

Probing structurally altered and aggregated states of therapeutically relevant proteins using GroEL coupled to bio-layer interferometry

Subhashchandra Naik,¹ Ozan S. Kumru,² Melissa Cullom,²
Srivalli N. Telikepalli,² Elizabeth Lindboe,¹ Taylor L. Roop,²
Sangeeta B. Joshi,² Divya Amin,¹ Phillip Gao,³
C. Russell Middaugh,² David B. Volkin,^{2*} and Mark T. Fisher^{1*}

¹Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, Kansas 66160

²Department of Pharmaceutical Chemistry, Macromolecule and Vaccine Stabilization Center, University of Kansas, Lawrence, Kansas 66047

³Protein Production Group, Structural Biology Center, University of Kansas, Lawrence, Kansas 66047

Received 6 May 2014; Revised 8 July 2014; Accepted 10 July 2014

DOI: 10.1002/pro.2515

Published online 14 July 2014 proteinscience.org

Abstract: The ability of a GroEL-based bio-layer interferometry (BLI) assay to detect structurally altered and/or aggregated species of pharmaceutically relevant proteins is demonstrated. Assay development included optimizing biotinylated-GroEL immobilization to streptavidin biosensors, combined with biophysical and activity measurements showing native and biotinylated GroEL are both stable and active. First, acidic fibroblast growth factor (FGF-1) was incubated under conditions known to promote (40°C) and inhibit (heparin addition) molten globule formation. Heat exposed (40°C) FGF-1 exhibited binding to GroEL-biosensors, which was significantly diminished in the presence of heparin. Second, a polyclonal human IgG solution containing 6–8% non-native dimer showed an increase in higher molecular weight aggregates upon heating by size exclusion chromatography (SEC). The poly IgG solution displayed binding to GroEL-biosensors initially with progressively increased binding upon heating. Enriched preparations of the IgG dimers or monomers showed significant binding to GroEL-biosensors. Finally, a thermally treated IgG1 monoclonal antibody (mAb) solution also demonstrated increased GroEL-biosensor binding, but with different

Additional Supporting Information may be found in the online version of this article.

Subhashchandra Naik and Ozan S. Kumru contributed equally to the work.

Grant sponsor: NIH; Grant number: R01AI090085. Grant sponsor: National Center for Research Resources Grant; Grant number: 5P20RR017708-10. Grant sponsor: National Institute of General Medical Sciences Grant; Grant number: 8P20 GM103420-10. Grant sponsor: NIH Biotechnology Training Grant; Grant number: 5-T32-GM008359. Grant sponsor: KUIC proof of concept Grant, KUMC Lied Basic Science fund, KUMC Biomedical Training Grant.

*Correspondence to: David B. Volkin, Department of Pharmaceutical Chemistry, Macromolecule and Vaccine Stabilization Center, University of Kansas, Lawrence, KS 66047. E-mail: volkin@ku.edu or Mark T. Fisher, Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City KS, 66160. E-mail: mfisher1@kumc.edu

kinetics. The bound complexes could be partially to fully dissociated after ATP addition (i.e., specific GroEL binding) depending on the protein, environmental stress, and the assay's experimental conditions. Transmission electron microscopy (TEM) images of GroEL-mAb complexes, released from the biosensor, also confirmed interaction of bound complexes at the GroEL binding site with heat-stressed mAb. Results indicate that the GroEL-biosensor-BLI method can detect conformationally altered and/or early aggregation states of proteins, and may potentially be useful as a rapid, stability-indicating biosensor assay for monitoring the structural integrity and physical stability of therapeutic protein candidates.

Keywords: protein aggregation; molten globule; bio-layer interferometry; GroEL; chaperonin; monoclonal antibody; stability; formulation

Introduction

The successful development of pharmacologically active protein therapeutics has exhibited an ever-expanding role in improving human health over the past few decades. It is estimated that there are about 150 protein-based drugs on the market with sales exceeding \$100 billion per year.¹ Notable successes include the design and development of insulin formulations of varying durations, and more recently, the development and application of a wide variety of novel anticancer therapeutics including monoclonal antibodies (mAbs). There are currently over 30 mAb based drugs approved for commercial use with hundreds more in clinical trials.²

Acquiring and maintaining correctly folded proteins is fundamentally important for successful results in the pharmaceutical sciences. Within the discovery phase of pharmaceutical research, this goal is crucial during the testing of a new drug's ability to interact with protein based targets and as part of the design of therapeutic strategies to correct aberrant protein folding encountered in human disease.^{3,4} During pharmaceutical development, ensuring the structural integrity of therapeutic proteins during their manufacturing scale-up, long-term storage, and administration to patients is a critical goal of formulation development^{5–7} and comparability assessments.^{8,9} Protein instability issues can affect the success of a protein drug's clinical development due to insolubility, aggregate formation, and chemical degradation along with concomitant loss of potency and increased potential for enhanced immune reactions.^{10–12}

During the storage of protein therapeutics, especially with high concentration mAb formulations for subcutaneous administration,¹³ aggregate formation is a common degradation pathway and typically results in amorphous protein complexes that vary in size, morphology, and composition.¹⁴ The aggregation degradation pathway leading to the formation of these various multimers often is initiated through a common intermediate where the initial stages of aggregate formation starts with structural perturbation events leading to the formation of non-native dimers or related preaggregate species.⁶ The detection

of these preaggregate species, especially in the context of a formulated pharmaceutical dosage forms, is currently challenging and not easily accomplished with current analytical technology.^{15,16}

In this work, we demonstrate the analytical development of a novel chaperonin-based, biolayer interferometry (GroEL-BLI) assay system that has the potential to enable pharmaceutical scientists to detect structurally altered and/or early aggregate species of virtually any therapeutic protein candidate that transiently exposes hydrophobic surfaces. We used several pharmaceutically relevant proteins as models to demonstrate the ability of this GroEL-BLI biosensor methodology to rapidly detect preaggregate/aggregate formation in protein solutions during slightly elevated (40–45°C) to moderate (55°C) thermal stress.

Results

Preparing and testing biotinylated GroEL

Prior to preparing GroEL BLI biosensors, it was necessary to determine if biotinylation affected the structural integrity, stability, and activity of GroEL. To address these questions, we first characterized the physical stability of wild-type and biotinylated GroEL proteins as a function of pH (3–8) and temperature (10–87.5°C) with a variety of biophysical techniques (Fig. 1). We also characterized the refolding activity of the two GroEL proteins as a function of temperature exposure at one pH (Supporting Information Fig. S1).

In terms of the physical stability data, CD spectra of both proteins displayed double minimums at 208 and 222 nm [Fig. 1(A–B)], which is indicative of α -helical secondary structure, as expected from the previously published X-ray crystal structure of GroEL.¹⁷ The CD signal for both proteins decreases at acidic pH indicating a loss of α -helical content. Monitoring the ellipticity at 222 nm as a function of temperature indicated that both proteins lost negative ellipticity at $\sim 60^\circ\text{C}$ [Fig. 1(C,D)], which corresponds to thermal unfolding, at pH values of 4–8. At pH 3, however, no changes in ellipticity versus temperature were observed indicating both proteins

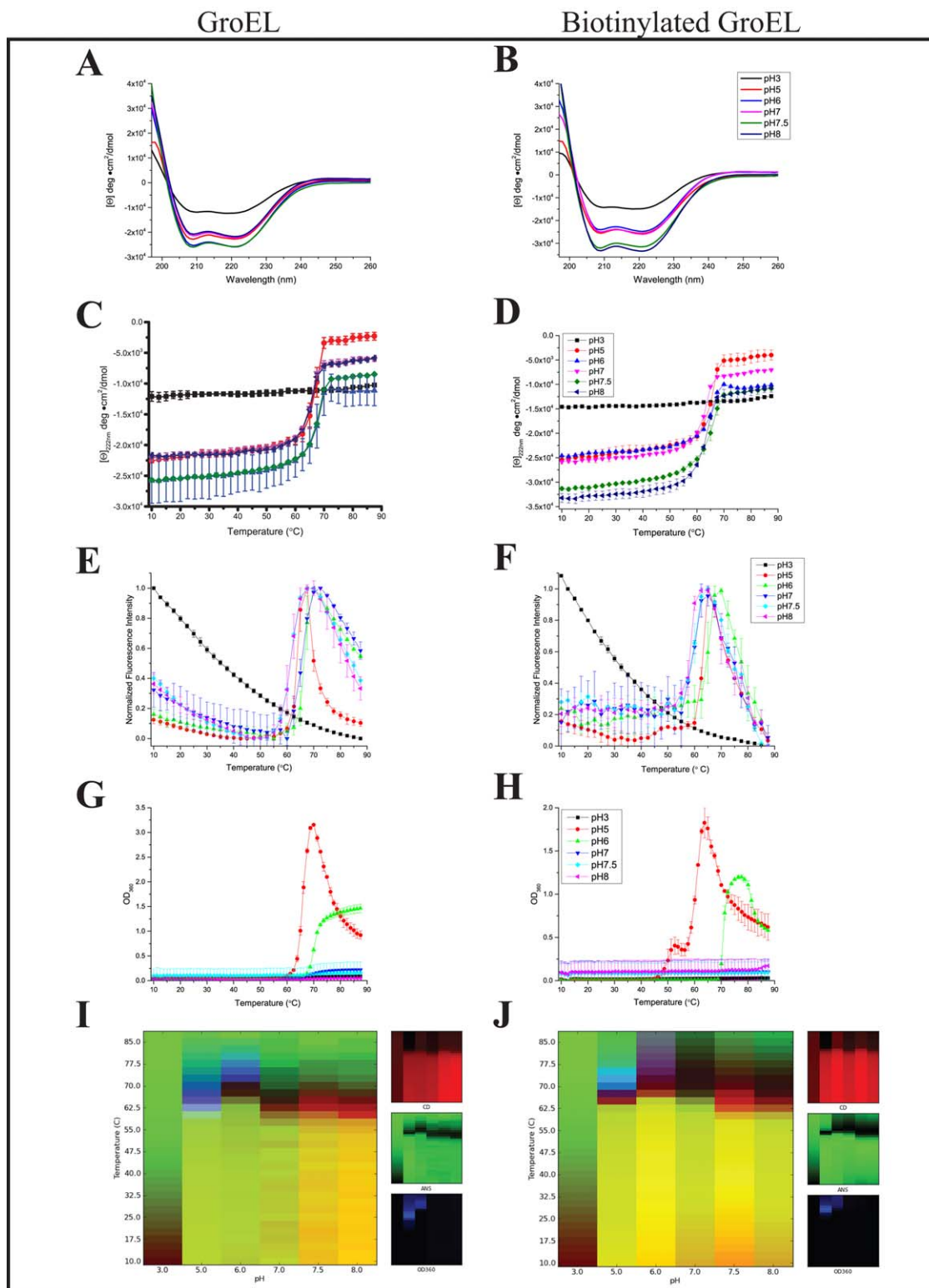


Figure 1. Biophysical characterization of stability profiles of GroEL and biotinylated GroEL as a function of temperature and pH. **(A, B)** Circular dichroism spectra at 10°C indicate an overall α -helical secondary structure. **(C, D)** When the CD signal is monitored at 222 nm as a function of temperature, the major loss in negative ellipticity initiates at $\sim 60^\circ\text{C}$. **(E, F)** The major increase in normalized ANS fluorescence intensity is observed at $\sim 55^\circ\text{C}$, with the exception of pH 3, which decreases as a function of temperature. **(G, H)** There were no measureable changes in turbidity as a function of temperature with the exception of pH 5 and 6. **(I, J)** A three-index EPDs were generated that incorporated biophysical stability data from CD at 222 nm, ANS fluorescence, turbidity measurements as a function of pH and temperature. The EPDs appear similar indicating similar physical stability profiles. All measurements are an average of duplicate experiments and the error bars representing the data range. Buffer baselines were subtracted from all measurements.

