3) was shown to bind the GroEL-biosensor even at low temperatures (25^{o}C). During thermal stress (50°C, one hour), increased Bis-3 binding to GroEL-biosensors was observed prior to aggregation by SEC or MFI. Transmission electron microscopy (TEM) analysis of Bis-3 pre-aggregate-GroEL complexes revealed, in some cases, potential hydrophobic interaction sites between the Fc domain of the bispecific antibody and GroEL protein. The automated BLI method not only enables detection of transiently formed pre-aggregate species that initiate protein aggregation pathways, but also permits rapid mAb formulation stability assessments at low volumes and low protein concentrations.

Introduction

Patients worldwide depend on therapeutic protein drugs to treat a wide number of diseases such as diabetes, cancer, immune disorders and infections[1]. The number of protein-based medical drug products on the market is increasing rapidly, especially with the expanded use of monoclonal antibodies (mAbs)[2]. The complexity of biopharmaceutical drug candidates is also increasing to now include bispecific antibodies, antibody-drug conjugates, single chain fragments variable (scFv) and modified natural enzymes[3]. To ensure safety and efficacy of protein therapeutic drugs across their shelf-life, key biological and structural characteristics that lead to product degradation need to be well understood and controlled when formulating a new biopharmaceutical candidate. To this end, critical quality attributes[4], such as potency, solubility and physicochemical stability, are used to define the overall stability profile of the protein drug candidate across the entire shelf-life. Numerous strategies now exist where directed engineering methods are implemented to rationally mutate suspect surface residues that decrease aggregation while not impacting biological activity (e.g., maintaining target affinity of mAbs while improving stability)[5]. In other instances, purposeful engineering and improvement of antigen binding or Fc receptor binding sites, can have the undesired effect of increasing aggregation[5] or altering the conformational dynamics and physical stability[6], of the engineered antibodies.

Protein-based drugs can be exposed to many types of environmental stresses (e.g., temperature, agitation, light, etc.) during manufacturing, long-term storage, distribution and administration[7]. These stresses can cause proteins to aggregate to varying extents, depending on the specific protein and formulation conditions, thereby reducing potency and/or increasing

immunogenicity risk upon patient administration. Aggregation is a complex multistep pathway in which native protein can form dynamic, folded or partially unfolded transient species that lead to the formation of reversible and nonreversible intermediates or small aggregate clusters[8]. Elucidation of the exact steps of the aggregation pathway for a particular protein, formulation and stress can be experimentally and theoretically challenging. One important analytical challenge is the ability to monitor and capture any initially formed, structurally altered intermediates (i.e., pre-aggregates), which are transiently present and reversible in nature. Since commonly used analytical methods to characterize protein therapeutics are unable to specifically detect this initial step, it is difficult to prevent or limit its occurrence during formulation development or even during initial production since an effective analytical assay that detects these transient states and reports on early stage stabilization by excipients is lacking.

A recent strategy has been developed where a chaperone GroEL biolayer interferometry (BLI) biosensor is constructed to detect dynamic hydrophobic transients that occur prior to the formation of larger scale aggregation[9]. This detection scheme is based on the observation that chaperone proteins in cells capture and prevent or reverse misfolding of cellular proteins and are important in maintaining protein homeostasis within the cell[10]. The GroEL (hsp60) chaperonin is made up of 14 identical subunits with a molecular mass of 58K each that assemble into two stacked heptameric rings. Within the center of these tail to tail assembled rings, a central hydrophobic, 45 Å wide, protein-binding cavity is present that can easily accommodate aggregation prone hydrophobic regions of dynamically fluctuating, partially folded or misfolded proteins. Upon ATP binding, the GroEL binding cavity switches to a

hydrophilic interior allowing the bound partially folded or even fully folded protein to be released into solution. The hydrolysis of ATP resets the system back to its higher affinity protein capture state[11]. The form of the chaperonin that is used to construct the biolayer interferometry biosensors is the nucleotide free form of GroEL since its binding affinity for hydrophobic partially folded proteins is high, sometimes approaching antigen antibody affinities[11.] Previous work in our laboratories has shown that a biotinylated GroEL-streptavidin-BLI biosensor is able to capture and detect pre-aggregate species during stress with several pharmaceutically relevant protein molecules (polyclonal Ab, IgG1 mAb and a heparin binding growth factor, FGF-1) before irreversible aggregation could be detected by size exclusion chromatography (SEC)[9]. In addition to increased binding during various stress conditions, the protein molecules also showed some binding under unstressed temperature conditions (25^{o}C). This detection/binding of pre-aggregates was verified to be specific for the chaperonin binding site because the binding was most often reversed by adding ATP to the GroEL-protein substrate complex that was immobilized on the BLI biosensor. In addition, preliminary TEM data of released GroEL complexes from the biosensor surface exposed to stressed mAb solution distinctly showed the presence of bound densities attributed to captured antibody proteins[9].

In this work, we further explore and develop the utility of this analytical technology[9] by using GroEL-BLI biosensors linked to an automated BLI technology platform (the Octet® 96 well system). The automated method was used to detect the formation of the pre-aggregate species for larger sets of various mAbs (both IgG1 and IgG4), as well as a bispecific antibody, to assess aggregation propensity of multiple samples simultaneously. This series of pharmaceutically relevant mAbs showed variable rates of aggregation when exposed to a variety of different

stresses (e.g., acidic pH, Gdn-HCl, temperature cycling), conditions shown previously to generate pre-aggregate species with other mAbs[12-14]. The GroEL biosensor results were compared with the rate and extent of soluble aggregates and sub-visible particulates formation as measured by SEC and MFI, respectively. TEM analysis was also used as an orthogonal method to structurally detect pre-aggregate mAb species that complexed to the GroEL protein (both in solution and released from the biosensor surface) with the goal of specifically pinpointing particular hydrophobic regions on the Ab structure that leads to aggregation and product degradation.

Materials and Methods:

Materials: Two IgG1 monoclonal antibodies (mAb-E & mAb-J), an IgG4 monoclonal antibody (mAb-D), and a bispecific antibody (Bis-3) were provided by MedImmune (Gaithersburg, Maryland). The mAb-E was provided as a 100 mg/mL protein solution in 25 mM Histidine, 8% Trehalose pH 6.0, mAb-J as a 150 mg/mL protein solution in 25 mM Histidine, 100 mM Arginine-HCl, 90 mM Sucrose, pH 6.5, mAb-D as a 50 mg/mL protein solution in 50 mM Acetate, 100 mM NaCl, pH 5.5, and Bis-3 as a 4.2 mg/mL protein solution in 20 mM Sodium Phosphate, 235 mM Sucrose, pH 7.2. The concentration of the various Abs and GroEL was measured after dialysis (into 5 mM citrate-phosphate buffer (CP) containing either 150 or 500 mM NaCl at pH 7.4 as described below) with an Agilent 8453 UV-visible spectrophotometer (Palo Alto, California). The absorption values at 280 nm were averaged for triplicate analysis and extinction coefficients of 1.42 (mAb-E), 1.56 (mAb-J), 1.68 (mAb-D), and 1.54 $(mg/mL)^{-1}$ cm^{-1} (Bis-3) were

used to calculate the protein concentration. FGF-1, used as an assay control in this work, was provided by Professor Mike Blaber at Florida State University.

Streptavidin (SA) biosensors were purchased from Fortébio (Menlo Park, California). Ninety six well black polypropylene plates were purchased from Greiner (Monroe, North Carolina). An XL25 dual channel pH meter, sodium chloride, guanidine hydrochloride, potassium chloride, EDTA, and LC-MS grade water were obtained from Fisher Scientific (Fair Lawn, New Jersey). Sodium phosphate dibasic (anhydrous), citric acid (anhydrous), and magnesium chloride were purchased from Acros Organics (Fair Lawn, New Jersey). Glycerol, Trizma base, ATP, and BSA were obtained from Sigma Aldrich (St. Louis, Missouri).

Methods:

Biolayer Interferometry- Samples were analyzed by biolayer interferometry using an Octet RED96 system (Fortebio, Menlo Park, CA) with the sample holder set at 25^{o}C, even for cases where samples were initially subjected to thermal stress (as described below). Biotinylated GroEL-Streptavidin biosensors were used to monitor the presence of pre-aggregates in antibody solutions using 9 steps to produce a sensogram as described below. Before a sensogram is obtained, each streptavidin biosensor is first hydrated for 10 min in GroEL buffer (50 mM Tris, 50 mM KCl, 10 mM $MgCl_2$, and 0.5 mM EDTA, pH 7.5) prior to start of the study. The automated nine step procedure is as follows: (1) an initial reference baseline of 30 sec is performed with GroEL buffer alone, (2) Biotinylated GroEL (b-GroEL-1mg/ml – 1 to 3 biotin adducts per GroEL oligomer [8]) is loaded onto the streptavidin biosensor for 170 sec followed by a (3) baseline determination that is performed in PBS. Once loaded, (4) a 220 sec blocking step was implemented to further diminish non-specific binding using BSA (1 mg/ml) as the blocking

agent. This blocking step was followed by a 30 sec baseline wash that is performed with buffer identical to that used for the mAb (5). In the testing phase, (6) the association of stressed or unstressed antibody sample (concentration ranges from 0.2 to 5.0 mg/ml) with the GroEL biosensor is performed for 5 min, (7) followed by monitoring the dissociation phase for 220 sec using a buffer identical to that used for the antibody sample. To reverse the specific binding of the stressed antibody from the GroEL biosensor, (8) three pulses of 20 m*M* ATP in an osmolyte mixture of 4M Urea, 4M glycerol is used to remove bound antibody from GroEL (return to baseline indicates specific binding), and (9) a final baseline is acquired for 60 sec using a buffer identical to that employed for the antibody and compared with the starting amplitude from step 5 (further described in Figure 1 in the Results section). As was shown previously, biotinylated GroEL remains tetradecameric and is fully functional under these solution conditions[15].

