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Global kinetic analysis of seeded BSA aggregation☆

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ABSTRACT

Accelerated aggregation studies were conducted around the melting temperature (T_m) to elucidate the kinetics of seeded BSA aggregation. Aggregation was tracked by SEC-HPLC and intrinsic fluorescence spectroscopy. Time evolution of monomer, dimer and soluble aggregate concentrations were globally analysed to reliably deduce mechanistic details pertinent to the process. Results showed that BSA aggregated irreversibly through both sequential monomer addition and aggregate–aggregate interactions. Sequential monomer addition proceeded only via non-native monomers, starting to occur only by 1–2 °C below the T_m. Aggregate–aggregate interactions were the dominant mechanism below the T_m due to an initial presence of small aggregates that acted as seeds. Aggregate–aggregate interactions were significant also above the T_m, particularly at later stages of aggregation when sequential monomer addition seemed to cease, leading in some cases to insoluble aggregate formation. The adherence (or non-thereof) of the mechanisms to Arrhenius kinetics were discussed alongside possible implications of seeding for biopharmaceutical shelf-life and spectroscopic data interpretation, the latter of which was found to often be overlooked in BSA aggregation studies.

1. Introduction

Protein aggregation has a known detrimental effect on the formulation stability of biopharmaceuticals (Arakawa and Kita, 2000; Vetri et al., 2007; Kayser et al., 2011; Bagger et al., 2007; Barreca et al., 2010; Bermudez and Forciniti, 2004; Roberts, 2003, 2007; Borgia et al., 2013; Andrews and Roberts, 2007; Brummitt et al., 2011a, 2011b; Demeule et al., 2007, 2009; Morris et al., 2009; Nicoud et al., 2014; Murphy and Roberts, 2013; Bernacki and Murphy, 2009; Fink, 1998; Maddux et al., 2014). Elucidating its underlying mechanisms is therefore vital for enhancement and accurate prediction of shelf-life in such formulations. Significant progress has been made in this regard since Oosawa et al.'s pioneering work (Oosawa et al., 1959; Oosawa and Kasai, 1962) but there is as yet no overall consensus on the underlying mechanisms of aggregation (Vetri et al., 2007; Borgia et al., 2013; Morris et al., 2009; Murphy and Roberts, 2013; Bernacki and Murphy, 2009; Fink, 1998; Cohen et al., 2012). One of the reasons for this is the range of mechanistic complications introduced by the existence of different types of the phenomenon (e.g. amorphous aggregation, amyloid fibrillation, etc. (Roberts, 2007; Borgia et al., 2013; Demeule et al., 2009; Morris et al., 2009; Murphy and Roberts, 2013; Fink, 1998, 2006; Cohen et al., 2012)). The interdisciplinary effort in this area has yielded numerous helpful kinetic models (see models summarised in (Kayser et al., 2011; Roberts, 2007; Morris et al., 2009; Bernacki and Murphy, 2009; Fink, 1998)), each attempting to account for a given type of aggregation. A kinetic model that accounts pictorially for the different types reported and/or modelled in the literature has also been developed (Roberts, 2007). Growth in the understanding of the kinetics of these types is likely to reveal which of the above approaches (i.e. separate models for different types or an overarching model for all types of protein aggregation) will be more suitable moving forward (Borgia et al., 2013; Cohen et al., 2012).

At present, one of the main obstacles for development of exhaustive kinetic models is the difficulty with detecting in isolation the many species involved in the aggregation process and accurately determining their concentration change with time (Roberts, 2003, 2007; Andrews and Roberts, 2007; Morris et al., 2009; Nicoud et al., 2014). This difficulty often necessitates compromises on physical accuracy through the use of assumptions and approximations. A particular case is the use of 'lumped' kinetic profiles in most if not all aggregation studies to date (Vetri et al., 2007; Kayser et al., 2011; Bagger et al., 2007; Borgia et al., 2013; Brummitt et al., 2011a, 2011b; Morris et al., 2009; Nicoud et al., 2014; Fink, 2006; Bhattacharya et al., 2011; Militello et al., 2003, 2004; Boye et al., 1996; Holm et al., 2007; Vetri et al., 2011; Vaiana et al., 2004; Buell et al., 2014), including the present one. As an example, it is common to track aggregation through a monomer loss profile under the assumption that all possible monomeric species — natively folded,

Abbreviations: BSA, bovine serum albumin; T_m, melting temperature; Trp, tryptophan; SEC-HPLC, size exclusion chromatography — high performance liquid chromatography; ODE, ordinary differential equation; AUC, area under the chromatogram; AUP, area under the peak.