were structurally perturbed at low pH. Perturbations in tertiary structure (i.e., surface hydrophobicity) were assessed using the extrinsic fluorescent dye ANS. Normalized ANS fluorescence intensity increased drastically at about 55–60°C in the presence of both GroEL and biotinylated GroEL [Fig. 1(E,F)] at pH values of 4–8. At pH 3, both proteins were structurally perturbed even at low temperature. The aggregation behavior of the two proteins was monitored via optical density (360 nm) as a function of temperature and pH. At pH 5 and 6, both proteins formed aggregates as a function of temperature [Fig. 1(G–H)], with no notable aggregation at more basic (pH 7 and 8) and acidic (pH 3) solution conditions, consistent with the known pI value of GroEL (pI = 4.7). It should be noted that both GroEL and biotinylated GroEL completely precipitated when exchanged into buffers at pH 4. Using these biophysical stability data sets, we constructed two EPDs for the wild-type and biotinylated GroEL proteins [Fig. 1(I–J)], as described in detail elsewhere,¹⁸ to summarize and visualize their physical stability profile. The two EPDs appeared similar in terms of the pH and temperature conditions demonstrating that biotinylation does not significantly affect the physical stability profile of GroEL. The EPDs contained three phases representing the native conformation from pH 5 to 8 and 10–55°C, a molten globule species from pH 5 to 8 and 60–70°C, and an unfolded/aggregated species at all temperatures at pH 3 and pH range of 5–8 at temperatures >70°C.

We then compared the partitioning and refolding efficiency of the two GroEL proteins using a green fluorescence protein (GFP) refolding assay. In this assay, acid denatured GFP is allowed to partition onto GroEL at neutral pH, which prevents GFP from refolding and regaining its inherent fluorescence. Furthermore, upon addition of ATP, functional GroEL releases GFP resulting in refolding and a corresponding increase in native fluorescence. As shown in Supporting Information Fig. S1, it was observed that both the wild-type and the biotinylated GroEL showed similar acid denatured GFP capture/partitioning efficiencies and ATP induced GFP refolding profiles when GroEL was subjected to temperatures ranging from 20 to 65°C. When GroEL was incubated at 80°C (prior to returning to ambient temperature for performing the GFP refolding assay), both wild-type and biotinylated GroEL samples were unable to bind unfolded GFP. In these instances, the GFP refolding preceded as if no GroEL was present. The near identical capture/refolding efficiencies of biotinylated and native GroEL indicated that biotinylation did not affect GroEL activity. In addition, there is also a large amount of direct experimental evidence, both from this lab and others, that demonstrates that

immobilization of GroEL onto surfaces, both through specifically engineered sites (known biotin attachment site) and random coupling (amine coupling or antibody capture) results in functionally competent immobilized GroEL.^{19–23}

Optimizing loading of streptavidin biosensor tips with biotinylated GroEL

The interaction of partially unfolded protein substrates with GroEL can be followed using Biolayer Interferometry (BLI) via two different methodologies. First, one can immobilize the test substrate proteins onto the biosensor tip and allow them to interact with wild-type GroEL in solution. This method would be preferred when the test substrate proteins are highly aggregation prone leading to rapid loss in solution. Alternatively, if the test substrate proteins display sufficient physical stability (i.e., substrate proteins are in dynamic equilibrium with partially folded states that are relatively stable and soluble), one can immobilize the biotinylated GroEL onto the BLI streptavidin biosensor tips and allow the immobilized GroEL to interact and capture transient hydrophobic species of the test substrate protein as they appear in solution. This second approach was selected as the preferred method for this work since the goal was to test the stability of pharmaceutically relevant proteins during storage under accelerated stress conditions.

Although biotinylated GroEL easily immobilizes onto the commercially available BLI streptavidin tips, the amount loaded onto the tips had to be optimized to minimize nonspecific binding of unrelated proteins. For example, solutions of varying concentrations of biotinylated GroEL (from 0.005 to 1 mg/mL) were used for immobilization onto the streptavidin BLI biosensors. These biosensor tips were tested for nonspecific binding using a constant 1 mg/mL concentration of bovine serum albumin (BSA). In the absence of GroEL, BSA readily binds to the streptavidin BLI biosensors. Thus, the observed BSA binding is assumed to represent nonspecific interactions with the streptavidin coated BLI tip surface (since the BSA is not biotinylated). As shown in Figure 2, we observed that BSA binding (nonspecific binding to tips, right Y-axis) decreased with increasing biotinylated GroEL immobilization (specific binding to streptavidin; left Y-axis). In addition, BSA binding to streptavidin tips did not show characteristic dissociation from the GroEL-biosensors when the biosensor tip was moved to buffer alone (data not shown), consistent with the known inability of BSA to bind to GroEL.^{24–26}

The observed decrease in nonspecific BSA binding with respect to increasing GroEL loading concentration occurs presumably since bound biotinylated GroEL diminishes the exposed streptavidin-tip surface area. At concentrations larger than 1 mg/mL

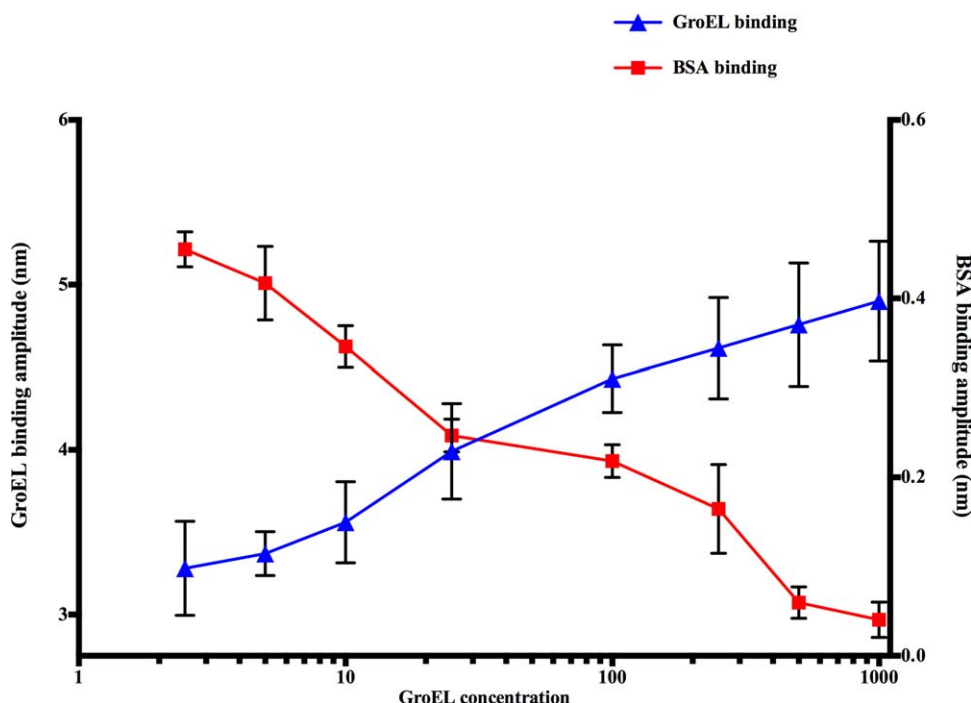


Figure 2. Optimizing biotinylated GroEL loading onto streptavidin biosensors. GroEL loading onto the streptavidin biosensor tips was optimized to decrease nonspecific binding to the tip surfaces. Different concentrations of biotinylated GroEL ranging from 5 to 1000 $\mu\text{g/mL}$ were loaded onto streptavidin tips and the binding amplitudes from nonspecific binding of a fixed concentration of BSA (1 mg/mL) to the GroEL laden tips was recorded. The amount of non-specific binding decreases with increasing concentration of GroEL binding to the tips.

biotinylated GroEL, BSA binding was substantially diminished (BLI signal < 0.01 nm). The nonspecific nature of this BSA binding to the sub-optimally loaded biotinylated GroEL tips (Fig. 2) was confirmed by subsequent incubation with an ATP containing solution where no accelerated decrease in the BLI signal was observed (data not shown), as would be expected for GroEL mediated binding (i.e., ATP binding to GroEL leads to protein substrate release.^{27,28} Thus, to minimize nonspecific binding of protein substrates (i.e., preaggregate or aggregate species) to the biosensors, subsequent studies were carried out with streptavidin tips optimally loaded with biotinylated GroEL concentrations ≥ 1 mg/mL. In addition, the extent of specific versus nonspecific binding to GroEL was evaluated by incubating the captured species on the GroEL biosensor tip with ATP containing solutions and monitoring the rate and extent of substrate dissociation as described below.

Detecting conformationally altered and heparin stabilized FGF-1 using a GroEL-BLI biosensor

FGF-1 is small, heparin binding growth factor (MW ~ 16 kDa) that has been evaluated as a possible therapeutic agent for wound healing and the treatment of ischemic diseases.²⁹ FGF-1 is an inherently conformationally unstable protein (thermal unfolding temperature, T_m of ~ 30 – 40°C as measured by different biophysical methods) and is dramatically structurally stabilized upon addition of heparin and

related sulfated polyanions ($T_m > 60^\circ\text{C}$).^{30,31} We took advantage of this well characterized system to evaluate the GroEL-BLI binding response to structurally altered protein substrates. We hypothesized that since FGF-1 exists as a molten globule in the absence of heparin,³² under ambient solution conditions, greater binding amplitudes would be observed. On the other hand, in the presence of heparin, these binding amplitudes should be significantly diminished. The experimental setup is illustrated in Figure 3(A) and includes loading of biotinylated GroEL onto the streptavidin biosensor tips, incubation with FGF solutions, buffer washing and ATP pulse stages (see Methods section).