GroEL-BLI Method Optimization- GroEL-BLI biosensor tips are pretreated with BSA to decrease possible nonspecific binding of mAb to any bare streptavidin biosensor surfaces. To insure that non-specific binding does not occur, the response of 1 mg/ml BSA binding to the streptavidin tip was examined with increasing concentration of GroEL loading . During step 2, concentrations of 0, 2.5, 5 ,10, 25, 100, 500, 1000 µg/mL GroEL were loaded onto individual biosensors in the automated OCTETRed96 system to evaluate loading capacity. Although BSA binding diminishes as GroEL binding increases (see Figure 2 in Naik et al., 2014)[9], as a precautionary step, during automated step 4, 1 mg/mL BSA was included in the buffer to block any nonspecific binding sites on the biosensor surface (Blocking step Figure 3A). The BSA blocking step may seem redundant when GroEL loading has been optimized (see also Figure 2

in reference 9), but in cases where GroEL loading has not been optimized (lower GroEL binding on the Biosensor), inclusion of the BSA blocking step simply insures that nonspecific binding of the test antibody sample does not occur. In fact, BSA loading onto bare streptavidin tips also leads to diminished non-specific Ab binding (Supplemental Figure S1). As a control, Step 6 was performed using a stable nonstressed antibody to evalute the amount of nonspecific/background binding occuring for each mAb prior to exposure of antibody solutions to stress conditions. A final concentration of 500 µg/mL GroEL was used (step 2) for future experiments.

Size Exclusion Chromatography (SEC)- The monomer, aggregate and fragment content of stressed antibody samples was assessed using a 7.8 x 30 cm^2 TOSOH TSK-Gel BioAssist G3SW_{XL} column and 6.0 mm ID x 4.0 cm TSK-Gel SW_{XL} guard column (TOSOH Biosciences, King of Prussia, Pennsylvania). Prior to analysis, the column was pre-equilibrated with 7-column volumes of mobile phase composed of 0.2 M sodium phosphate, pH 6.8. Removal of insoluble aggregates from the stressed samples was accomplished by centrifugation at 14,000 x *g* for five min before injection onto the column. Elution of the various species from the column was assessed by monitoring the UV absorbance at 214 and 280 nm (as described previously)[16] using a Shimadzu high-performance liquid chromatography system equipped with a photodiode array detector. Molecular weight standards (Biorad Laboratories, Hercules, California) were used to assess the efficiency of separation. Injection of antibody stored in the formulation buffer was also completed on the day of each time-point to assess day-to-day variability and column lifespan. Peaks corresponding to aggregates, monomer, and fragments were selected and the area under each peak was quantified using the LC Solutions data analysis package.

Microflow Imaging (MFI) - A DPA-4200 microflow imaging system (Protein Simple, Santa Clara, California) was used to count an image micron-sized subvisible particles in the size range of 2-100 µm. Stressed and control samples were added directly to the MFI without any prior dilution as described in detail elsewhere[17]. Buffer controls containing no protein, were also analyzed and subtracted from the protein-containing samples.

Transmission electron microscopy (TEM) - A JEOL JEM-1400 TEM operating at 100KV equipped with an AMT digital camera system was used to visualize the GroEL and GroEL-complexes. To form the GroEL-mAb complexes, mAb-D and Bis3 samples were heated at 50°C for 60 min in solution with a selected molar ratio of GroEL. To analyze biosensor released GroEL-Ab complexes, the Biosensor was loaded with the 60 min stress Ab sample and the complex was released onto EM grids by reducing the S-S biotinylated GroEL-Ab complexes with 50 mM dithiothreitol (DTT)[9]. To assess complexes that form on the GroEL biosensor, S-S biotinylated GroEL was used since the complex is easily removed from the biosensor surface using 50 m*M* containing DTT solution. Two µL of the sample was placed on carbon coated Cu 300 square mesh copper grids (Electron Microscopy Sciences) that were glow discharged prior to use. The samples were incubated on the grids for 10 min, followed by a 3X wash with deionized water. The grids were then negatively stained with filtered (0.02 micron Whatman filters) 0.75% uranyl formate and imaged.

Sample preparation and temperature stress treatment of mAbs- To determine potential candidates for further analysis, long term thermal stress experiments were performed. Stock solutions of various mAbs that were initially stored at -80°C were dialyzed into 5 mM citrate-

phosphate buffer (CP) containing either 150 mM or 500 mM NaCl at pH 7.4. The mAbs were then diluted using the CP buffer to achieve a protein concentration of 5 mg/mL (mAb-E, D, and J). The samples were filtered using 0.22 µm syringe filters in a laminar flow hood (Millipore, Billerica, Massachusetts). Aliquots of 1.5 mL were placed into 3 mL borosilicate glass type I vials and capped with rubber stoppers (West Pharmaceutical Services, Exton, Pennsylvania). Samples of all three mAbs were stored at 4, 50, 55, and 60 °C for various times in triplicate. Samples were then analyzed by GroEL-BLI after storage for two weeks.

Sample preparation and stress treatment by pH shift with mAb-D- The mAb-D stock solution was diluted using the CP buffer with 150mM NaCl to achieve a protein concentration of 5 mg/mL. The samples were then stressed by shifting the pH to 4.0 for 1 minute using 0.5 M HCl and then increasing the pH back to 7.4 by titrating with a 1 M Na phosphate stock solution. Using a concentrated stock solution allows one to adjust the pH of the solution with minimal dilution. A second set of samples were generated by shifting the pH to 5.0 and back to 7.4 after 1 min incubation. Once the pH reached 7.4, the samples were analyzed immediately using the GroEL-BLI, SEC and MFI.

Sample preparation and stress treatment by thermal cycling of mAb-D- The mAb-D stock solution was diluted using the CP buffer with 150 mM NaCl to achieve a protein concentration of 5 mg/mL. mAb-D was stressed by thermal cycling from 25 to 45°C and back to 25°C at 3°C per sec resulting in ~68 complete thermal ramps in 15 min and ~ 540 complete thermal ramps over a two 2 h period. The samples were then analyzed using GroEL-BLI, SEC, and MFI.

Sample preparation and stress treatment by addition of Guanidine HCl with mAb-D- A stock solution of 5 M guanidine was prepared in 5 mM CP, 150 mM NaCl at pH 7.4. The stock mAb-D was diluted using the corresponding CP buffer and the guanidine hydrochloride stock solution to achieve a protein concentration of 5 mg/mL and 0.5 M guanidine HCl. The mAb-D control samples were also prepared using only CP buffer with 150 mM NaCl. The samples were filtered using 0.22 µm syringe filters (Millipore, Billerica, Massachusetts). Aliquots of 1.5 mL were placed into 3 mL borosilicate glass type I vials and capped with rubber stoppers (West Pharmaceutical Services, Exton, Pennsylvania). Triplicates of the perturbed samples were analyzed over 24 h by GroEL-BLI, SEC and MFI at time points of 8 and 68 min, 2, 5, and 24 h.

Sample preparation and stress treatment by 50°C incubation of Bis-3 and mAb-D- The mAb-D stock solution (50 mg/mL) was diluted using the CP buffer with 150 mM NaCl to achieve a protein concentration of 5 mg/mL. The stock solution of Bis-3 (4.2 mg/mL) was diluted to a final concentration of 0.2 mg/mL using 20 mM sodium phosphate, 235 mM sucrose at pH 7.2. mAb-D and Bis-3 solutions were stressed over 1 h at 50°C. At time points of 0, 5, 10, 20, 30, and 60 min (mAb-D) and 0, 10, 20, 30, 45, 60 min (Bis-3), the samples were analyzed using GroEL-BLI, SEC, and MFI.

P-value calculation- p-values were calculated using the t-Test: paired two sample for means data analysis function from Excel software (Microsoft, Redmond WA). The alpha value was 0.05 with N=3 for all sample sets.