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intermediate(s) and unfolded — are reactive with respect to aggregation. The compromise that may be introduced by such lumping is often furthered by basing modelling on mathematical fits made to single 'kinetic profiles' (Bernacki and Murphy, 2009), e.g. only monomer loss or only aggregate formation (Vetri et al., 2007, 2011; Kayser et al., 2011; Borgia et al., 2013; Brummitt et al., 2011a, 2011b; Morris et al., 2009; Fink, 2006; Bhattacharya et al., 2011; Holm et al., 2007; Buell et al., 2014). This one-sided approach can limit the deduction of mechanistic details pertinent to aggregation because it prevents proper establishment of the link between reactant loss and product formation for the myriad of interactions during aggregation (Bernacki and Murphy, 2009). Simultaneous fitting of multiple kinetic profiles, termed 'global analysis' from hereon (Nicoud et al., 2014; Bernacki and Murphy, 2009; Cohen et al., 2012; Vanstokkum et al., 1995), can help overcome this problem by producing common kinetic parameters to describe the time evolution of various species' concentrations and thus linking monomer loss to the formation of aggregate species (Bernacki and Murphy, 2009).

Global analysis can overcome, partially at the least, physical accuracy problems associated with analysis of single kinetic profiles (Bernacki and Murphy, 2009). For instance, 'higher-order' analysis that defines aggregation in terms of monomer loss to the nth degree (Buswell and Middelberg, 2003; Clenet et al., 2014) has little physical grounding compared to defining aggregation as the collection of many second-order and/or pseudo-first-order reactions, a substantial portion of which may not even involve monomers for many proteins (Cohen et al., 2012). Modelling concurrently even a small number of these reactions, even if using assumptions like lumping, would produce a physically more meaningful fit (Nicoud et al., 2014; Bernacki and Murphy, 2009; Cohen et al., 2012; Vanstokkum et al., 1995) and provide reliable mechanistic insight. As another example, 'over-fitting', whereby a single kinetic profile such as monomer loss is fit by the fluctuation of a multitude of parameters (Borgia et al., 2013), may produce mathematically good fits but the reliability of mechanistic insight it may provide is questionable if the large number of fit parameters is not statistically justified (Murphy and Roberts, 2013; Bernacki and Murphy, 2009). In comparison, global analysis would make the statistical justification of the same number of parameters more plausible because more profiles are being fit with the same number of parameters and as such there is less 'room' for these parameters to fluctuate in, making them physically more realistic (Nicoud et al., 2014; Bernacki and Murphy, 2009; Cohen et al., 2012; Vanstokkum et al., 1995). But perhaps the greatest benefit of global analysis is the ability to directly eliminate many potential kinetic models when fitting experimental data; it has been shown that analysis of only monomer loss kinetics when modelling can be misleading because many models can fit such data with equal precision and that, in comparison, simultaneous analysis of just two kinetic profiles can eliminate many of these models, which fail to approach the accuracy with which they can fit a single kinetic profile (Bernacki and Murphy, 2009).

Herein, a global analysis study of seeded BSA aggregation kinetics is reported as a model. Seeded aggregation was studied purposefully to better reflect post-production conditions since biopharmaceuticals may have 'seeds' for aggregation as a result of rigorous purification processes (Bermudez and Forciniti, 2004; Demeule et al., 2009; de Frutos et al., 1998; Maruyama et al., 2001). Due to the limitations of the principal method, SEC-HPLC, the time evolution of only three lumped species' concentrations were reliably tracked; namely, monomers (all monomeric species treated as one), dimers and aggregates (all soluble aggregates larger than dimers treated as one). Fluorescence spectroscopy was used to probe conformational/morphological changes (Murphy and Roberts, 2013) in these species as a function of temperature. Based on the findings, a kinetic model was developed, common kinetic parameters for time evolution of monomer, dimer and soluble aggregate concentrations were derived and mechanistic insights about the aggregation process were drawn.