We tested FGF-1 that was incubated either at ambient temperature or at 40°C for 5 min both with and without heparin [Fig. 3(B)], and observed a significantly diminished binding of FGF-1 to the GroEL loaded biosensor in the presence of heparin. In addition, greater binding amplitudes were observed after incubation at 40°C than at ambient temperature, but only in the absence of heparin [Fig. 3(C)]. Upon addition of ATP/osmolyte solution, there was an initial increase in binding amplitude reflecting a change in the solution refractive index, followed by a gradual decrease in the BLI signal that is most apparent in the absence of heparin. After the final buffer rinse, the binding amplitude was reduced by $\sim 80\%$ from the end of the association phase (Fig. 3, Step 6). As an additional control, we tested the effect

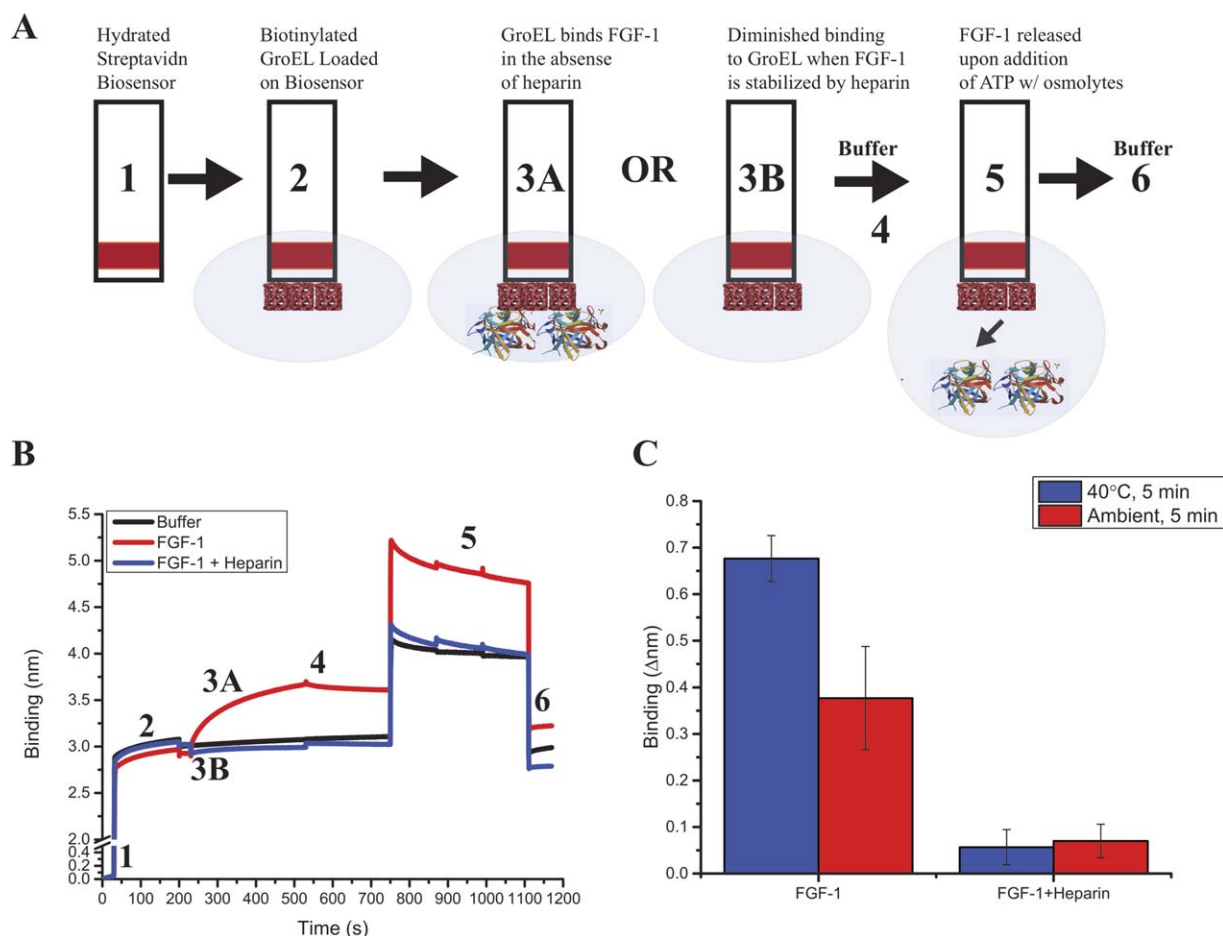


Figure 3. Assessment of FGF-1 stability in the presence and absence of heparin by the GroEL-BLI biosensor assay. (A) The experimental steps of a typical BLI experiment are shown. The step numbers correspond with the step numbers shown on the BLI sensograms in (B). (B) A representative BLI sensogram of FGF-1, with and without heating at 40°C for 5 min, followed by assessment by GroEL-BLI assay. Note, the diminished binding in presence of 3:1 (w/w) ratio of heparin:FGF-1. (C) The normalized binding amplitudes of FGF-1 to GroEL after the association phase was quantified. The bar chart is an average of three independent experiments with the error bars representing the standard deviation.

of heparin on the substrate binding and release of GFP protein to GroEL and found heparin had no effect (Supporting Information Fig. S2). Taken together, these results show that although the GroEL-BLI method is able to specifically detect conformationally altered states of FGF-1, some of the bound protein substrate to the GroEL biosensor is not readily released by our standard ATP incubation procedure. Possible reasons for this observation include tight binding of the protein substrate species to GroEL or some nonspecific interactions with the biosensor (see Discussion section).

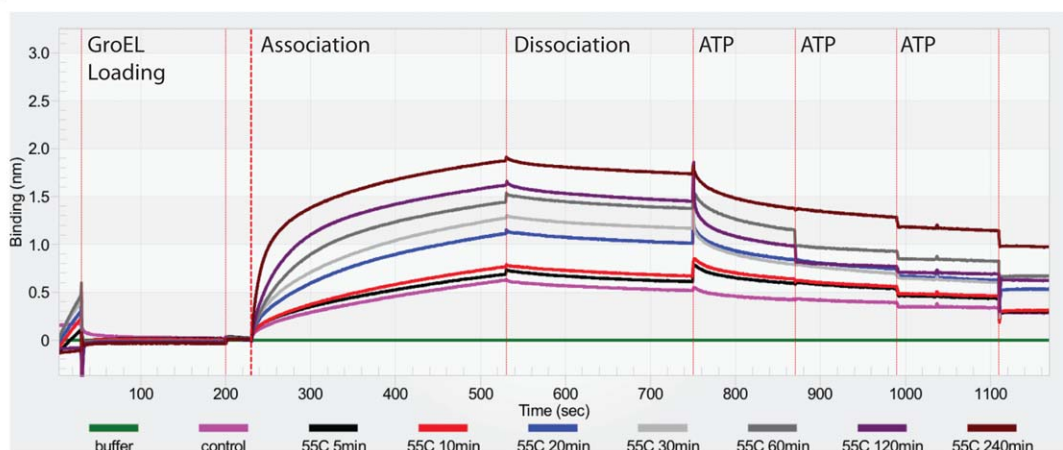
Correlation of GroEL-BLI binding with SEC using a human polyclonal IgG subjected to thermal stress

To determine the levels of preaggregate and/or aggregate species formed in a stressed protein solution, and to compare results of the GroEL-BLI method with a commonly used analytical method for monitoring protein aggregate formation, we analyzed

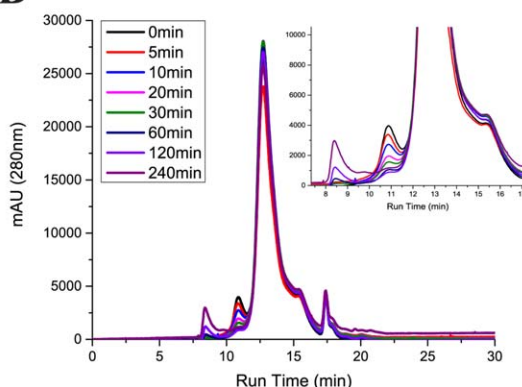
a polyclonal IgG preparation by a combination of size exclusion chromatography (SEC) and GroEL-BLI. We also heated the poly-IgG at 55°C over a 4 h time course, and analyzed the levels of soluble aggregates by SEC and the BLI binding amplitudes.

When the poly-IgG samples were analyzed by analytical SEC, 6–8% dimer content was observed at time zero [Fig. 4(B)]. This result is consistent with the known presence of ~10–20% dimers in polyclonal human IgG preparations due to the presence of idiotype/anti-idiotypic antibody complexes,³³ although it is possible some of the dimer species were formed due to intermolecular disulfide interchange reactions. Upon heating at 55°C, there was a gradual disappearance of this dimeric species. After 60 min of heating at pH 6.0, a larger “multimeric” species begins to accumulate with increasing levels noted over time by SEC [Fig. 4(B)]. There was no loss of total peak area measured by SEC indicating no formation of insoluble aggregates over the time course of this study (data not shown).

A



B



C

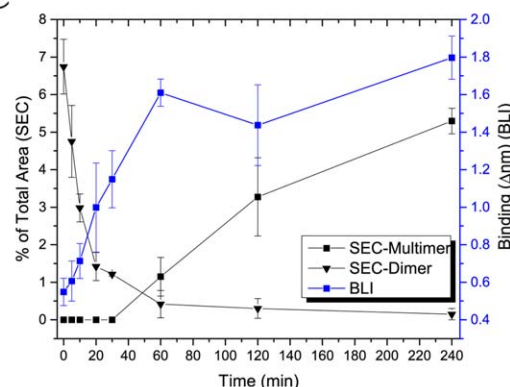


Figure 4. Correlation of GroEL-BLI assay and SEC readouts upon heating of a polyclonal human-IgG solution. (A) Representative sensograms of poly-IgG solution (0.5 mg/mL, pH 6) that was subjected to heating at 55°C over a 4 h time course. Sensograms were corrected for buffer contributions and aligned to the association phase. (B) Representative SEC chromatograms of the same poly-IgG solution heated over a 4 h time course. (C) Comparison of GroEL-BLI binding amplitudes and integrated SEC dimer and multimer areas as a function of heating time at 55°C. Data are an average of three independent experiments with the error bars representing the standard deviation.

The BLI sensorgrams indicate increased binding amplitude as a function of heating time as shown in Figure 4(A). At the end of the association phase, a reading of ~0.6 nm binding amplitude was observed even when no heat was applied to the sample [Fig. 4(A)]. This observed binding at time zero suggests that there is structurally altered or non-native dimeric species present in the starting preparation (as observed by SEC analysis; see above). Upon heat treatment, additional increases in binding amplitude were recorded indicating formation of additional non-native species due to heat treatment. Upon the addition of ATP, a partial reduction in binding was observed, indicating release of the poly-IgG species from GroEL at each time point, along with some species not readily released (perhaps due to either tight binding of the protein substrate species to GroEL or some nonspecific interactions with the biosensor tip because of the formation of highly aggregated species upon prolonged heating; see Discussion section).

A comparative summary of the SEC and BLI results are shown in Figure 4(C). The BLI binding signal was observed to be elevated at time zero and further increased upon heating with the signal leveling off after about 60 min of heating. Concomitantly, as monitored by SEC, the IgG dimer decreased from ~8% to less than 1% over the first 60 min of heating followed by the appearance of the larger molecular weight multimeric species. Taken together, these data demonstrate the GroEL-BLI assay showed changes in the poly-IgG binding profile to the biosensor concurrently with changes in the levels of soluble dimeric and multimeric aggregate species as measured by SEC.