Results and Discussion

Automated GroEL-BLI assay optimization and initial assessment of aggregation propensity of three mAbs

In this work, the utility and pharmaceutical applicability of a previously reported GroEL-BLI biosensor assay[9] to monitor the formation of pre-aggregate species found in various therapeutic mAb candidates exposed to different environmental stresses were evaluated. A key analytical advancement in this work was the development and implementation of an automated BLI platform (OctetRed96 system). By moving from the single channel BLI system described previously[9] to an automated, multichannel platform (96 well plate), this work demonstrates the use of the GroEL biosensor as a part of formulation development of mAbs and other therapeutic protein candidates. Other improvements in the assay described in this work include the addition of a BSA pretreatment step to further minimize non-specific binding of proteins to the biosensor surface even with variable GroEL loading, and more routine implementation of various controls (including a relatively unstable control protein, FGF-1, and non-stressed mAbs as positive and negative controls). Moreover, by using an automated platform, many more replicates can be run for each sample to further ensure the reproducibility of each set of experiments. The automated GroEL-BLI system was then examined with 4 different therapeutic mAb candidates (two IgG1 molecules, one IgG4 molecule and one bispecific mAb) exposed to different environmental stresses previously shown to induce formation of aggregates using various mAbs[12-14].

As an initial step to optimize the assay and minimize any potential nonspecific binding to the biosensor, concentrations of 0-1 mg/mL of b-GroEL (0, 2.5, 5 ,10, 25, 100, 500, 1000 µg/mL) were loaded onto the biosensor. This step was followed by a blocking step with concentrations

of 0-1 mg/mL bovine serum albumin (BSA). A non-stressed mAb sample was then exposed to the biosensor. The conditions with the least amount of non-specific binding during the association phase were chosen for future studies and consisted of loading with 0.5 mg/mL GroEL and 1 mg/mL BSA (See Supplemental Figure S2).

Following adaptation to the Octet multichannel system and assay optimization, a screening of three pharmaceutically relevant mAbs (mAb-D, E, and J) was performed. Thermal stresses were used to probe each mAb at temperatures approaching their T_{onset} values, at the T_{onset}, and above the T_{onset} (mAb-D: 58.9°C ± 0.5; mAb-E: 56.3°C ± 0.08; mAb-J: 55.3°C ± 0.2 as obtained by differential scanning calorimetry, data not shown). All three mAbs were stressed at temperatures of 50, 55, and 60°C (along with a 4°C control) for 14 days in solutions containing different salt concentrations (150 mM and 500 mM NaCl). Figure 1 represents the average binding amplitude of the different samples (each mAb at 5.0 mg/ml) from the GroEL-BLI biosensor. In general, it was seen that mAb-D showed the highest binding amplitude at both salt concentrations at each of the three elevated temperatures. In addition, increasing the NaCl concentration from 150 to 500 mM did not have any major effect on the IgG binding amplitudes. The slight decrease in binding amplitude seen from 50°C to 60°C can be due to the smaller pre-aggregate species forming larger sized aggregates in solution (from the increase heat stress) and these larger aggregates cannot be captured by the GroEL as effectively. This decrease in GroEL biosensor capture efficiency could be due to the diminished exposure of hydrophobic patches upon aggregation or the decreased collisional frequency with the biosensor due to a decrease in diffusion or a combination of both these aggregate properties.

These results show that different mAbs can be compared for their aggregation propensity in different solutions using this GroEL-BLI biosensor method in an automated platform.

GroEL-BLI, SEC and MFI analysis of mAb-D aggregation under various stress conditions

To further evaluate the utility of this approach, mAb-D was further exposed to four other environmental stresses involving acidic pH exposure, thermal cycling, Gdn-HCl perturbation, and 50°C incubation as outlined by the flowchart illustrated in Figure 2 (conditions 1, 2, 3, 4, respectively). These experimental conditions were selected based on previous reports for their ability to initiate formation of early intermediates in the mAb aggregation pathways[12-14]. Figure 3A shows a representative GroEL-BLI biosensor sensogram of mAb-D stressed under one of the four conditions (condition 4, 50°C for 60 min). The association step shows triplicate runs of mAb-D stressed solution with the dissociation step conducted in mAb-D formulation buffer alone. The final baseline determination was performed after using the mAb-D formulation buffer with added ATP to induce release of bound mAb-D from the GroEL. The sensogram trace returns at or near the original baseline (compare initial mAb binding reaction starting amplitude with last step baseline buffer return in Figure 3A), indicating specific binding to the GroEL biosensor. Figure 3B is an enlargement of the association and dissociation phases of the same sample after a 60 min thermal stress treatment. Similar GroEL-BLI experiments were then performed with mAb-D exposed to each of the four stress conditions (Figure 2) and the results are described below and summarized in Figure 3C. The GroEL-BLI results were compared to analysis of the same mAb-D samples by SEC and MFI as summarized in Figure 4A and 4B, respectively.

For condition 1, a brief decrease in solution pH to acidic conditions has been previously shown to cause the formation of a wide range of metastable aggregate species with various mAbs, produced by the unfolding of the Fab region[12]. The mAb-D solution was subjected to a brief exposure (1 min pH jump) by rapidly dropping to pH 4.0 followed by the pH return to 7.4 by readjusting with concentrated phosphate buffer. This pH pulse and return resulted in a significant increase (p-value of 0.0025) in the binding amplitude using the GroEL-BLI assay compared to the non-stressed mAb-D control (Figure 3C). This change in binding amplitude was also observed during a pH 5.0 pulse, but the BLI binding signal was diminished compared with the pH 4.0 pulse amplitude (p-value of 0.0037), thereby indicating that pH 4.0 pulse created a larger population of aggregates compared to the pH 5.0 pulse. When these samples were examined using size exclusion chromatography (SEC), a substantial increase in percent aggregation (soluble and insoluble) of ~18% for the pH 4.0 pulse samples and ~ 15% for the pH 5.0 samples was observed compared to the control sample that contained ~0.2% aggregate (Figure 4A). An increase in formation of both soluble and insoluble aggregates (loss of total area in the SEC chromatogram) with a concomitant decrease in the monomer peak was observed. The samples were assessed for formation of subvisible particles by Microflow Imaging (MFI), with both pH pulsed samples showing an increase in total particle concentration compared to the non-stressed mAb-D control (Figure 4B). The particle size distribution of the control and stressed mAb-D samples showed the majority of particles to be in the size range of 2-10 µm (data not shown).

For condition 2, mAb-D was exposed to multiple thermal cycles from 25 to 45°C and back to 25°C. The heating phase during thermal cycling has been shown previously to partially

unfold the Fab domain of an antibody while the cooling phase minimizes protein aggregation[14]. To determine if these conditions could lead to the formation of pre-aggregate species with mAb-D, the thermal cycling was performed from 25°C to 45°C and back to 25°C at 3°C per second over the course of 15 min and 2 h and resulted in an increase in the binding amplitude using the GroEL-BLI assay compared to the mAb-D non-stressed control (Figure 3C). When the samples were examined using SEC, a relatively small increase in total aggregation was seen. There was ~0.5% increase in aggregation seen for the 15 min thermal cycled samples and ~1% increase for the 2 h cycled samples compared to the non-stressed mAb with ~ 0.2% total aggregation (Figure 4A). This was also accompanied by a loss in total peak area, indicating the formation of insoluble aggregates that did not pass through the column matrix. The samples were assessed by MFI with both 15 min and 2 h samples showing an increase in subvisible particle concentration compared to the non-stressed mAb-D control (Figure 4B). The particle size distribution of the control and stressed mAb-D samples showed the majority of particles to be in the size range of 2-10 µm (data not shown).

Exposing mAb-D to conditions 1 and 2 (Figure 2) showed concomitant formation of pre-aggregate species (GroEL-BLI biosensor), aggregates (SEC) and subvisible particles (MFI). These results with mAb-D can be compared to previous work done on other mAbs[12 12]. For example, a brief low pH shift[12] resulted in polydisperse mAb aggregates from dimers to aggregates of 10 µm in diameter as assessed by SEC and light obscuration. Similar results were observed with mAb-D with aggregates of varying size (from dimer to subvisible particles of 2-10 µm). For the thermal cycling method[14], however, the heating pulse was reported to lead to partial unfolding of the Fab region allowing Fab-Fab interactions to occur. The cooling period caused the

refolding and minimization of detectable aggregation for the examined mAb. Thermal cycling led to an increase in the amount of fragments with a concomitant decrease in the presence of dimers observed by SEC. In contrast, in this study, mAb-D showed a steady increase in the level of insoluble aggregates and increased amounts of subvisible particles after multiple thermal cycling events. These contrasting results could be due to differences in the inherent stability or dynamics of the two mAbs, or possibly differences in experimental solution conditions.

