BIOTECHNOLOGY

Equilibrium Studies of Protein Aggregates and Homogeneous Nucleation in Protein Formulation

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ABSTRACT: Shaking or heat stress may induce protein aggregates. Aggregation behavior of an IgG1 stressed by shaking or heat following static storage at 5 and 25°C was investigated to determine whether protein aggregates exist in equilibrium. Aggregates were detected using different analytical methods including visual inspection, turbidity, light obscuration, size exclusion chromatography, and dynamic light scattering. Significant differences were evident between shaken and heated samples upon storage. Visible and subvisible particles (insoluble aggregates), turbidity and *z*-average diameter decreased whilst soluble aggregate content increased in shaken samples over time. Insoluble aggregates were considered to be reversible and dissociate into soluble aggregates and both aggregate types existed in equilibrium. Heat-induced aggregates had a denatured protein structure and upon static storage, no significant change in insoluble aggregates content was shown, whilst changes in soluble aggregates content occurred. This suggested that heat-induced insoluble aggregates were irreversible and not in equilibrium with soluble aggregates. Additionally, the aggregation behavior of unstressed IgG1 after spiking with heavily aggregated material (shaken or heat stressed) was studied. The aggregation behavior was not significantly altered, independent of the spiking concentration over time. Thus, neither mechanically stressed native nor temperature-induced denatured aggregates were involved in nucleating or propagating aggregation.
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Keywords: IgG; protein aggregation; spiking; nucleation; seeding; visible and subvisible particles; soluble aggregates; mechanical stress; heat stress; denaturation; shaking; size exclusion chromatography; light obscuration; turbidity; dynamic light scattering

INTRODUCTION

The phenomenon of protein aggregation is a common issue within biotherapeutics and it is becoming increasingly important to understand the interactions, causes, and analyses of protein aggregates. The control of aggregate formation is crucial as it has been reported that protein aggregates may have a lower activity compared to the native protein.[1] In addition, some types of aggregates are considered to have the potential to generate an immune response[2–4] or anaphylaxis[2,5] although other authors have reported

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that aggregated rFVIII showed no enhanced immunogenicity.[6] However, it is currently not clear whether aggregates or which type of protein aggregates may be causing an immune response.

"Protein aggregates" were defined previously[7] as a combination of protein species of higher molecular weight such as oligomers or multimers instead of the desired defined species (e.g., a monomer) and are therefore an universal term for all kinds of not further defined multimeric species that are formed by covalent bonds or noncovalent interactions. Within this definition a protein aggregate consists of two or more proteins molecules where the individual protein molecule may either be native, unfolded or partially unfolded.[8,9]

Noncovalent protein aggregation is due to protein–protein interaction and may be due to weak forces such as van der Waals interactions, hydrogen bonding, hydrophobic and electrostatic interactions.[10] The simplified model of protein aggregation[8] in Figure 1 indicates that aggregation may proceed from native or unfolded state and that various sizes and types of aggregates may exist. The aggregation process may in general lead to smaller (soluble) or larger (insoluble) aggregates or precipitation.[8,9,11,12] The authors consider aggregates as soluble when they are separated from the monomer peak in a standard size exclusion high performance liquid chromatography (SE-HPLC) as oligomers including dimers, whereas insoluble aggregates cannot be detected by SE-HPLC as these aggregates are prevented from entering the SE-HPLC column, for example by a precolumn, being mechanically stuck in the column bed or by sample preparation such as centrifugation. Insoluble aggregates may be assessed by, for example, visual inspection or light obscuration techniques, though these methods cannot specifically measure protein-derived particles as these methods cannot distinguish the source of particle being measured.

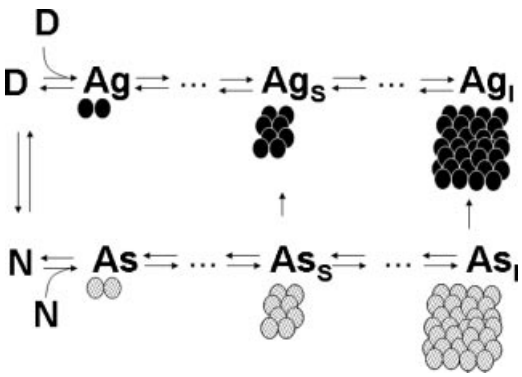

Figure 1. Simplified model of proposed protein aggregation and association mechanisms representing denatured protein molecules (●) and native protein molecules (◍). (D), Denatured state; (Ag), aggregate formation; (Ag_S), soluble aggregates; (Ag_I), insoluble, macroscopic aggregates; (N), native state; (As), protein association; (As_S), soluble protein association; (As_I), insoluble, macroscopic protein association.

Different stress applied to a protein could result in the formation of various different types of noncovalent aggregates. Heat-induced soluble, nonnative aggregates form as the protein undergoes unfolding with increasing temperature.[13] In contrast, mechanical stress results in noncovalent native soluble and insoluble aggregates.[9] However, published reports on aggregation pathways of pharmaceutically relevant proteins such as monoclonal antibodies are still very limited. It is considered that protein aggregates may exist in an equilibrium state, as depicted by the arrows in Figure 1. The equilibrium between different sizes and types of aggregates may be shifted by temperature, surface area changes, freezing/thawing, organic solvents, exposure to interfaces/surfaces or denaturing chemicals and may also be affected by parameters such as pH, ionic strength, buffer composition, or surfactant concentration.[7] However, the equilibrium between various aggregate sizes and forms such as soluble and insoluble aggregates especially is poorly understood and was therefore in the scope of this study.

Protein aggregation has also been described to follow a nucleation–propagation polymerization mechanism.[13–16] Glass nanoparticles seeded or spiked to protein formulations were shown to act as heterogeneous nuclei and induce native protein aggregates, however subsequent growth of aggregates was limited in the case of recombinant human platelet-activating factor acetylhydrolase.[14] Other sources of heterogeneous nuclei may include particles shed from pumps.[16] It was shown that spiking an unstressed monoclonal antibody with pumped stressed buffer resulted in the formation of subvisible native-like protein particles after storage at 25°C. Nucleation may also be generated from protein that has an altered monomeric structure or by multimers.[13,15] In seeding experiments with heat (65°C) and acidic conditions (pH 3.5) induced soluble, nonnative