To better understand the nature of the interaction of the different polyclonal IgG species with the GroEL biosensor, the polyclonal IgG preparation was further purified using preparative FPLC by collecting monomer and dimer-enriched aliquots as described in the Methods section. The enriched monomer gave a predominant single peak when

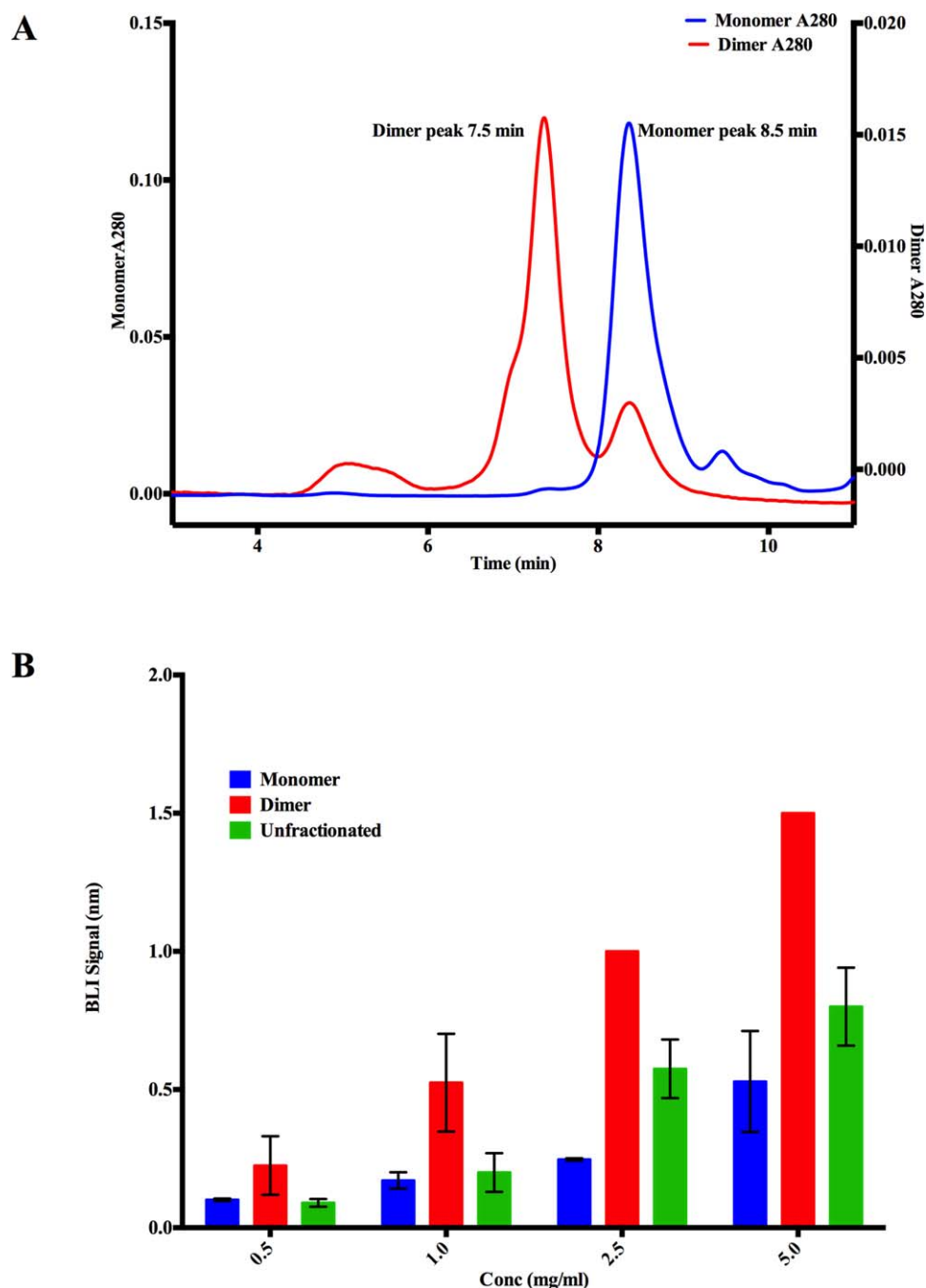


Figure 5. Comparing binding amplitudes of enriched polyclonal human antibody dimer and monomer species to biotinylated GroEL tips. (A) The polyclonal antibody preparation was separated using preparative size exclusion FPLC to obtain monomer-enriched samples. The dimer species from another polyclonal antibody aliquot was enriched by mild heating and prolonged incubation and similarly isolated by preparative SE-FPLC to obtain dimer-enriched fractions. The binding of these enriched fractions to GroEL (loaded at 2.5 mg/mL concentration) streptavidin biosensors was measured at room temperature (25°C). (B) At low concentrations, the binding of the monomer-enriched fraction was observed to be similar to that of the unfractionated sample (which is predominantly monomer). Enriched-dimer fraction was observed to bind with higher binding amplitudes than the enriched monomer fractions. Due to limitations in isolating and obtaining higher concentrations of the enriched dimer species, the 2.5 and 5 mg/mL studies were performed once.

analyzed by SEC [Fig. 5(A), blue trace]. The enriched dimer peak showed mostly dimer with the presence of some monomer and multimers [Fig. 5(A), red trace]. The unfractionated polyclonal sample, along with isolated monomer and dimer-enriched

fractions from preparative SEC, were then incubated with biotinylated GroEL-streptavidin biosensors at various concentrations [Fig. 5(B)]. The three samples showed differential binding to GroEL biosensor in a concentration dependent manner [Fig. 5(B)].

The concentration dependent binding of the monomer-enriched fraction was observed to be overall similar to that of the unfractionated polyclonal antibodies at lower concentrations, with lower binding amplitudes at the higher protein concentrations (as may be expected since the unfractionated material contains higher percentage of dimer). On the other hand, it was observed that the enriched dimers displayed higher binding amplitudes to the GroEL-BLI biosensor. The increase in BLI amplitude from dimer binding is most likely due to the larger mass of the dimers. In addition, the dimers may have more hydrophobic surface area that can interact with GroEL at time zero compared to monomers. Multimers observed in the enriched dimer preparation were further isolated and examined for their ability to bind the GroEL biosensor; and as shown in Supporting Information Figure S4, the multimers do not show any notable binding to the biotinylated GroEL. At lower stress temperatures (42°C), the polyclonal IgG binding signal shows a complete reversible dissociation with ATP addition and return to the original baseline (Supporting Information Fig. S5). As controls, nonspecific polyclonal IgG binding to streptavidin tips was not reversed upon ATP addition. Furthermore, the signal amplitudes using tips with streptavidin alone were substantially higher than the binding amplitudes with the GroEL biosensors for both unstressed and low heat stressed polyclonal IgG biosamples. Because of the inherent heterogeneity of the polyclonal antibody preparation, it is difficult to distinguish between these species in terms of describing the interaction of specific IgG species with the GroEL. Therefore, subsequent experiments were performed with a more highly purified monoclonal IgG1 antibody as described in the following section.

Analysis of an IgG1 mAb solution subjected to mild heat stress

Similar GroEL-biosensor binding studies along with SEC analysis were performed using an IgG1 mAb containing lower levels of non-native dimer (~2% by analytical SEC). It was observed that at the end of the association phase, there was some initial binding [Fig. 6(A), purple trace] at room temperature. This binding amplitude (~0.3 nm for mAb) was lower as compared to the binding amplitude observed with a similar concentration of the polyclonal IgG that was not subjected to heat stress [~0.6 nm for polyclonal; Fig. 4(A)], consistent with the lower dimer content observed by SEC. We also observed that the buffer wash resulted in decreased BLI binding amplitude followed by a more rapid return to baseline with the ATP wash [Fig. 6(A)]. These results indicate the presence of a species that predominantly binds GroEL, which may be accompanied by some amount of weaker or nonspecific binding under these conditions.

To determine the effect of mild temperature exposure, the IgG1 mAb solution at 2.5 mg/mL was incubated in GroEL buffer with 150 mM NaCl, pH 7.5 at 42°C for 5 and 15 min. These samples were then equilibrated back to room temperature and allowed to interact with the biotinylated GroEL BLI biosensors. These heat-incubated mAb samples showed progressively higher binding amplitudes [Fig. 6(A), red and green traces] indicating potential higher instability within the heat-treated samples. An aliquot of the same mAb samples was also loaded onto an SEC column and the area under the curve (AUC) of the monomer, dimer, multimer, and fragment species was measured [Fig. 6(B)]. Upon heating, small changes in the dimer and multimer contents were noted by SEC, with slight increases in the multimer peak. However, the multimer levels were low and probably below the limit of quantitation for a typical SEC experiment to monitor protein aggregation.³⁴

To further characterize the nature of the interaction between the IgG mAb and GroEL, a reversible biotinylated GroEL biosensor was developed to release the GroEL-mAb complexes that were formed upon binding the heat-treated mAb to the GroEL-BLI biosensor. For this reversible reaction, the GroEL was biotinylated using LC-biotin with a cleavable S-S bond (Methods section). The S-S biotin GroEL was tested for binding and partitioning studies with GFP as a test substrate, and like the biotinylated GroEL alone, this modified GroEL species was also found to be fully functional with respect to substrate capture and release (Supporting Information Fig. S2). The S-S-biotin GroEL was loaded onto streptavidin BLI tips and then dipped into a 2.5 mg/mL mAb solution incubated at 42°C for 15 min (and then allowed to equilibrate to room temperature). A control experiment was also performed in which the S-S-biotin GroEL streptavidin tips were dipped into buffer alone. Following incubation with the heated mAb solution, the tips were washed with buffer to remove nonspecifically bound mAbs, and the bound mAbs were eluted from the tip using DTT to reduce the S-S- biotin linkage (see Methods section). As an additional control, similar treatments of the mAb with the DTT reducing agent did not show any extensive changes in monomer, dimer or fragment populations as measured by SEC (see Supporting Information Fig. S3).

The GroEL samples were then analyzed by TEM as shown in Figure 7. For the biotinylated GroEL alone sample, both top (open barrel) and side views of GroEL protein were observed [Fig. 7(A)]. For the GroEL incubated with the heated mAb, distinct protein particles bound to the top of the GroEL barrel, where the GroEL binding site is located, are evident [Fig. 7(B)]. The TEM views preferentially show primarily side views of the GroEL when