In summary for conditions 1 and 2, mAb-D formed irreversible aggregates under the conditions of acidic pH exposure and temperature cycling as measured by SEC and MFI. Concomitantly, an increase in binding amplitude was observed using the GroEL biosensor. Thus, the GroEL biosensor was detecting either formation of the pre-aggregate species and/or formation of the irreversible aggregates. It was difficult to distinguish these two possibilities under these two experimental conditions because of the formation of multiple types of aggregates during these stresses. Indeed previous results indicate that the GroEL-BLI biosensor binds to stable IgG dimers[8]. Thus, the biosensor results correlate with SEC and MFI data indicate that the GroEL biosensor method can potentially be used as a rapid general assessment of protein aggregation propensities which bodes well implementation of a routine automated assay setup (8 or 16 channel Octet systems and other higher HDXOctet 96 channel BLI throughput systems).

In contrast to the mAb-D aggregation profile observed with conditions 1 and 2, conditions 3 and 4 (see Figure 2) showed a different profile in which the GroEL-BLI biosensor detects hydrophobic substrates (pre-aggregate species) prior to the detection of irreversible aggregate formation as measured by SEC and MFI. For example, Condition 3 consisted of

perturbing mAb-D with 0.5 M guanidine HCl for various times. Exposure to intermediate concentrations of Gdn-HCl has previously been shown to partially unfold the CH_2 domains of a mAb and lead to aggregate formation[13]. Therefore, perturbation of mAb-D with 0.5 M Gdn-HCl was performed to evaluate the ability of these conditions to generate aggregate and/or pre-aggregate species. A significant increase in the binding amplitude using the GroEL-BLI assay (compared to the mAb-D non-stressed control) was observed (Figure 3C). The association phase during the GroEL-BLI study show a slight increase in binding at the 24 h time point compared to earlier times, which showed no significant differences (p-value=0.059). Based on SEC, the mAb-D samples produced less than 1.5% total aggregation (approx. equivalent to the amount observed in the unstressed mAb-D control where no significant GroEL biosensor binding was observed, Figure 4A). Similarly, when the samples were also analyzed by MFI, no notable increase in total subvisible particle concentration (compared to the non-stressed mAb-D control) at the early time points (8 and 68 min, and 2 h) were observed, although the longer time points under these same conditions (5 h and 24 h) showed a small increase in subvisible particle concentration compared to the control (Figure 4B).

Under the final stress condition examined (Condition 4, see Figure 2), mAb-D was incubated at 50°C for various times up to an hour. For the GroEL-BLI results, Figure 3C shows increasing binding amplitudes for the stressed mAb-D samples at 50°C for up to one hour. The stressed mAb-D samples were also evaluated for the presence of aggregates using SEC as well as for the formation of larger, subvisible particles by MFI. Figure 4A displays the percent total aggregate formation as determined by SEC for mAb-D samples for various times along with an unstressed control. It can be seen that no detectable increase in aggregate is observed. Figure

4B shows the concentration of subvisible particles in the same samples as measured by MFI. Results indicate that no increase in particle formation is observed between 10 and 60 min of incubation at 50°C. The particle size distribution of the control and stressed mAb-D samples showed the majority of particles in the range of 2-10 µm (data not shown).

TEM analysis was used to visualize the GroEL-mAb-D complexes that were formed after exposing the mAb-D sample to heating at 50°C for 60 min (Condition 4). Figure 5 shows TEM images of the different preparations, including 0.05 µM GroEL in the solution as a control (Figure 5A), solution based interaction of the stressed 0.05 µM mAb-D with 0.05 µM GroEL (Figure 5B), dissociation of –S-S- biotinylated-GroEL (SS-b-GroEL) from the streptavidin biosensor with a DTT control (Figure 5C), and dissociation of the mAb-D-GroEL complex from the streptavidin biosensor surface using DTT (Figure 5D). Complexes between the GroEL and mAb-D (or potentially fragments of mAb-D), after temperature exposure, can be seen using both approaches (Figure 5B and 5D) indicating the binding of structurally altered forms of mAb-D species to GroEL upon exposure of the mAb-D to elevated temperature. This is manifested as extra density bound to the GroEL binding site.

Exposing mAb-D to conditions 3 and 4 (Figure 2) showed formation of pre-aggregate species (GroEL-BLI biosensor) prior to formation of aggregates (SEC) and subvisible particles (MFI). These results with mAb-D can be compared to the previous work reported on other mAbs[13 18]. Mehta et. al. 2014[13] showed unfolding of the CH_2 domain was the interaction site for aggregation of this particular mAb when exposed to 1.2-1.6 M Gdn-HCl. At lower denaturant concentrations, the mAb remained in its native state. In the case of mAb-D in our study, when exposed to 0.5 M Gdn-HCl, SEC and MFI showed no increased amounts of aggregation

compared to the control. Interestingly, the GroEL-BLI biosensor method was able to detect the formation of pre-aggregates. For thermal stress, Hawe et. al. (2009)[18] showed that thermal incubation of a mAb solution (at a few degrees below its visible aggregation temperature) resulted in formation of small aggregates around 30 nm and subvisible particles below 25 μm. During the thermal stress of mAb-D in this work, performed at ~9 degrees below its T_{onset} value, no increase in aggregates was detected using SEC and nor MFI, yet the GroEL-BLI system does detect early structurally altered forms of mAb-D. These results demonstrate that the different environmental stresses lead to the formation of differing levels of mAb-D pre-aggregate, soluble aggregate and subvisible particle species which can be elucidated using a combination of the GroEL-BLI biosensor, SEC and MFI analyses.

Automated GroEL-BLI, SEC and MFI analysis of Bis-3 bispecific antibody under thermal stress conditions

To further examine the ability of the automated, optimized GroEL-BLI biosensor to detect formation of pre-aggregate species, solutions of a pharmaceutically relevant bispecific Ab (Bis-3) were examined, before and after thermal stress, by a combination of the GroEL-BLI biosensor assay, SEC and MFI. Bis-3 solutions at a low protein concentration (0.2 mg/ml) were incubated at 50°C (for up to 60 min), a temperature well below the T_{onset} of the bispecific antibody (as determined by differential scanning calorimetry where Bis-3 exhibited a multiphasic DSC thermogram with a Tm_1 value of ~64°C). Figure 6A shows representative GroEL-BLI biosensor sensograms of the association and dissociation phase of Bis-3 samples before and after an exposure to elevated temperature (50°C, 10 min) followed by a return to

25^{o}C prior to measurement. Figure 6B shows the association and dissociation phase of Bis-3 samples after 60 min at 50°C. The dissociation step was performed with the formulation buffer alone. Three pulses of a 20 mM ATP containing solution (with elevated values due to high refractive index of solution due to the glycerol) released the captured Bis-3 specifically bound to the GroEL as evidenced by the return to the original GroEL loaded baseline. For each of the time points, the average binding amplitude (from three replicates) for the association, dissociation, and final baseline were plotted as a bar graph in Figure 6C. An increase in binding amplitude of the unstressed Bis-3 sample (at room temperature) over the buffer control (no Bis-3) is observed indicating the Bis-3 molecule has an exposed hydrophobic GroEL binding interaction site even at room temperature. In contrast, neither the IgG1 nor the IgG4 mAbs showed any such GroEL-BLI binding signal at room temperature. Interestingly, the Bis3 sample concentration tested (0.2 mg/ml) was significantly lower than the mAb-D sample tested (5 mg/ml), yet showed a larger binding amplitude during the association phase.

When the same temperature stressed Bis-3 samples were examined for irreversible aggregate formation using SEC, no substantial increase in percent aggregation was observed (both for soluble aggregates in the chromatogram and insoluble aggregates as indicated by total area recovered), compared to the control Bis-3 sample (both contained ~0.45% aggregate) (Figure 7A). The Bis-3 thermally stressed samples also showed no increase in the levels of subvisible particles as assessed by MFI measurements over the unstressed Bis-3 control (Figure 7B). For both the unstressed and thermally stressed samples, the particle size distribution within the 2-10 µm size range remained the same (data not shown). These results indicate that these thermally stressed Bis-3 samples contained an easily detectable amount of pre-aggregate

species by the GroEL-BLI biosensor prior to any detectable formation of irreversible aggregates as determined by SEC and MFI.

TEM was used to visualize the GroEL-Bis-3 complexes. Figure 8 shows representative TEM fields of Bis-3 GroEL complexes generated from both solution formed and biosensor released complexes. The first two image fields represent the complexes that were formed in solution with the GroEL alone (Figure 8A) and GroEL-Bis-3 complexes (Figure 8B) which were heated at 50°C for 60 min. The field in Figure 8B represents a solution containing equal molar amounts of Bis-3 and GroEL; see methods section). In Figure 8C and D, the images were generated by performing the GroEL-BLI assay on streptavidin biosensors loaded with S-S biotinylated GroEL, and then dissociating GroEL alone (Figure 8C) and GroEL-Bis3 complexes (Figure 8D) by reducing the S-S linkage with DTT from three separate biosensors (to enhance complex concentrations, see methods section). In Figure 8D, the released GroEL-Bis-3 complex was formed by heating the Bis-3 sample at 50^{o}C for 60 min followed by an immersion of the GroEL biosensor into this solution for 5 min. After this immersion phase, the complex was then released from the biosensor with DTT and visualized using negative staining TEM. For both approaches to form the GroEL-Bis 3 complexes, the TEM images clearly show that the GroEL binding site is occupied by extra protein density with prominent extensions. In Figure 8B, some GroEL complexes show clear interactions between the GroEL binding site and the Fc portion of the bispecific mAb with the Fab domains pointing away from the GroEL binding site (Figure 8B). In Figure 8D, the biosensor released complexes also show clear extensions but the complexes are not as resolved as in the GroEL-Bis-3 solution complexes (Figure 8B).