2. Materials and methods

2.1. Materials

BSA (Product #A7906), potassium phosphate dibasic trihydrate, potassium phosphate monobasic and phosphoric acid were purchased from Sigma-Aldrich (Missouri, USA) at the highest grade available and used with no further purification. Polyamide filters with 0.45 μm pore size and 47 mm diameter were obtained from Sartorius AG (Goettingen, Germany). Deionised water was used throughout all experiments.

2.2. Melting temperature studies

For all aggregation studies, a 20 mg/mL stock solution of BSA in sterilised 150 mM potassium phosphate buffer (pH 6.5) was prepared, filtered and kept refrigerated at 4 °C. The existence of seed aggregates in unstressed samples (Arakawa and Kita, 2000; Bagger et al., 2007; Vaiana et al., 2004; de Frutos et al., 1998; Brahma et al., 2005; Barone et al., 1992; Honda et al., 2000; Yohannes et al., 2010) was checked using SEC-HPLC as outlined below. To determine the T_m, a 0.2 mg/mL BSA solution was prepared from the stock. Unfolding was tracked through intrinsic trp fluorescence. The steady-state fluorescence of the samples was measured using a Horiba Jobin Yvon Fluorolog FL-322 (New Jersey, USA) and its associated software, FluorEssence for Windows V3.5. A Hellma quartz microcuvette (New York, USA) was used for all measurements. The microcuvette was filled with much more sample than usual (1 mL) and used with a lid to minimise sample loss through evaporation during heating. The sample chamber was piezoelectrically heated to temperatures from 25–97 °C in 3 °C increments. The samples were allowed to equilibrate for 1 min at each temperature point before their spectra were taken. Samples were excited at 295 nm and emission was scanned from 300–500 nm. Both excitation and emission slits were kept at 2 nm. All necessary corrections and blank subtractions were performed for all spectra. Data analysis was done with Igor Pro 6 (Oregon, USA).

2.3. Accelerated BSA aggregation studies

For accelerated aggregation studies, the 20 mg/mL stock solution was used. 30 μL aliquots of this solution were incubated at 60, 65, 70 and 75 °C for varying time periods to thermally induce aggregation. An Applied Biosystems 2720 thermal cycler (California, USA) was used for incubation to prevent inhomogeneous heating and sample evaporation. Rigorous disturbance of the samples through vortexing, centrifugation and shaking was avoided throughout all experiments to prevent possible aggregation and/or disassembly of formed aggregates (Demeule et al., 2009; Buell et al., 2014). Mixing for uniformity was only performed by gently pipetting samples up and down. Incubated samples were placed in ice for 10 min to quench aggregation before a triplicate of 5 μL injections into the HPLC column was performed for each sample.

SEC-HPLC experiments were conducted using an Agilent 1200 series (California, USA) with the Agilent ChemStation for LC software (Rev.B.02.01). A Tosoh TSKgel Super SW3000 SEC column was used with a Super SW guard column (P/N 18762) (Ohio, USA) to separate the different species. The column was saturated with a concentrated BSA solution during assay optimisation stages to curb protein–column interactions prior to the actual aggregation experiments. The auto sampler and column were maintained at 4 °C and 22 °C respectively and absorbance was monitored at 280 nm. Sterilised 150 mM potassium phosphate buffer (pH 6.5) was used as the mobile phase.

If samples precipitated, the insoluble aggregates were prevented from entering the column in the interest of maintaining column integrity (Brummitt et al., 2011a, 2011b). Also, if and when macroscopic changes in the form of precipitation and/or gelation were associated with a discrepancy in chromatograms, namely with considerable losses