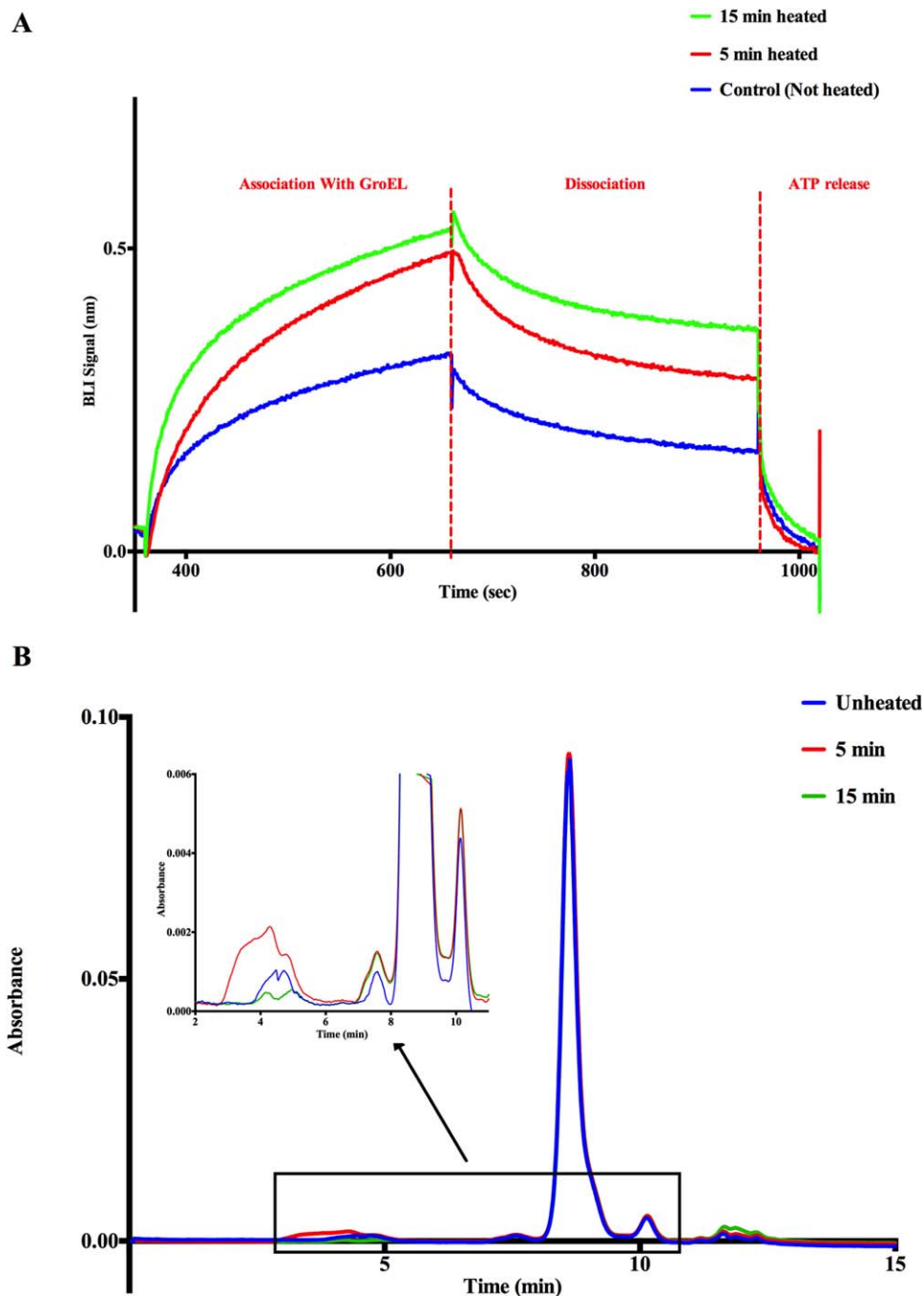


Figure 6. Effect of heating on an IgG1 monoclonal antibody as measured by GroEL-BLI assay and SEC. The IgG1 mAb solution (2.5 mg/mL in GroEL buffer, 150 mM NaCl, pH 7.5) was heated to 42°C for 5 and 15 min, allowed to equilibrate to room temperature and then incubated with biotinylated-GroEL streptavidin biosensors. (A) mAb binding to biosensor immobilized GroEL was measured and binding amplitudes were observed to increase with heating time including a fraction of the mAb binds to GroEL without heat treatment. The rate of release of samples from GroEL biosensor tip shows a dramatic increase when ATP is added, indicative of specific binding to GroEL. (B) The SEC profile, including expansion of aggregation peaks in the SEC profile, showed an increase between 5 and 15 min (~0.1% increase in AUC). When the BLI signal was compared to peak area changes in SEC monomer and dimer contributions, BLI signals show substantial increases with time compared with the corresponding area changes in monomer or dimer peaks by SEC.

complexed with heated protein, with the bound particles projecting from the GroEL binding cavity. These electron micrographs provide a visual conformation of GroEL binding to a hydrophobic species from the heated mAb solution.

Discussion

In the cell, macromolecular crowding can result in protein concentration levels that approach 300 mg/mL.³⁵ Molecular chaperones are constantly involved in preventing *in vivo* intracellular aggregation

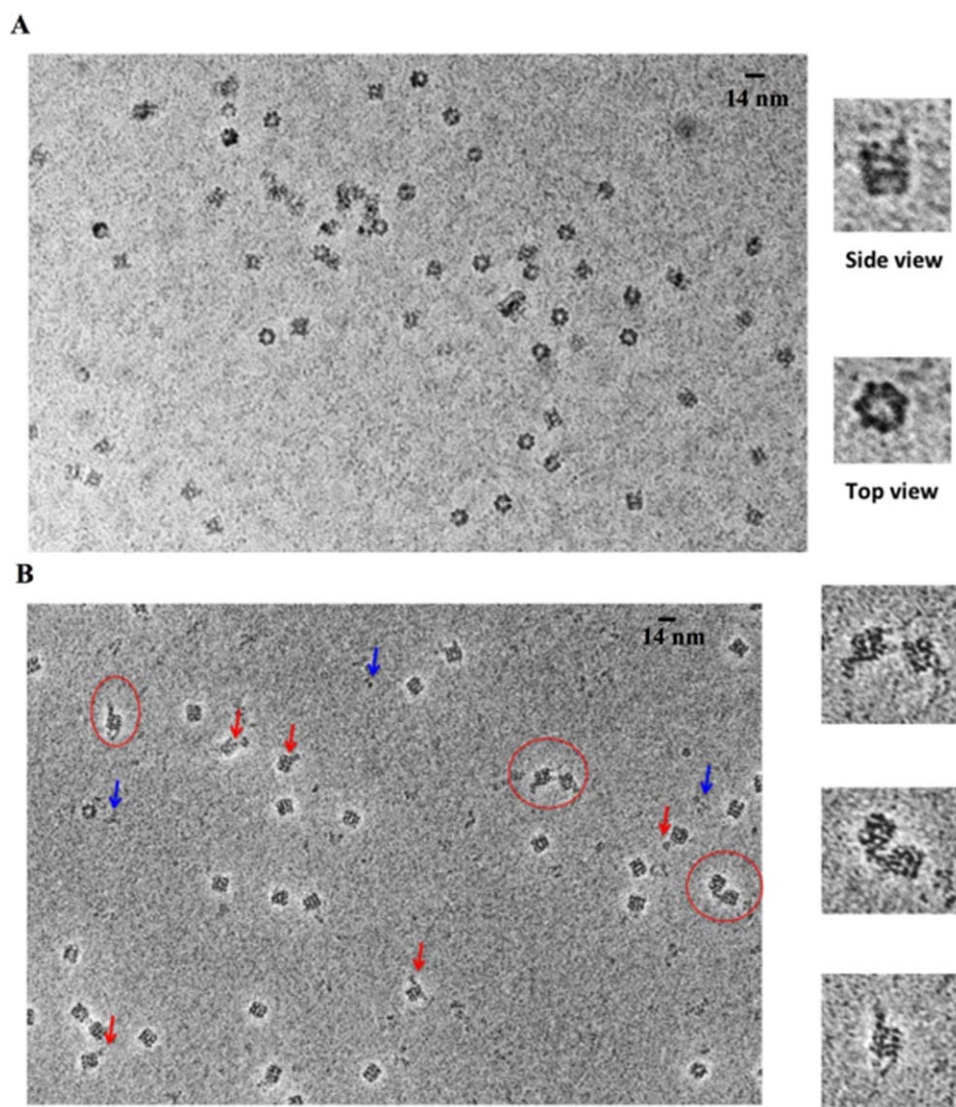


Figure 7. TEM micrographs of heated treated IgG1 mAb bound to GroEL biosensor tips. Solutions (GroEL buffer, 150 mM NaCl pH 7.5) without (A) and with (B) 2.5 mg/mL of IgG1 mAb were both heated at 42°C for 15 min, incubated in the presence of streptavidin biosensors coupled with reversible S-S-biotinylated GroEL. Following association phase, samples were exposed to a dissociation phase with GroEL buffer, and the biosensor tip was then immersed into a buffer containing 50 mM DTT to covalently release GroEL and GroEL-mAb complexes from the biosensor tip (Methods section). The GroEL complexes were wicked onto TEM grids, stained with uranyl acetate and observed under TEM. GroEL incubated with buffer alone show the characteristic top view of GroEL molecule along with a few side views (A, right panels). In contrast, GroEL incubated with heat treated mAb solution have predominantly side views and show GroEL bound to species that might potentially be mAb preaggregate species (B, red arrows and right panels). The micrographs also shows the presence of some free mAb molecules (B, Blue arrows), potentially due to some dissociation of the mAb from GroEL during the dissociation phase.

within this crowded environment. In a pharmaceutical setting, development of subcutaneously administered protein formulations often requires high concentrations (up to 200 mg/mL) that also creates a crowded environment, but this time within a primary container (e.g., vials, cartridges, prefilled syringes) used for storage and administration.³⁶ In this case, the problem that needs to be addressed involves the prevention of deleterious protein reversible self-association interactions as well as irreversible aggregation when formulated at high

concentrations. Minimizing and preventing protein aggregation is particularly important to eliminate possible immune side-reactions, ensure adequate dosing and delivery of the drug, and increase the shelf life of therapeutic protein drug candidates.

Although protein aggregation can be caused by a combination of conformational instability (i.e., exposure of interior apolar residues) and colloidal interactions (i.e., tendency of protein molecules to interact in solution), protein aggregation pathways typically begin with the formation of transient

exposure of hydrophobic surfaces, arising predominantly through the misfolding of individual protein molecules.³⁷ From a formulation development perspective, it is highly desirable to detect the formation of these initial misfolded preaggregates (i.e., conformationally altered monomers) along with the small aggregation prone oligomers (e.g., conformationally altered dimers).³⁷ Thus, the successful development of analytical approaches to detect these initial misfolded species is a major hurdle to the better prediction of protein aggregation rates.⁶ Currently, self-interaction chromatography is potentially a very specific method for detecting preaggregate species. For example, one recent report shows that this type of detection can be achieved using biolayer interferometry and attached monoclonal antibody platforms.³⁸ Although this method may be useful in detecting self-associating antibodies, one is still unable to determine the exact molecular alterations that result in an interacting species (e.g., which region of an IgG mAb interacts or undergoes conformational alterations). Furthermore, one must always be aware of the possibility of experimental complexities (e.g., the attachment procedure to the biolayer tips may compromise the conformation and/or orientation of the immobilized mAb).

In this work we demonstrated that the chaperonin GroEL, in concert with Biolayer interferometry, is a potentially useful detection system that can capture the preaggregate and/or the initial aggregate populations defined by partially folded protein monomers and dimers in formulations of therapeutically relevant proteins (provided that hydrophobic surfaces are stably or transiently exposed). This represents a new analytical tool to monitor formation of conformationally altered monomers and/or early aggregate species (e.g., dimers in this work). Since the BLI detection system depends on changes in protein thickness on an immobilized GroEL biosensor biolayer, changes at this interface due to the capture of partially unfolded IgG monomers and dimers were readily observed. This interaction is certainly not unprecedented in the chaperonin field since chemically unfolded monoclonal IgG Fab domains and GroEL were first demonstrated to form complexes by Schmidt and Buchner over 20 years ago.³⁹ Our work extends this previous observation and shows that the dip and read format of a GroEL chaperonin-dependent Biolayer interferometry biosensor system can detect either partially folded mAb monomers or initial dimeric aggregates from polyclonal IgGs induced by various stress conditions. Curiously, initial evaluation of multimeric sized fractions from polyclonal IgGs do not seem to be able to bind to GroEL (Supporting Information Fig. S4). This observation supports the notion that conformationally altered dimeric and/or monomeric sized IgG1 species are primary interacting with GroEL. As part

of our future work, we plan to evaluate the ability of isolated and purified Fc and Fab preparations to bind GroEL biosensors to better understand the nature of the interaction of IgGs with GroEL under pharmaceutical conditions (also see below).

In terms of broad binding capabilities, GroEL is perhaps the optimal chaperone that can be used to detect the appearance of partial exposure of apolar regions (partial unfolding) since the nucleotide free form of the GroEL chaperone has the highest affinity for hydrophobic partially folded proteins. Specifically, the GroEL chaperonin oligomer contains a very large hydrophobic binding site that rings the opening of the large 45 Å wide interior central protein binding cavity. This large binding site can bind to and accommodate very large protein domains.^{17,40} Because of the tight binding interactions sometimes approach antibody antigen binding affinities,⁴¹ hydrophobic proteins remain bound to the immobilized GroEL in the absence of ATP. Fortunately, the double heptamer barrel shape of the biotinylated GroEL will invariably expose at least one if not both of its binding sites since this large barrel structure ($14 \times 14 \text{ nm}^2$) is presumably randomly orientated on the streptavidin BLI tip.