Bispecific antibodies are designed by recombinant DNA technologies to recognize two different antigens to increase effectiveness of the therapeutic protein for certain targets[19]. Due to their asymmetrical, multi-domain nature, such engineered antibody molecules can have very different pharmaceutical properties than IgG mAbs including *in vivo* half-life and *in vitro* stability[19]. When examining Bis-3 using the GroEL-BLI assay, it was first noted that there was an increase in binding amplitude of unstressed Bis-3 sample (at room temperature) that was not seen with the three other IgG mAbs. Importantly, this GroEL-BLI detection signal with Bis-3 occurred at a ~25-fold lower protein concentration than with the mAb-D samples. This result indicates the Bis-3 molecule has a structurally altered, enhanced hydrophobic nature at room temperature compared to the mAbs. In addition, the Bis-3 samples were also inherently less stable than the IgG1 and IgG4 mAbs when the Bis-3 samples were exposed to limited thermal stresses (i.e., 10 min at 50°C) and the binding amplitudes remained relatively constant over the 60 min sampling period. This constant signal may indicate that the population of pre-aggregate species reaches an equilibrium state after thermal stress that does not diminish even after the samples are returned to ambient temperatures for the GroEL BLI biosensor measurements. This enhanced signal with the GroEL biosensor for both unstressed and thermally stressed Bis3 samples was obtained without any appreciable increase in the formation of irreversible aggregate species as measured by SEC and MFI. One additional difference between the Bis-3 and mAb-D is that Bis-3 had a significantly higher binding amplitude in the GroEL-BLI assay, probably indicating a higher population of the pre-aggregate species. This is also consistent with the increased population of complexes seen with TEM.

Previous results by Naik et al. (2014) [9] showed that the GroEL-BLI biosensor could detect the existing and thermally stressed formation of hydrophobic patches on polyclonal-IgG and mAb samples, with binding amplitudes seen to increase with the time of thermal stress. Likewise, the mAb-D examined in this work also showed time dependent increases in GroEL binding and hence detection as the thermal stress times increased. In contrast, this trend was not observed for the Bis-3 Ab as extended stress times at 50^{o}C did not show any time dependent increases in GroEL binding over the 60 min sampling period but rather manifested a steady, consistent binding response. In the samples examined in this work, the GroEL binding hydrophobic species (pre-aggregates) remained in solution even after the temperature is returned to ambient temperatures prior to analysis. This is another unexpected advantage of this method because this enhanced detection scheme could be useful in determining conditions that can inhibit the formation of or alternatively reverse the accumulation of these potentially metastable, kinetically trapped, partially unfolded, hydrophobic states.

Conclusions

In this work, a previously described GroEL-BLI based method[9] was further optimized and automated (using an Octet system, 96 well plate format) to bind and detect partially structurally altered intermediates (pre-aggregates) formed in stressed solutions of several different therapeutic Ab candidates including IgG1, IgG4 and bispecific antibodies. Transmission electron microscope (TEM) images of stressed Ab samples bound to the GroEL showed the formation of Ab pre-aggregate-GroEL complexes. The utility of this approach for use in protein formulation development was explored with various mAbs (IgG1 and IgG4) exposed to various

stress conditions as well as by using a relatively less stable bispecific antibody (Bis-3). For the latter, significant binding amplitudes were observed with the GroEL-BLI biosensor at room temperature at significantly lower protein concentrations (i.e., unstressed conditions), compared to the other mAbs, indicating its relatively enhanced hydrophobic nature, and by correlation, aggregation propensity. When Bis-3 was incubated at 50°C, increases in GroEL-BLI binding amplitude were observed before any increases were seen for irreversible aggregate/particle formation as measured by SEC and MFI. When examining the more stable mAb-D (IgG4), upon exposure to different stresses including elevated temperatures, acidic pH, and addition of guanidine-HCl, the GroEL-BLI biosensor could detect pre-aggregate formation before, or in some cases concomitantly with, irreversible aggregate formation as measured by SEC and MFI. Thus in this case, the GroEL-BLI biosensor can supplement information gained from more traditional aggregation detection methods to better detect pre-aggregate species that may be involved in the formation of longer term deleterious protein aggregates.

The GroEL biosensor technology provides protein specific information about the presence of potentially aggregation prone species. In some instances, this technology reports the formation of pre-aggregate transient species (GroEL binders) before larger scale aggregation is even detected. The specificity of this GroEL-Ab interaction can be confirmed by ATP induced reversal of Ab binding and the GroEL-Ab complexes can be easily visualized by TEM analysis. This method can rapidly assess solution stability and pre-aggregate formation within the relatively short time window of minutes. This rapid detection of pre-aggregate species using an automated platform can be particularly useful for protein formulation development. This early BLI detection method can be used to identify stabilizing conditions and excipients

that diminish pre-aggregate formation which may correlate with aggregation profiles during long term stability studies.

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Figure 1: Screening of the aggregation propensity of mAb-D (red), mAb-E (blue), and mAb-J (green) performed using the optimized, automated GroEL-BLI biosensor assay. The mAb solutions were incubated at temperatures of 4, 50, 55, and 60°C for 2 wk. The average binding amplitude of the different samples are shown with mAb-D showing the highest amplitude at both salt concentrations and at each of the three elevated temperatures. Error bars represent one standard deviation (N=3). The mAbs were formulated at 5 mg/mL in 5 mM citrate-phosphate buffer (CP) containing either 150 mM or 500 mM NaCl at pH 7.4.

Figure 2: Flow chart of the various stresses performed on mAb-D to assess aggregation propensity by a variety of analytical methods (GroEL-BLI, SEC and MFI). Condition 1 was performed by rapidly dropping the pH of the solution from 7.4 to pH 4.0 or 5.0, holding for 1 min and then returning to pH 7.4 with the addition of 1 M phosphate buffer. For condition 2, thermal cycling was performed from 25°C to 45°C back to 25°C for times between 15 min to 2 h at 25°C. Condition 3 consisted of addition of 0.5 M GdnHCl and incubation from 8 min to 24 h. Condition 4 was a 50°C incubation at various time points over 60 min. The aggregation of mAb-D solutions after exposure to each of the four conditions was examined using the GroEL-BLI biosensor, SEC, and MFI. The mAbs were formulated at 5 mg/mL in 5 mM citrate-phosphate buffer (CP) containing either 150 mM NaCl at pH 7.4.

Figure 3: GroEL-BLI biosensor analysis of mAb-D after exposure to different stresses a 50°C for 60 min. (A) Representative sensogram of mAb-D stressed at 50°C for 60 min in the GroEL-BLI assay showing association and dissociation phases, followed the addition of ATP and subsequent return to baseline (indicating specific GroEL binding; see text). B) Expanded view of the association and dissociation phases of the sensograms, and (C) Comparison of the binding amplitudes at the association phase of each of the mAb-D samples. Error bars represent one standard deviation (N=3). The mAbs were formulated at 5 mg/mL in 5 mM citrate-phosphate buffer (CP) containing 150 mM NaCl at pH 7.4.

Figure 4: Effect of various stress conditions on mAb-D aggregation and particle formation as measured by SEC and MFI, respectively. (A) Percent total aggregation (soluble and insoluble) of mAb-D before and after various stresses as measured by SEC, and (B) concentration of subvisible particles of mAb-D samples before and after various stresses as measured by MFI. Error bars represent one standard deviations (N=3). The mAbs were formulated at 5 mg/mL in 5 mM citrate-phosphate buffer (CP) containing 150 mM NaCl at pH 7.4.

Figure 5: Representative TEM images of GroEL alone and GroEL complexed with mAb-D stressed at 50°C for 60 min. Enlarged images of GroEL molecule (blue boxs) are shown to the right. (A) GroEL alone in solution, (B) GroEL-mAb-D complexes formed in solution, (C) GroEL released from the BLI biosensor surface with 50 mM DTT, and (D) GroEL-stressed mAb-D complexes released from the biosensor with 50 mM DTT. See methods section for further detail.

Figure 6: GroEL-BLI biosensor analysis of a bispecific Ab (Bis-3) during exposure to 50°C over 60 min. A) An expanded association and dissociation phase of the sensograms of 50°C stressed Bis-3 sample for 10 min in triplicates (green, red, and teal) and the unstressed sample (Blue) (B) An expanded association and dissociation phase of the sensograms of 50°C stressed Bis-3 sample for 60 min in triplicate (green, red, and teal) and buffer (Blue), and (C) comparison of binding amplitudes in the GroEL-BLI assay of each of the Bis-3 samples at the association, dissociation, and final baseline return after ATP. The baseline binding amplitude returning to near zero values after ATP exposure indicates specific binding to GroEL. Error bars represent one standard deviation (N=3). The Bis-3 was formulated at 0.2 mg/mL in a 20 mM sodium phosphate buffer containing 235 mM Sucrose at pH 7.2.

