**Protein Unfolding, Interactions, and Aggregation at High Pressure**

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Monoclonal antibodies (MAbs) are a relatively recent class of drugs that have introduced new curative strategies in an emerging precision medicine paradigm. MAbs have high and tunable specificity for antigens, along with potency to effectuate robust immune responses in the treatment of many life-threatening diseases and health conditions. Protein aggregation is a common degradation pathway for MAb drug products during multiple steps of manufacturing and storage. Aggregated protein must be tightly controlled due to potential adverse effects on efficacy and safety.

Bulk freezing, at temperatures as low as -80 °C, can mitigate protein aggregation, though degradation may still occur through cold denaturation, interactions at ice-liquid interfaces, cryo-concentration effects, and pH shifts. Hydrostatic pressure has been previously used to prevent water from crystallizing at sub-zero °C temperatures, allowing for the characterization of protein structural perturbations that potentially induce aggregation. While this approach enables the solution to maintain a liquid phase below 0 °C, there is the obvious convolution of pressure effects and cold denaturation on protein unfolding and aggregation. As an environmental variable, pressure has potential to be used in conducting accelerated aggregation studies analogous to temperature accelerated studies, thereby deconvoluting pressure- / cold-denaturation effects. Novel techniques are introduced in this thesis to gain a molecular understanding of pressure-induced unfolding and aggregation. The resulting models and characterization tools offer mechanistic insight into the aggregation process that would otherwise not be considered in temperature-based formulation development.

Since pressure can affect the relative population of partially unfolded species, it is necessary to perform *in situ* structural and folding characterization of these intermediate states. The effects of high pressure and low temperature on the stability of two uniquely behaving MAbs were examined in this work. Fluorescence and small-angle neutron scattering were used to monitor the *in situ* effects of pressure to infer shifts in tertiary structure, and characterize aggregation prone intermediates. Partial unfolding was observed for both MAbs, to different extents, under a range of pressure / temperature conditions. Several combinations of pressure and temperature were also used to discern the respective contributions of the isolated MAb fragments (Fab and Fc) to unfolding and aggregation. The results showed that there was not a simple correlation between the folding stability of the full MAb and either the Fc or Fab fragment across all cases, indicating a multifaceted connection to full MAb unfolding and aggregation behavior.

Direct measurement of protein aggregation or shifts in protein-protein interactions at high pressure remains limited by the availability of analytical methods. To further improve the fundamental understanding of pressure effects on aggregation, a new high pressure light scattering apparatus is introduced as a suitable technique for the investigation of the shape, size,

and interactions of MAb monomers and aggregates during pressure incubations and pressure-cycling. The light scattering geometry was designed for minimal customization of commercially available equipment such as the high-pressure cell, laser, and scattered light detectors, to allow for a low barrier of implementation. The ability of the high pressure light scattering apparatus to measure the influence of pressure on protein-protein interactions was assessed using lysozyme, a relatively well-characterized protein under hydrostatic pressure. Overall, the apparatus serves as a convenient and reproducible method that complements current small angle neutron / x-ray instrumentation. The results address a growing demand to characterize protein aggregates and aggregation-prone partially unfolded intermediates, particularly at high pressure conditions.

Biophysical properties provide insight into aggregation propensity and the mechanistic steps, but are not sufficient on their own to predict aggregation rates and extent. Therefore, MAb samples were incubated under a systematic combination of high pressures, temperatures, and solution conditions to characterize aggregate species and measure aggregation rates. Resulting aggregate morphologies and formation mechanism were monitored with circular dichroism, background membrane particle counting / sizing, and size exclusion chromatography. Studies performed at isothermal conditions under a range of pressures show that both the extent of aggregation and the underlying pathway(s) change as a function of pressure. The experimental framework provides a means to conduct accelerated stability at high pressure in an orthogonal manner to experiments conducted at high temperatures.

In summary, this thesis demonstrates the complex relationship between *in situ* high-pressure biophysical properties and short- to medium- term high-pressure stability studies for two therapeutic MAbs. The novel approaches and instrumentation offer molecular-scale insight into how aggregation rates, mechanisms, and conformational stability are assessed during high pressure accelerated stability studies in connection with those conducted at high or sub-zero °C temperatures. These techniques could be used in future studies to support formulation development of biotherapeutics beyond proteins with new mechanistic information related to pressure-induced unfolding and aggregation.