As the first stage of the development of a GroEL-BLI assay, the physical stability and refolding activity of the biotinylated GroEL was examined and found to be virtually identical to its unmodified wild-type counterpart (Fig. 1 and Supporting Information Fig. S1). The biotinylated GroEL was then used to develop a BLI assay where nonspecific BSA binding was shown to be diminished when the concentration of biotinylated GroEL solutions for tip loading was optimized (Fig. 2). The GroEL biosensors were then used to probe the presence of preaggregates/aggregates in therapeutically relevant protein solutions. In addition, the nature of the interaction of these species with a GroEL biosensor was evaluated. As controls, the nonspecific binding of our test proteins was examined using streptavidin tips alone. In these instances, binding could not be easily reversed when ATP was added to the streptavidin tips nor did we observe any substantial dissociation after the tips were dipped into buffer alone (Supporting Information Fig. S5). In addition, biotinylated GroEL did not dissociate from the BLI tips in the presence of ATP (control trace (black) in Fig. 3).

The interaction of different proteins (FGF-1, polyclonal IgGs and a monoclonal IgG1), exposed to different thermal treatments leading to some degree of protein unfolding and aggregation, with the GroEL-BLI biosensors was examined. First, the binding of the conformationally unstable protein FGF-1 (acidic fibroblast growth factor) to GroEL, and diminished binding in the presence of the stabilizing polyanion ligand heparin, demonstrates that this interaction is sensitive to the presence of the FGF-1 molten

globule population. Since the FGF-1 binding onto the GroEL biosensor could be diminished with the ligand, heparin, this binding detection method could potentially also be used to readily assess the efficacy of excipients on the stability of protein therapeutics assuming the excipients do not affect GroEL binding. In this case, the interaction of a generic substrate such as a partially folded green fluorescence protein was unaffected by the presence of heparin (Supporting Information Fig. S2). In addition, GroEL binding of preaggregate species has been shown previously to be unaffected by the presences of low concentrations of excipient mixtures.⁴²

We then evaluated two different antibody solutions to correlate the appearance of preaggregate/aggregate species by GroEL-BLI biosensor binding with the time-dependent appearance of larger molecular weight protein aggregates (using SEC) during exposure to moderate or elevated heat stress conditions. The conditions used in this work ranged from slightly elevated physiological temperatures (42°C) with a purified IgG1 mAb species (Fig. 6) and polyclonal IgG (Supporting Information Fig. S5) to higher temperature stress (55°C) with the polyclonal human Abs preparation (Fig. 4). In these instances, an increase in the binding amplitudes preceded the appearance of larger aggregated species (either dimeric or multimeric species) as assessed by SEC. For example, the GroEL biosensor enabled us to probe the conformational properties of polyclonal IgG at time zero as well as follow the time dependent increase in binding as the polyclonal IgG species were heat stressed. In addition, the polyclonal IgG partitioning was observed for both isolated monomer species and in solutions containing enriched polyclonal IgG dimers. During heating, dimer populations from the higher temperature stressed polyclonal IgG populations actually decreased as the chaperonin dependent BLI signal increased, perhaps indicating that the GroEL-BLI biosensor was binding dissociated transients prior to multimer formation. The exact nature of this binding interaction of GroEL with different species formed during the heating of polyclonal IgG solution is difficult to determine due to the heterogeneous nature of this sample. Thus, lower temperature stress with heterogeneous polyclonal IgG and homogeneous IgG1 monoclonal antibody was also examined during exposure to milder heating conditions. The GroEL binding reactions with IgG1 Ab appear to be specific since these bound species showed accelerated dissociation kinetics from the GroEL-BLI biosensor in the presence of ATP. Depending on the osmolyte solution used in combination with the ATP, in some instances, one could easily discern that the dissociation rate was dramatically accelerated with ATP (Fig. 6). Under lower temperature heat stress conditions (~42°C) the baseline return of the BLI signal to the original values

were essentially complete. The most important demonstration of specific binding was realized when GroEL-mAb complexes were removed, deposited on TEM grids, and were directly visualized using negative stain electron microscopy resulted in the appearance distinct GroEL-mAb substrate complexes (Fig. 7). These GroEL-mAb complexes were readily observed using the reversible S-S biotinylation procedure (Methods section). Interestingly, the mAb binding was consistently localized at the GroEL binding site located at the ends of the barrel structures (Fig. 7).

For experiments with FGF-1 and polyclonal human IgG preparations, ATP binding to GroEL decreased the chaperonin affinity for partially folded proteins, leading to a detectable acceleration in the dissociation rate of the bound protein substrates. Nonetheless, in the IgG polyclonal sample that was stressed at higher temperatures, the substrate dissociation did not return to baseline. In this instance, more complete dissociation may require additional ATP pulses since these elevated heat stressed species may have more exposed hydrophobic surfaces resulting in tighter binding to GroEL. In fact, during purification of GroEL in our labs, methanol or acetone washes are required to remove contaminants tightly bound to the GroEL that could not be removed by ATP addition or ATP osmolyte addition alone. In some instances, tighter binding interactions between the protein substrate and GroEL require the combined binding free energies from ATP and the cochaperonin GroES to relieve the added structural constraints imposed by more extensive binding.^{43,44} Recently, it has been recognized that in some instances, the C terminal tails may also contribute to GroEL-substrate binding.⁴⁵ Since it is likely this will vary depending on the specific protein, formulation, and environmental stress being studied, method development activities for specific proteins for specific stability monitoring applications will require optimization of these assay parameters.

In terms of future work, we plan to explore the extent in which BLI GroEL biosensor can be used as a “general detector” of a wide range of potential preaggregate species in different protein solutions exposed to different environmental stresses (i.e., heat, agitation, light, freeze-thaw, etc.) in different formulations. We plan to further evaluate the BLI measurements in a more quantitative way (i.e., relative binding kinetics) and correlate these BLI measurements with a variety of other analytical techniques used to monitor protein aggregate formation across a larger size range (e.g., not only SEC, but also light scattering, digital imaging methods, etc.). Since IgG1 mAb-GroEL complexes can be directly visualized by TEM, future experiments could be aimed at structurally classifying and characterizing these complexes using single particle

analysis. The goal of these future experiments will be to reconstruct the GroEL-mAb populations, raising the possibility that one may be able to better elucidate protein aggregation pathways by pinpointing the regions on the IgG molecule that are susceptible to partial unfolding during environmental stress. It will also be of interest to evaluate the extent to which different types and classes of monoclonal antibodies, exposed to different environmental stresses, will differ in terms of their GroEL biosensor binding behavior. In addition, inhibition of BLI GroEL biosensor binding could be a useful approach to screen different excipients to identify stabilizers of various proteins exposed to different environmental stresses.

Materials and Methods

Materials

The high purity nucleotide free high affinity form of GroEL was produced in our lab as described previously.⁴⁶ Biotinylation of the GroEL was carried out as described below. The IgG1 monoclonal antibody was prepared in our labs as described below. FGF-1 was provided by Dr. Michael Blaber, Florida State University School of Medicine, and was prepared as described previously.³⁰ Polyclonal human antibody preparation and green fluorescent protein (GFP) were purchased from MP biochemicals (Santa Ana, CA) and Calbiochem (Billerica, MA), respectively. The polyclonal antibody was further purified in our labs as described below. Analytical grades of Tris, MgCl₂, KCl, and EDTA from Fisher Scientific (Pittsburgh, PA) and Na₂HPO₄, NaH₂PO₄, NaCl from Sigma (St. Louis, MO) were used for preparing various buffers and solutions.

GroEL was biotinylated using an EZ-link Sulfo-NHS LC biotinylation kit (Pierce, Rockford, IL), following the manufacturer's instructions. Briefly, 15 μ M of GroEL (tetradecamer) in pH 7.5 PBS was incubated for 3 h at room temperature with 300 μ M of the biotinylation reagent. After 3 h, the excess biotinylation reagent and the reaction products were removed by dialysis using a 10 kDa MWCO Slide-A-Lyzer dialysis cassette (Pierce Scientific, Rockford, IL) against a total of 1 L of GroEL buffer (50 mM Tris, 50 mM KCl, 10 mM MgCl₂, 0.5 mM EDTA, pH 7.5) for 16 h at 4°C. The GroEL buffer has been previously developed to specifically enhance the activity of GroEL and to facilitate protein substrate capture and refolding.⁴⁷ The biotinylation was quantified using a biotin quantitation kit (Pierce Scientific, Rockford, IL) and following the manufacturer's instructions. The labeling ratio was calculated to be three biotin molecules per GroEL oligomer. Similarly, the reversible S-S biotin GroEL was developed by incubating 15 μ M GroEL (tetradecamer) in PBS buffer, pH 7.5 with 300 μ M of EZ-Link

Sulfo-NHS-SS-Biotin (Pierce Scientific, Rockford, IL) for 3 h at room temperature. The excess biotinylation reagent and the reaction products were removed by dialysis against GroEL buffer, as described above. The biotinylation ratio was quantified to be three reversible S-S biotin molecules per GroEL oligomer using the biotin quantification kit (Pierce Scientific, Rockford, IL).

An IgG1 mAb was produced in our labs from CHO cells. The cell-free supernatant was collected, and loaded onto a protein A column (Pierce Scientific, Rockford, IL). The bound antibody was eluted from protein A following the manufacturer's instructions. The eluted fractions containing the mAb was further enriched and concentrated using 300 and 100 kDa Amicon ultrafiltration cassettes (Millipore, Billerica, MA) that were prewashed with pH 7.5 PBS buffer (10 mM Na₂HPO₄, 2 mM NaH₂PO₄, 2.7 mM KCl, 140 mM NaCl from Sigma, St. Louis, MO). The IgG1 mAb was shown to be ~97% pure by SDS-PAGE and SE-HPLC and was stored in pH 7.5 PBS buffer at 4°C.

The monomer and dimer species in the polyclonal human antibody preparation were isolated by Preparative Size Exclusion Fast Protein Liquid Chromatography (SE-FPLC) using a BioLogic DuoFlow system (BioRad, Richmond, CA) equipped with a Superose 12 10/300 GL size exclusion column (10 $\times$ 300 mm, particle size 11 μ m, GE Healthcare, Piscataway, NJ) and a BioLogic QuadTec UV/Visible absorbance detector (BioRad, Richmond, CA). The "GroEL buffer" (50 mM Tris, 50 mM KCl, 10 mM MgCl₂, 0.5 mM EDTA, pH 7.5) with an additional 150 mM NaCl was used as the running buffer at a flow rate of 1 mL/min. Five milligrams of poly-IgG in pH 7.5 PBS buffer were loaded onto the column, followed by the collection of 0.5 mL fractions. Fractions corresponding to the IgG monomer peaks as monitored by both UV absorbance at 280 nm and SDS-PAGE were pooled and concentrated using 100 kDa MWCO Amicon ultrafiltration cartridges prewashed with GroEL buffer. Since it was difficult to purify sufficient amounts of the dimer species for analysis (the polyclonal IgG preparation contains ~4–5% dimers as measured by preparative SEC column), the protein solution was incubated under mild heating conditions of 37°C for 48 h, followed by incubation at room temperature for a further 120 h. This treatment led to an increase in the dimer population in the polyclonal IgG to about 15%. This heated sample was then loaded on the SE-FPLC and the dimer fractions collected and pooled as described above.