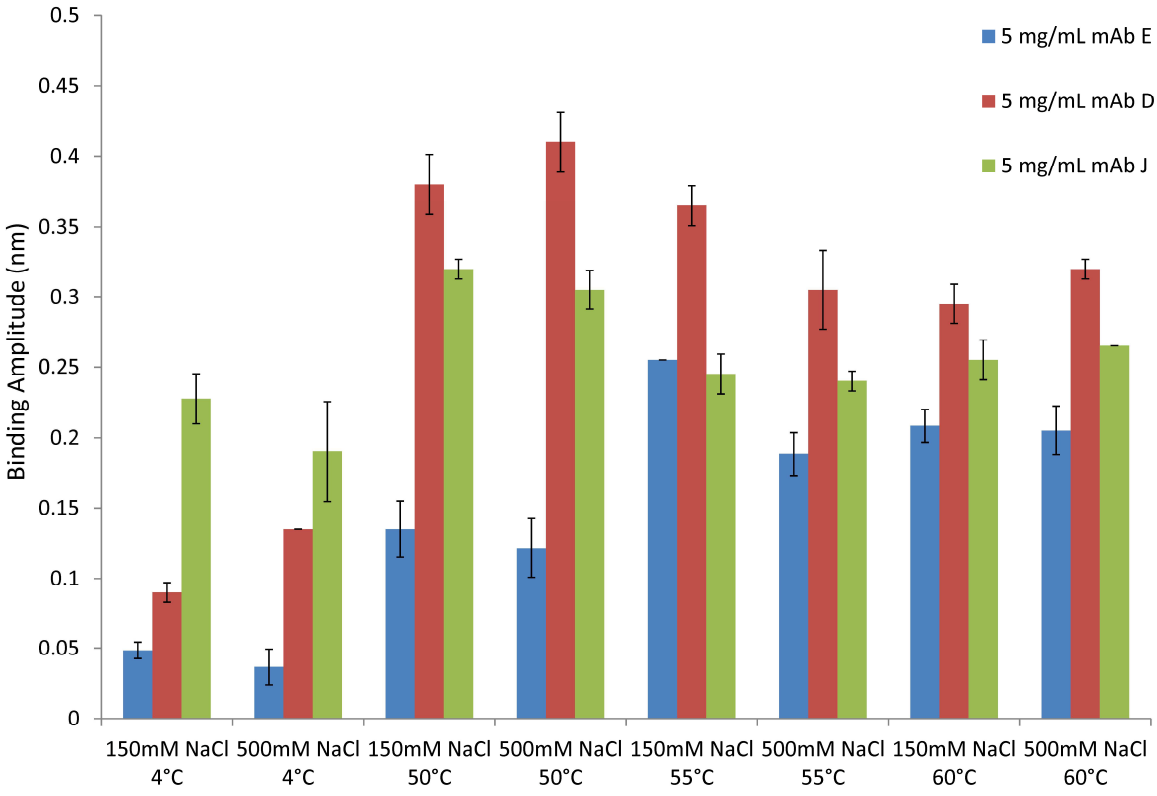
Figure 7: Effect of elevated temperature (50°C) on aggregation and subvisible particle formation of the bispecific antibody Bis3 as measured by SEC and MFI, respectively. (A) Percent total aggregation (soluble and insoluble) of Bis-3 before and after temperature stress as measured by SEC, and (B) concentration of subvisible particles of Bis3 samples before and after thermal

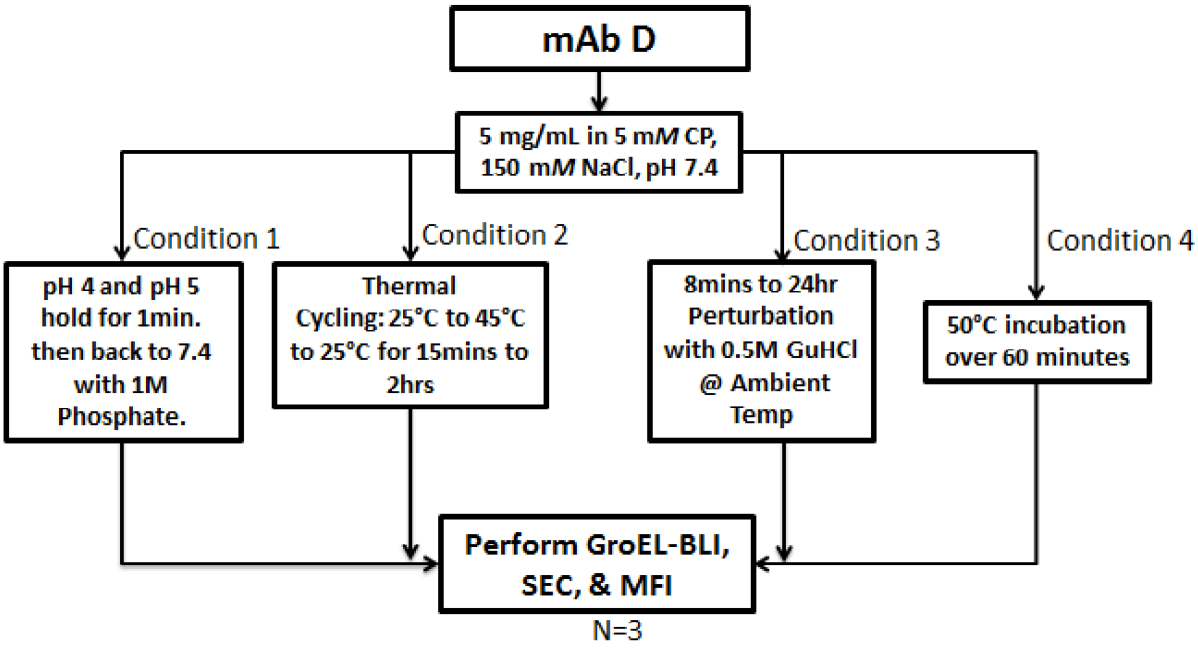
stress as measured by MFI from ≥2 <100 . Error bars represent one standard deviation (N=3). The Bis-3 was formulated at 0.2 mg/mL in a 20 mM sodium phosphate buffer containing 235 mM Sucrose at pH 7.2.

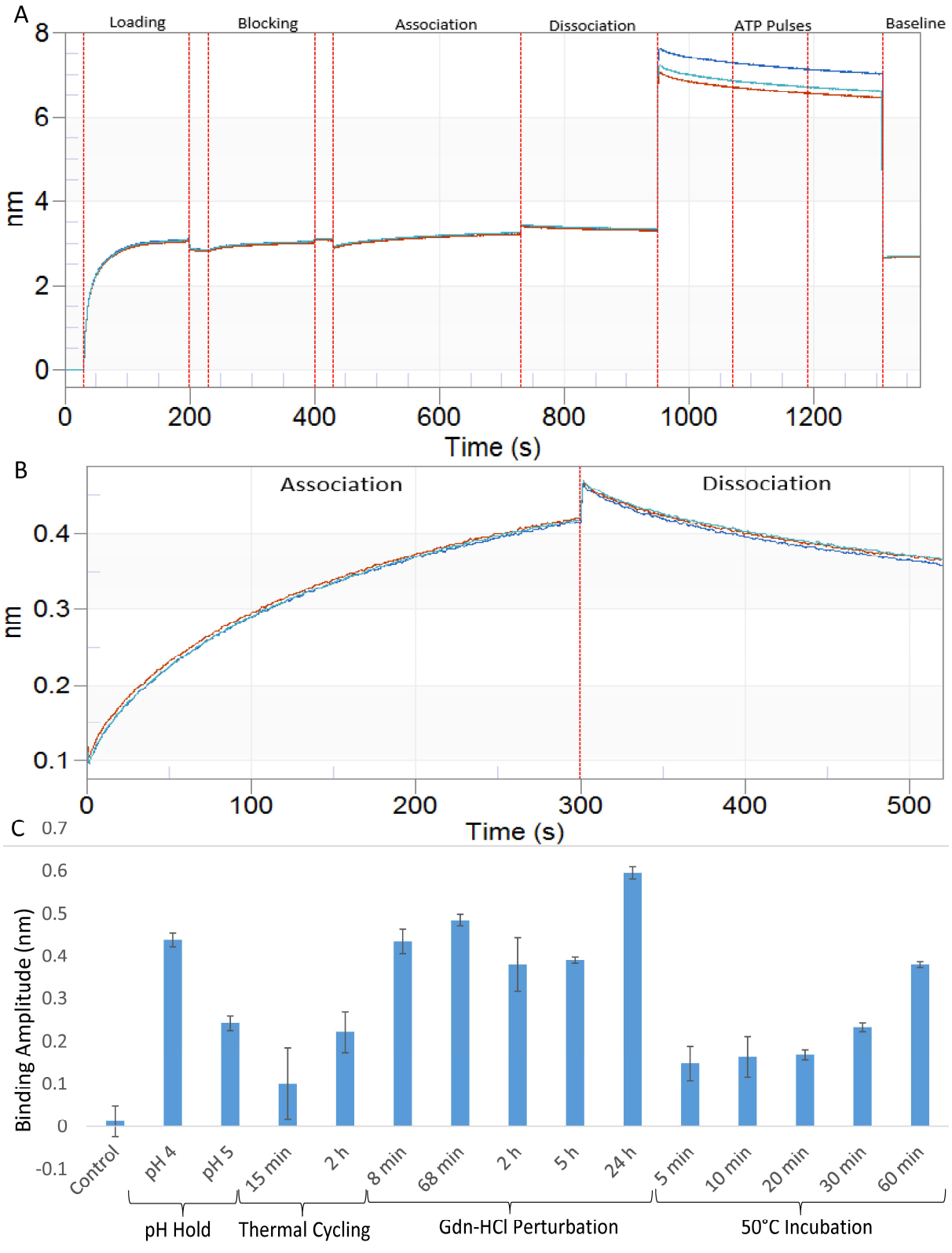
Figure 8: Representative TEM images of GroEL alone and GroEL complexed to the bispecific antibody Bis-3 stressed at 50°C for 60 min. representative enlarged images of GroEL from each TEM images (blue boxs) are shown to the right. (A) GroEL alone in solution, (B) GroEL-stressed Bis-3 complexes formed in solution, (C) GroEL released from the BLI biosensor surface with 50 mM DTT, and (D) GroEL-stressed Bis-3 complexes released from the biosensor with 50 mM DTT. See methods section for further detail.

Supplemental Figure S1: Optimization of the GroEL-BLI biosensor method. The binding amplitudes of the different concentrations of b-GroEL was plotted vs BSA (1 mg/mL). Unstressed mAb-D was exposed to the GroEL loaded tip with 150 mM NaCl and the binding amplitude was measured. (n=2-4, mean ± SD) at room temperature (25^{o}C).

Supplemental Figure S2: Optimization of the GroEL-biosensor method. The binding amplitudes of the different concentrations of b-GroEL and BSA (1 mg/mL) were summed together for total binding onto streptavidin tips. Unstressed mAb-D was exposed to the GroEL loaded tip with 150 and 500 mM NaCl and the binding amplitude was measured. (n=2-4, mean ± SD) at room temperature (25^{o}C).



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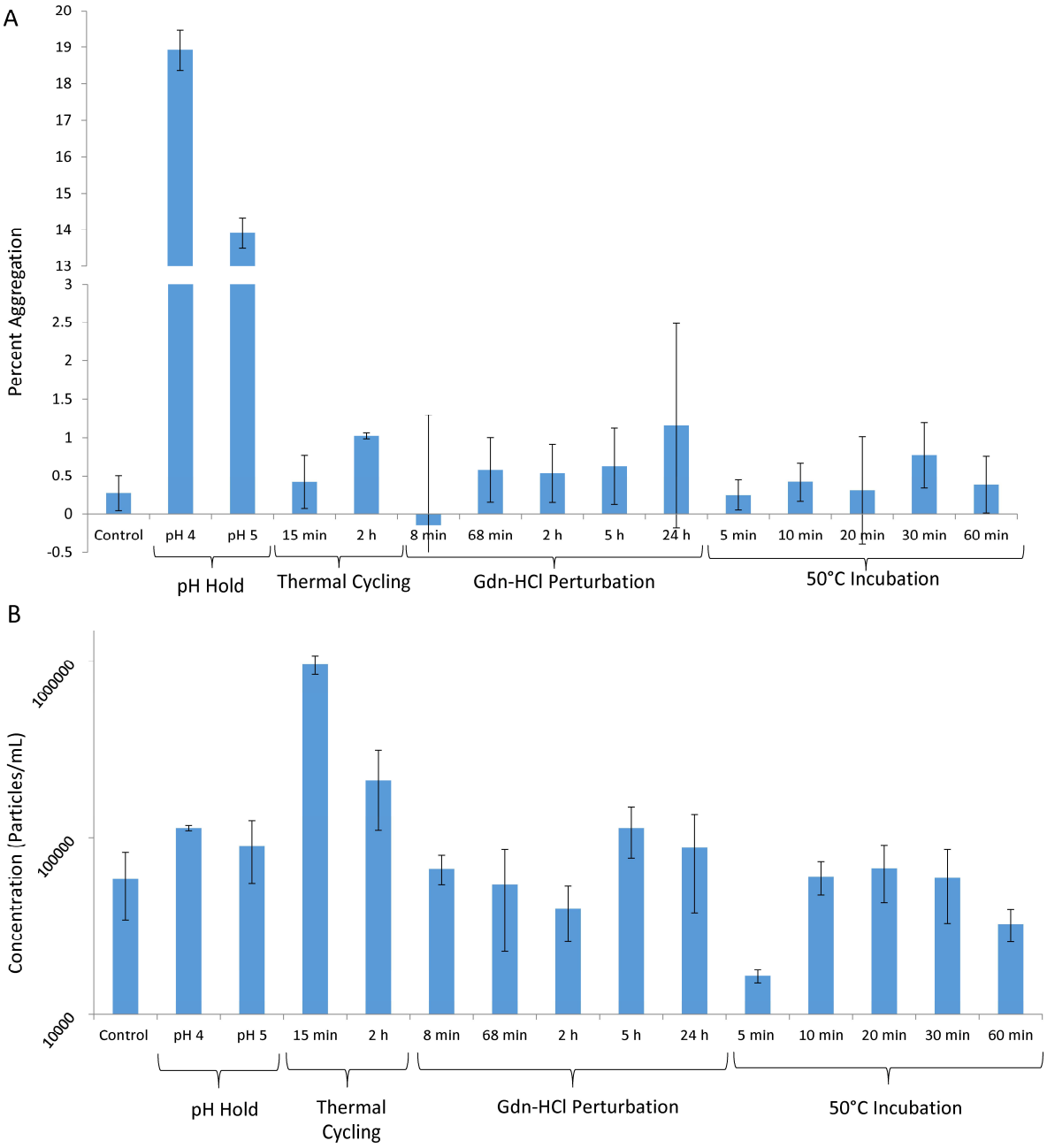
50°C Incubation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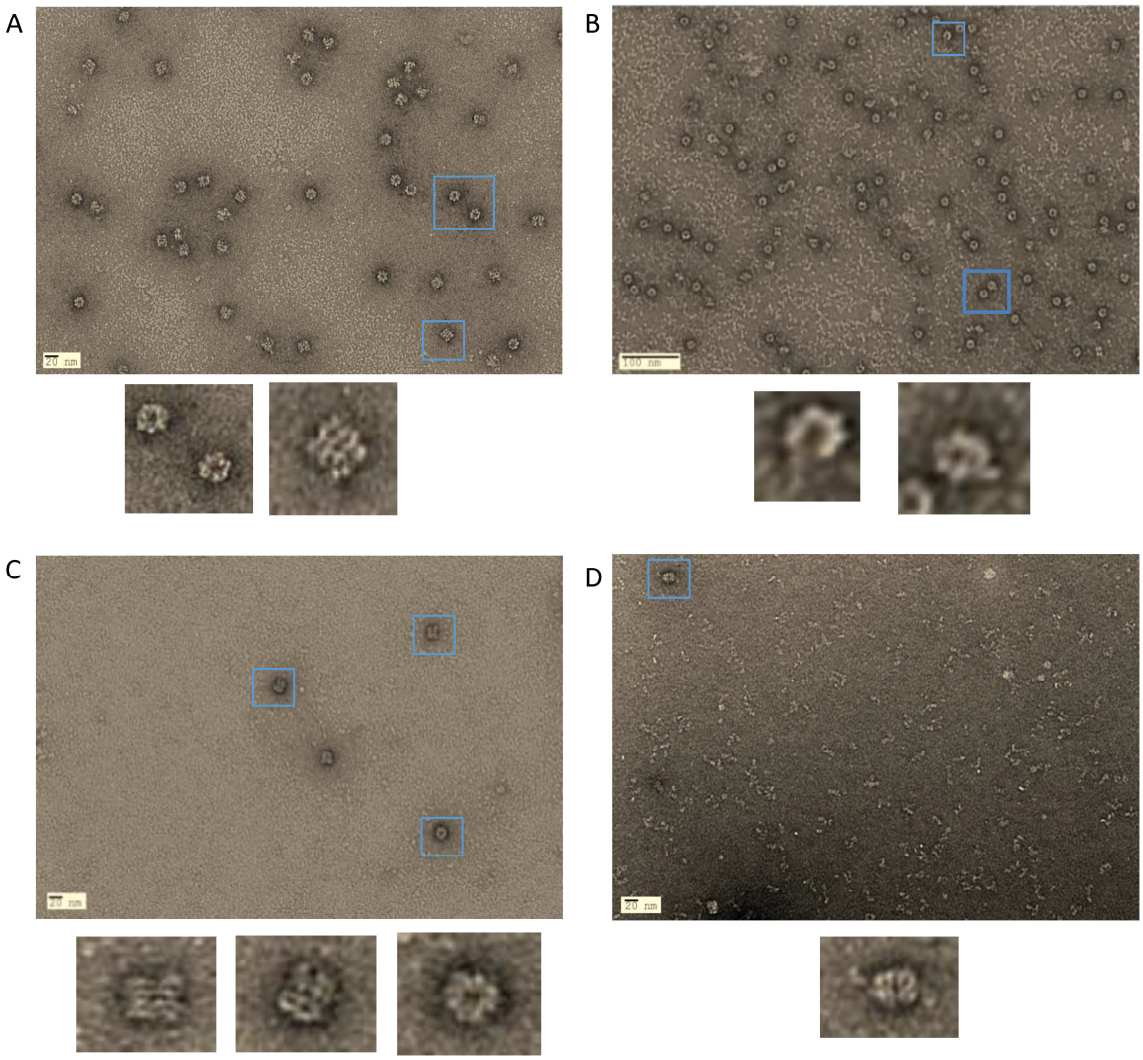