Methods

Biophysical characterization of GroEL and biotinylated GroEL. *Far-UV circular dichroism spectroscopy.* Circular dichroism analysis was

performed using a Chirascan-plus CD spectrometer (Applied Photophysics, Leatherhead, UK) equipped with a 4-cell position temperature controlled peltier unit (Quantum Northwest, Liberty Lake, WA). Far-UV CD spectra of GroEL and biotinylated GroEL at 0.1 mg/mL were collected from 260 to 200 nm using 0.1 cm path length quartz cuvettes (Starna Cells, Atascadero, CA). Data were acquired every 1 nm for 0.5 s. Ellipticity at 222 nm was monitored as a function of temperature from 10 to 87.5°C in 2.5°C increments using a heating rate of 1°C/min and allowing the sample to equilibrate at 1 min at each temperature. Data were then subjected to a three-point Savitzky-Golay smoothing filter using the Chirascan software. The ellipticity value of the buffer alone was subtracted from all measurements.

Turbidity. The optical density at 360 nm was monitored as a function of temperature using a Cary-100 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA) equipped with a 12-cell temperature controlled peltier unit. Duplicate samples of GroEL and biotinylated GroEL at 0.1 mg/mL were analyzed using 1 cm quartz cuvettes. The temperature was ramped from 10 to 87.5°C in 2.5°C increments using a heating rate of 1°C/min and a 1 min equilibration time at each temperature. The optical density of the buffer alone was subtracted from all measurements.

Extrinsic fluorescence. Accessibility of exposed hydrophobic moieties of GroEL and biotinylated GroEL as a function of temperature was assessed using 8-Anilino-1-naphthalenesulfonate as a probe (ANS, Sigma, St Louis, MO). Fifty micromolar ANS was added to a 0.1 mg/mL protein solution and incubated in the dark for at least 5 min at 10°C. Fluorescence data were obtained using a Photon Technologies International QM-40 spectrofluorometer (PTI, Birmingham, NJ) equipped with a 4-cell peltier holder (Quantum Northwest, Liberty Lake, WA). Duplicate samples were excited at 372 nm and the emission spectrum was monitored from 400 to 600 nm while scanning every 1 nm and using a 0.5 s integration time. Spectra were collected as a function of temperature (10–87.5°C). The excitation and emission slits were both set at 2 nm. The spectra were collected every 2.5°C with a 3 min equilibration time at each temperature. The emission values of the buffer containing 50 μ M ANS were subtracted from all measurements. Normalized fluorescence intensity at 480 nm was plotted as a function of temperature.

Empirical phase diagram construction. The three-index empirical phase diagrams (EPDs) were constructed as described previously^{18,48} to summarize and facilitate the analysis of the biophysical stability data sets from different instruments (CD signal at 222 nm, ANS fluorescence intensity, and OD₃₆₀). Calculations were performed and data visualization phase diagrams were prepared using

in-house laboratory software (Middaugh Suite) as described in detail elsewhere.¹⁸

GFP refolding assay. GFP refolding was used to test the GroEL partitioning and refolding efficiencies. Green fluorescent protein (GFP) was treated using 125 mM HCl (Sigma, St. Louis, MO) to obtain unfolded GFP. Wild-type or biotinylated GroEL was heated from 10 to 80°C in 2.5°C increments using a heating rate of 1°C/min and each sample was allowed to equilibrate for 1 min at each temperature. Aliquots were withdrawn at 20°, 35°, 50°, 65°, and 80°C and the GroEL samples were allowed to equilibrate to ambient temperature. Denatured GFP at 0.5 μ M was incubated with 1 μ M (tetradecamer) of biotinylated GroEL or wild type GroEL from each temperature point in GroEL buffer, pH 7.5. As a control, 0.5 μ M of denatured GFP alone was diluted with GroEL buffer, pH 7.5. The GFP fluorescence was measured using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA) with excitation and emission wavelengths set at 395 and 510 nm, respectively. Ten millimolar ATP (pH adjusted to 7.5) was then added to each GroEL-GFP mixture. The GFP refolding and the resulting increase in fluorescence was then measured over 30 min as described above. In addition, the GFP refolding assay was also used to determine if heparin interfered with FGF-1 binding to GroEL as described in the text.

Bio-layer interferometry (BLI). BLI detects changes in mass (protein thickness) on a biosensor tip where changes in the reflectance interference wave pattern between the sample and an internal reflectance reference layer result in a phase shift (Δ in nanometers), and these changes can be followed in real-time in both kinetic and quantitative modes.⁴⁹ Moreover, because BLI is a rapid dip and read procedure, it is a particularly attractive technique for high-throughput development.⁴⁹ BLI experiments were performed using a single channel BLItz system (ForteBio, Menlo Park, CA) using streptavidin biosensors. The BLItz platform stirring speed gave optimal signal responses at 2000 rpm and above. The biotinylated GroEL was loaded onto the streptavidin biosensors (Forte Bio, Menlo Park, CA) using the following methodology. Biosensors were first hydrated for at least 10 min in GroEL buffer followed by loading the biotinylated GroEL onto the streptavidin tips at increasing concentrations (from 0.005 to 1.0 mg/mL) with a 5 min incubation time at each protein concentration. The tips were then washed briefly (30 s) with the GroEL buffer to remove nonspecifically bound GroEL and each GroEL loaded biosensor tip was then incubated with 1 mg/mL bovine serum albumin (BSA) (Sigma, St. Louis, MO) in GroEL buffer for 5 min to

determine the extent of nonspecific binding. The amplitude of BSA binding to the biotinylated GroEL loaded tips after 5 min was recorded and plotted as a function of GroEL loading to determine the optimal loading concentration.

The BLI runs were performed at room temperature as follows (although some experiments had some specific alterations to this general protocol; see below): a buffer baseline for 30 s was run followed by biotinylated GroEL loading for 170 s (in GroEL buffer). Another baseline was run for 30 s in buffer containing at least 0.1M NaCl to remove excess biotinylated GroEL followed by an association phase (experimental sample) for 300 s. The addition of NaCl to the buffer was essential to decrease electrostatic interactions between GroEL and the proteins (data not shown). The association phase was followed by a dissociation phase (buffer alone) for 220 s. To dissociate bound substrate from GroEL biosensor, 3 pulses of 20 mM ATP in GroEL buffer were run for 120 s each, and the end run was a return to a 60 s wash with buffer alone. The BLI method for FGF-1, poly-IgG, and the IgG mAb were each optimized and are as follows:

BLI analysis of FGF-1. FGF-1 in 50 mM sodium phosphate, 0.1M NaCl, 10 mM ammonium sulfate, 2 mM dithiothreitol (DTT) (pH 7.5), referred to as "crystallization buffer" was assayed by GroEL-BLI. FGF-1 samples at 0.3 mg/mL with and without heparin at a 3:1 (w/w) heparin:FGF-1 ratio were prepared and then incubated at room temperature and at 40°C for 5 min in crystallization buffer, followed by quenching on ice for at least 5 min before analysis by BLI at ambient temperature. The ATP release steps were performed using 20 mM ATP in an osmolyte mixture that consisted of 4M urea and 4M glycerol in GroEL buffer at ambient temperature. The biosensor hydration, dissociation, and final buffer wash steps were performed in crystallization buffer.

BLI analysis of Poly-IgG. Human poly-IgG (MP Biomedicals, Santa Ana, CA) at 0.5 mg/mL concentration was diluted in 20 mM citrate-phosphate buffer (pH 6.0) with a total ionic strength of 0.15 (adjusted with NaCl), placed in 5 mL glass vials, and capped with rubber stoppers (West Pharmaceuticals, Exton, PA). The vial solutions were heated at 55°C and aliquots were removed at 0, 5, 10, 20, 30, 60, 120, and 240 min, quenched on ice for at least 5 min prior to analysis by BLI (and SE-HPLC). The BLI ATP release steps were performed in buffer containing 20 mM ATP and 1M sucrose in GroEL buffer. All experiments were performed at ambient temperature. The biosensor hydration, dissociation, and final buffer wash steps were performed in 20 mM citrate-phosphate buffer (pH 6.0) with a total ionic strength of 0.15.

BLI analysis of IgG1 mAb. The IgG1 mAb stock solution was diluted in GroEL buffer containing

150 mM NaCl. This diluted solution (2.5 mg/mL) placed in 0.6 mL microcentrifuge tubes was then heated on an orbital heating block maintained at 42°C for 5 min or 15 min. After heating, the mAb solutions were allowed to equilibrate back to room temperature. Biotinylated GroEL streptavidin biosensors were then dipped into an aliquot of the mAb solution and the binding amplitude recorded. The BLI experiments were carried out as described above using the general BLI analysis protocol.

Size exclusion high performance liquid chromatography (SE-HPLC). Analytical SE-HPLC analysis of the polyclonal human antibody preparations was performed using a Shimadzu prominence UFLC HPLC system equipped with a diode array detector (Shimadzu Corporation, Kyoto, Japan) at KU, Lawrence laboratories. A TSKgel BioAssist G3SWxl gel filtration column (Tosoh Corporation, Tokyo, Japan), 7.8 mm × 3.0 cm with a 5 μm particle size, along with the corresponding guard column (6.0 mm × 4.0 cm, 7 μm particle size), was operated at ambient temperature and was equilibrated with at least 10 column volumes of 200 mM sodium phosphate, pH 6.8 that was sterile filtered (0.22 μm pore size). Twenty-five micrograms of each sample were injected with a total run time of 30 min. Peak integrations were performed using the dual wavelength method (OD 280 nm and OD 214 nm), as described previously.³⁴

For the IgG1 mAb experiments, analytical SE-HPLC was performed using a Waters 600S LC system equipped with a 996 photodiode array detector at KUMC, Kansas City laboratories. Ten microliter of the aliquot was loaded onto a Phenomenex BioSep Sec S3000 column (300 × 7.8 mm, 5 μm particle size) with GroEL buffer containing 150 mM NaCl as the running buffer.