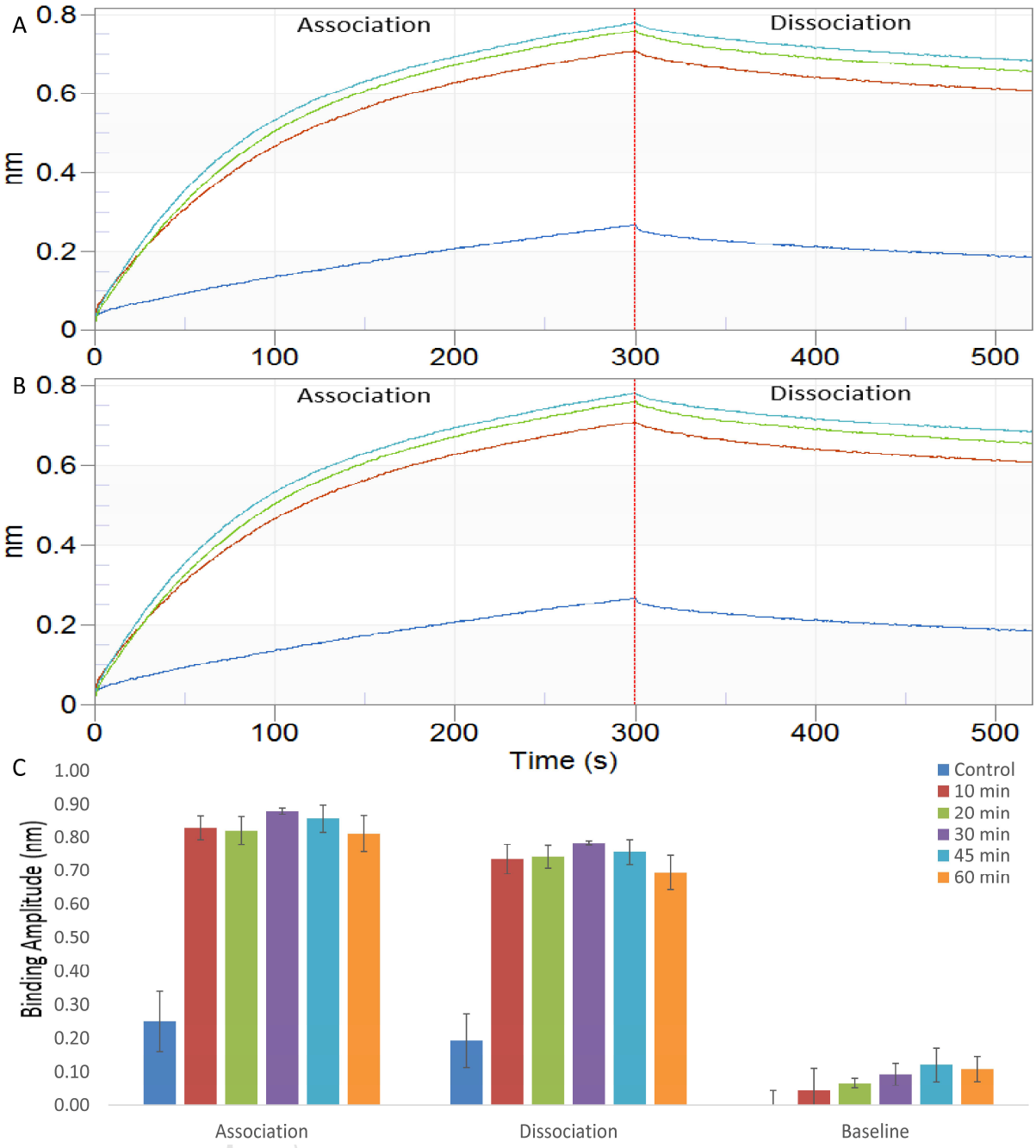

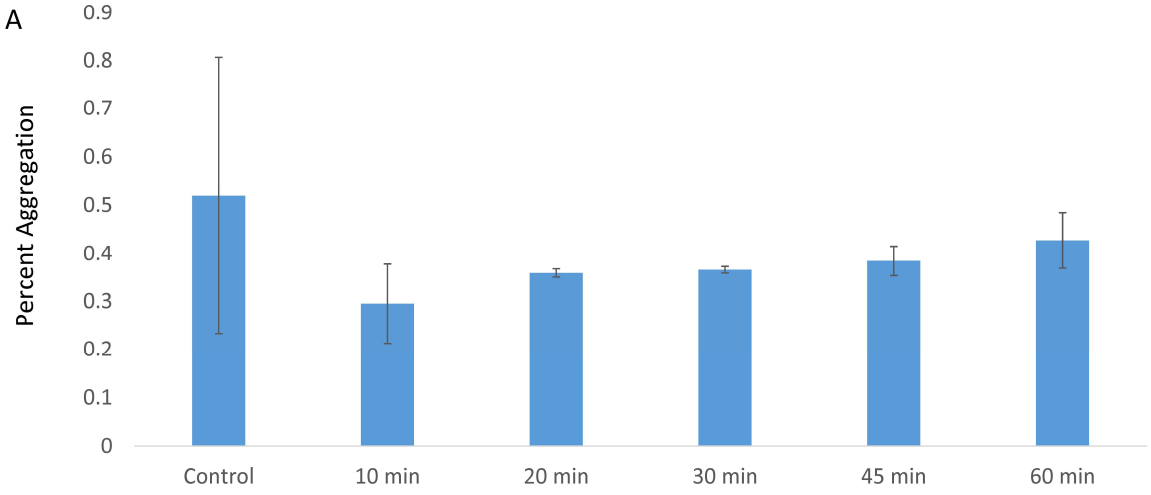
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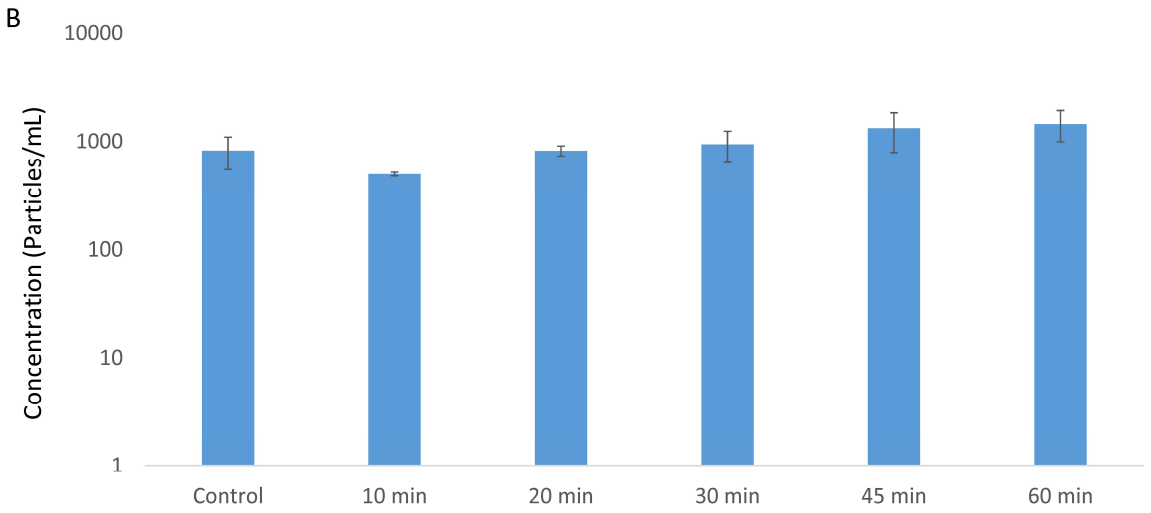
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