Transmission electron microscopy (TEM). BLI tips containing either mAb-S-S biotinylated GroEL complex or S-S biotinylated GroEL alone was used for TEM sample preparation. To prepare the mAb-GroEL complex, mAb solution heated at 42°C for 15 min was incubated with the S-S biotinylated GroEL streptavidin biosensors for 5 min. The excess bound mAbs were removed by washing the biosensor tips with GroEL buffer containing 150 mM NaCl in a dissociation phase (220 s). The control tips containing GroEL alone was prepared by incubating the S-S biotinylated GroEL streptavidin biosensors for 5 min with GroEL buffer containing 150 mM NaCl. The immobilized complexes (GroEL-mAb or GroEL) were removed from the BLI biosensor tips by immersing the tip directly into 2 μL of GroEL buffer containing 50 mM dithiothreitol (DTT, Sigma, St. Louis, MO). The DTT cleaves the S-S biotin bond and results in the elution of the biotinylated GroEL

into the DTT-buffer solution. A control run with IgG1 mAb treated under these same conditions and analyzed by SEC as discussed in the text indicates that very little change in size distribution occurred (Supporting Information Figure section). The 2 μ L solution containing biosensor released GroEL was wicked onto carbon-coated Cu 300 mesh EM grids (Electron Microscopy Science) that were glow-discharged prior to use. The samples were allowed to incubate for 1 min on the grids and then the grids were briefly washed by immersion into three separate water droplets. After washing, the grids were negatively stained using 1% uranyl acetate (pH 7). Samples were then imaged using a JEOL-1200 EXII transmission electron microscope at 100 keV housed at the University of Kansas Medical Campus (KUMC) as described previously.⁵⁰

References

- Xu J, Ge X, Dolan MC (2011) Towards high-yield production of pharmaceutical proteins with plant cell suspension cultures. *Biotechnol Adv* 29:278–299.
- Nelson AL, Dhimolea E, Reichert JM (2010) Development trends for human monoclonal antibody therapeutics. *Nat Rev Drug Discov* 9:767–774.
- Stefani M, Dobson CM (2003) Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. *J Mol Med (Berl)* 81:678–699.
- Ringe D, Petsko GA (2009) What are pharmacological chaperones and why are they interesting? *J Biol* 8:80.
- Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS (2010) Stability of protein pharmaceuticals: an update. *Pharm Res* 27:544–575.
- Roberts CJ, Das TK, Sahin E (2011) Predicting solution aggregation rates for therapeutic proteins: approaches and challenges. *Int J Pharm* 418:318–333.
- Chaudhuri R, Cheng Y, Middaugh CR, Volkin DB (2014) High-throughput biophysical analysis of protein therapeutics to examine interrelationships between aggregate formation and conformational stability. *AAPS J* 16:48–64.
- Lubiniecki A, Volkin DB, Federici M, Bond MD, Nedved ML, Hendricks L, Mehndiratta P, Bruner M, Burman S, Dalmonte P, Kline J, Ni A, Panek ME, Pikounis B, Powers G, Vafa O, Siegel R (2011) Comparability assessments of process and product changes made during development of two different monoclonal antibodies. *Biologicals* 39:9–22.
- Federici M, Lubiniecki A, Manikwar P, Volkin DB (2013) Analytical lessons learned from selected therapeutic protein drug comparability studies. *Biologicals* 41:131–147.
- Gibson TJ, McCarty K, McFadyen IJ, Cash E, Dalmonte P, Hinds KD, Dinerman AA, Alvarez JC, Volkin DB (2011) Application of a high-throughput screening procedure with PEG-induced precipitation to compare relative protein solubility during formulation development with IgG1 monoclonal antibodies. *J Pharm Sci* 100:1009–1021.
- Filipe V, Jiskoot W, Basmeleh AH, Halim A, Schellekens H, Brinks V (2012) Immunogenicity of different stressed IgG monoclonal antibody formulations in immune tolerant transgenic mice. *MAbs* 4:740–752.
- Wang W, Singh SK, Li N, Toler MR, King KR, Nema S (2012) Immunogenicity of protein aggregates—concerns and realities. *Int J Pharm* 431:1–11.
- Shire SJ, Shahrokh Z, Liu J (2004) Challenges in the development of high protein concentration formulations. *J Pharm Sci* 93:1390–1402.
- Narhi LO, Schmit J, Bechtold-Peters K, Sharma D (2012) Classification of protein aggregates. *J Pharm Sci* 101:493–498.
- Zolls S, Tantipolphan R, Wiggenhorn M, Winter G, Jiskoot W, Friess W, Hawe A (2012) Particles in therapeutic protein formulations, Part 1: overview of analytical methods. *J Pharm Sci* 101:914–935.
- Wang T, Joshi S, Kumru OS, Telikepalli S, Middaugh CR, Volkin DB, Case studies applying biophysical techniques to better characterize protein aggregates and particulates of varying size. In: Narhi L, Ed. (2013) *Biophysics for therapeutic protein development*. New York: Springer, pp 205–243.
- Braig K, Otwinowski Z, Hegde R, Boisvert DC, Joachimiak A, Horwich AL, Sigler PB (1994) The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. *Nature* 371:578–586.
- Kim JH, Iyer V, Joshi SB, Volkin DB, Middaugh CR (2012) Improved data visualization techniques for analyzing macromolecule structural changes. *Protein Sci* 21:1540–1553.
- Phadtare S, Fisher MT, Yarbrough LR (1994) Refolding and release of tubulins by a functional immobilized groEL column. *Biochim Biophys Acta* 1208:189–192.
- Taguchi H, Ueno T, Tadakuma H, Yoshida M, Funatsu T (2001) Single-molecule observation of protein-protein interactions in the chaperonin system. *Nat Biotechnol* 19:861–865.
- Ueno T, Taguchi H, Tadakuma H, Yoshida M, Funatsu T (2004) GroEL mediates protein folding with a two successive timer mechanism. *Mol Cell* 14:423–434.
- Naik S, Haque I, Degner N, Kornilayev B, Bomhoff G, Hodges J, Khorassani AA, Katayama H, Morris J, Kelly J, Seed J, Fisher MT (2010) Identifying protein stabilizing ligands using GroEL. *Biopolymers* 93:237–251.
- Takei Y, Iizuka R, Ueno T, Funatsu T (2012) Single-molecule observation of protein folding in symmetric GroEL-(GroES)₂ complexes. *J Biol Chem* 287:41118–41125.
- Goloubinoff P, Christeller JT, Gatenby AA, Lorimer GH (1989) Reconstitution of active dimeric ribulose biphosphate carboxylase from an unfolded state depends on two chaperonin proteins and Mg-ATP. *Nature* 342:884–889.
- Martin J, Horwich AL, Hartl FU (1992) Prevention of protein denaturation under heat stress by the chaperonin Hsp60. *Science* 258:995–998.
- Murai N, Taguchi H, Yoshida M (1995) Kinetic analysis of interactions between GroEL and reduced alpha-lactalbumin. Effect of GroES and nucleotides. *J Biol Chem* 270:19957–19963.
- Fisher MT (1991) Differences in thermal stability between reduced and oxidized cytochrome b562 from *Escherichia coli*. *Biochemistry* 30:10012–10018.
- Jackson GS, Staniforth RA, Halsall DJ, Atkinson T, Holbrook JJ, Clarke AR, Burston SG (1993) Binding and hydrolysis of nucleotides in the chaperonin catalytic cycle: implications for the mechanism of assisted protein folding. *Biochemistry* 32:2554–2563.
- Xia X, Babcock JP, Blaber SI, Harper KM, Blaber M (2012) Pharmacokinetic properties of 2nd-generation fibroblast growth factor-1 mutants for therapeutic application. *PLoS One* 7:e48210.

30. Alsenaidy MA, Wang T, Kim JH, Joshi SB, Lee J, Blaber M, Volkin DB, Middaugh CR (2012) An empirical phase diagram approach to investigate conformational stability of "second-generation" functional mutants of acidic fibroblast growth factor-1. *Protein Sci* 21:418–432.
31. Mach H, Volkin DB, Burke CJ, Middaugh CR, Linhardt RJ, Fromm JR, Loganathan D, Mattsson L (1993) Nature of the interaction of heparin with acidic fibroblast growth factor. *Biochemistry* 32:5480–5489.
32. Mach H, Ryan JA, Burke CJ, Volkin DB, Middaugh CR (1993) Partially structured self-associating states of acidic fibroblast growth factor. *Biochemistry* 32:7703–7711.
33. Bolli R, Woodtli K, Bartschi M, Hofferer L, Lerch P (2010) L-Proline reduces IgG dimer content and enhances the stability of intravenous immunoglobulin (IVIG) solutions. *Biologicals* 38:150–157.
34. Bond MD, Panek ME, Zhang Z, Wang D, Mehndiratta P, Zhao H, Gunton K, Ni A, Nedved ML, Burman S, Volkin DB (2010) Evaluation of a dual-wavelength size exclusion HPLC method with improved sensitivity to detect protein aggregates and its use to better characterize degradation pathways of an IgG1 monoclonal antibody. *J Pharm Sci* 99:2582–2597.
35. Ellis RJ (2007) Protein misassembly: macromolecular crowding and molecular chaperones. *Adv Exp Med Biol* 594:1–13.
36. Minton AP (2005) Influence of macromolecular crowding upon the stability and state of association of proteins: predictions and observations. *J Pharm Sci* 94:1668–1675.
37. Roberts CJ (2007) Non-native protein aggregation kinetics. *Biotechnol Bioeng* 98:927–938.
38. Sun T, Reid F, Liu Y, Cao Y, Estep P, Nauman C, Xu Y (2013) High throughput detection of antibody self-interaction by bio-layer interferometry. *MAbs* 5:838–841.
39. Schmidt M, Buchner J (1992) Interaction of GroE with an all-beta-protein. *J Biol Chem* 267:16829–16833.
40. Chen L, Sigler PB (1999) The crystal structure of a GroEL/peptide complex: plasticity as a basis for substrate diversity. *Cell* 99:757–768.
41. Fenton WA, Horwich AL (1997) GroEL-mediated protein folding. *Protein Sci* 6:743–760.
42. Tieman BC, Johnston MF, Fisher MT (2001) A comparison of the GroE chaperonin requirements for sequentially and structurally homologous malate dehydrogenases: the importance of folding kinetics and solution environment. *J Biol Chem* 276:44541–44550.
43. Motojima F, Chaudhry C, Fenton WA, Farr GW, Horwich AL (2004) Substrate polypeptide presents a load on the apical domains of the chaperonin GroEL. *Proc Natl Acad Sci U S A* 101:15005–15012.
44. Taguchi H (2005) Chaperonin GroEL meets the substrate protein as a "load" of the rings. *J Biochem* 137:543–549.
45. Chen DH, Madan D, Weaver J, Lin Z, Schroder GF, Chiu W, Rye HS (2013) Visualizing GroEL/ES in the act of encapsulating a folding protein. *Cell* 153:1354–1365.
46. Voziyan PA, Fisher MT (2000) Chaperonin-assisted folding of glutamine synthetase under nonpermissive conditions: off-pathway aggregation propensity does not determine the co-chaperonin requirement. *Protein Sci* 9:2405–2412.
47. Mendoza JA, Rogers E, Lorimer GH, Horowitz PM (1991) Chaperonins facilitate the in vitro folding of monomeric mitochondrial rhodanese. *J Biol Chem* 266:13044–13049.
48. Alsenaidy MA, Jain NK, Kim JH, Middaugh CR, Volkin DB (2014) Protein comparability assessments and potential applicability of high throughput biophysical methods and data visualization tools to compare physical stability profiles. *Front Pharmacol* 5:39.
49. Concepcion J, Witte K, Wartchow C, Choo S, Yao D, Persson H, Wei J, Li P, Heidecker B, Ma W, Varma R, Zhao LS, Perillat D, Carricato G, Recknor M, Du K, Ho H, Ellis T, Gamez J, Howes M, Phi-Wilson J, Lockard S, Zuk R, Tan H (2009) Label-free detection of biomolecular interactions using BioLayer interferometry for kinetic characterization. *Comb Chem High Throughput Screen* 12:791–800.
50. Naik S, Brock S, Akkaladevi N, Tally J, McGinn-Straub W, Zhang N, Gao P, Gogol EP, Pentelute BL, Collier RJ, Fisher MT (2013) Monitoring the kinetics of the pH-driven transition of the anthrax toxin prepore to the pore by biolayer interferometry and surface plasmon resonance. *Biochemistry* 52:6335–6347.