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Identifying protein aggregation mechanisms and quantifying aggregation rates from combined monomer depletion and continuous scattering

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ABSTRACT

Parallel temperature initial rates (PTIR) from chromatographic separation of aggregating protein solutions are combined with continuous simultaneous multiple sample light scattering (SMSLS) to make quantitative deductions about protein aggregation kinetics and mechanisms. PTIR determines the rates at which initially monomeric proteins are converted to aggregates over a range of temperatures, under initial-rate conditions. Using SMSLS for the same set of conditions provides time courses of the absolute Rayleigh scattering ratio, $I_R(t)$, from which a potentially different measure of aggregation rates can be quantified. The present report compares these measures of aggregation rates across a range of solution conditions that result in different aggregation mechanisms for anti-streptavidin (AS) immunoglobulin gamma-1 (IgG1). The results illustrate how the two methods provide complementary information when deducing aggregation mechanisms, as well as cases where they provide new mechanistic details that were not possible to deduce in previous work. Criteria are presented for when the two techniques are expected to give equivalent results for quantitative rates, the potential limitations when solution non-idealities are large, as well as a comparison of the temperature dependence of AS-IgG1 aggregation rates with published data for other antibodies.

1. Introduction

Proteins are inherently labile molecules that maintain a dynamic equilibrium between folded and unfolded or partly unfolded states in solution, with only a marginal free energy bias towards folded states under native-favoring or folded-state favoring conditions [1]. Proteins such as monoclonal antibodies (MAbs) are one of the fastest growing classes of new therapeutic candidates in the pharmaceutical industry [2]. As part of the process for developing therapeutic protein drug products, one must assess and optimize their *in vitro* stability in the context of a number of degradation routes [3]. One of the most prevalent routes is non-native aggregation, which generally refers to processes by which an otherwise natively folded, monomeric protein can become incorporated into aggregates that are composed of partly or fully unfolded protein chains [4]. In many cases, the aggregates are stabilized by strong noncovalent contacts between hydrophobic amino acids, as well as hydrogen bonding between the amide backbone of neighboring proteins. As a result, such non-native aggregates often have a large degree of inter-protein beta-sheet structure, and are effectively irreversible under the solution conditions that they form [5,6]. While some of the early stages of non-native aggregation (hereafter referred to simply as aggregation) are often reversible, the net aggregation process is irreversible and this requires one to consider aggregation rates and mechanisms or pathways when designing rational strategies to control and improve protein stability [3,7].

There are too many possible aggregation mechanisms to realistically summarize within the scope of this report. However, work to date indicates that a number of therapeutic proteins such as MAbs [8–15], antibody fragments [16,17], and cytokines [18,19] share a relatively common set of possible aggregation pathways

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that are also adopted by non-therapeutic proteins [20–22]. Fig. 1 summarizes these schematically for the case of an antibody, and is adapted from Refs. [6,23]. Briefly, monomeric proteins can partly or fully unfold to reveal hydrophobic sequences that are able to form strong inter-protein contacts that stabilize aggregates – so called, aggregation-prone "hot spots" [24,25]. This unfolding process is reversible if the monomers are able to refold prior to encountering another protein. Under most conditions of practical interest, the temperature is sufficiently far below the midpoint unfolding temperature (T_m) that the unfolding transition(s) will equilibrate more rapidly than the time scales for subsequent aggregation events [7]. In this case, the fraction of the monomer population that comprises the (partly) unfolded or "reactive" (R) state is determined by the thermodynamics of unfolding. Therefore, the net rate of aggregation can change by orders of magnitude with a temperature change of only 5–10 °C, because the equilibrium constant for unfolding (K) has a large unfolding enthalpy (ΔH_{un}) associated with it [26,27].

Association of R monomers may involve reversible steps prior to nucleation of the smallest species that are effectively irreversible; termed nuclei and denoted by A_x (x = nucleus stoichiometry) in Fig. 1. Historically, many of the protein and peptide systems that were studied showed rapid "downhill" polymerization of these initially small aggregates. This led to the use of the term "nuclei", by analogy with nucleation and growth in phase transitions [7]. More recently, it has been shown that protein such as MAbs display a wider variety of behaviors. In some cases, they form irreversible dimers (x = 2) but those dimers grow very slowly, if at all [8,11,12,28]. In the nomenclature of ref. [11,12], this was termed nucleation-dominated (ND) aggregation, and corresponds to effectively stopping at stage (3) in Fig. 1. In other cases, nucleation is combined with significant consumption of monomer via growth by monomer-addition or "chain polymerization" (CP), which corresponds to the combination of stages (1) to (4) in Fig. 1 [10–12,29]. Finally, when aggregates associate rapidly with each other one can observe growth via association polymerization (AP) to form very large soluble species, as well as essentially phase separation (PS) of the aggregates [8,10–12,29–32].

Recently, the effects of solution conditions (pH, NaCl concentration, and buffer species) were determined systematically for the aggregation mechanisms of an immunoglobulin gamma-1 (IgG1) antibody that targets streptavidin [11,12], and that will serve as a test system in this report. For example, the mechanism shifted between ND, CP, and PS simply by changing the solution pH between 4 and 6 with 5 mM sodium citrate buffer [11,12]. That work utilized *ex vivo* laser scattering with size exclusion chromatography (SEC) for a single temperature for each solution conditions, and required multiple samples at each temperature. From a practical perspective, this required significant user manipulation, user time, sample material, and also did not address the question of whether the mechanisms change as a function of temperature. The present report focuses on an approach to circumvent those limitations by combining two recently developed methods to obtain temperature-dependent measures of aggregation rates: parallel-temperature initial rates (PTIR) with SEC [33], and simultaneous multiple-sample static light scattering (SMSLS) [34].

Parallel temperature initial rates (PTIR) analysis uses the following approach for quantifying degradation rates as a function of temperature; in this case the degradation route is aggregation. For context, in conventional approaches one determines monomer loss for many samples at predetermined incubation times for a single or small number of temperatures. In the PTIR method, one instead determines monomer loss for a single or small number of samples at many temperatures for the same incubation time. It has been shown elsewhere, that in the initial-rate regime the two approaches are quantitatively equivalent, but the PTIR method is more sample sparing and efficient [33]. Because aggregation is determined only for the initial rates regime, no specific aggregation model needs to be assumed, although they can be inferred later (see Discussion). The resulting aggregation rate coefficient (k_{obs}), or reduced initial-aggregation-rate (units of 1/time), is given generally by

$$k_{obs}(T) = \frac{1 - c_m(T)/c_{m,0}}{t} \quad (1)$$

and is based on the general result that many mechanisms reduce to zero-order kinetics in the limit of small extents of reaction [33,35]. In the above relations, t is the preselected incubation time, T is the incubation temperature, c_m is the concentration of monomer at T for that incubation time, and $c_{m,0}$ is the initial concentration prior to heating. In previous work, the ratio of c_m to $c_{m,0}$ was defined as the monomer fraction (m) [12,33].

The development of simultaneous multiple static light scattering (SMSLS) was based on the idea that the many methods monitor only a small number of particular time points due to the need for *ex situ* analysis, or they monitor a single sample in situ and do not take advantage of the ability to reconfigure the instrument to monitor multiple samples in parallel. SMSLS monitors many samples in parallel and allows one to take advantage of a similar parallel approach to what PTIR is based upon. In the limit of low protein concentration (more rigorously, the limit of negligible non-idealities from protein-protein and protein solvent/cosolute interactions), light scattering (LS) provides the weight-averaged molecular weight (M_w) for a given sample [34,36]. In addition, the weight-averaged molecular weight necessarily

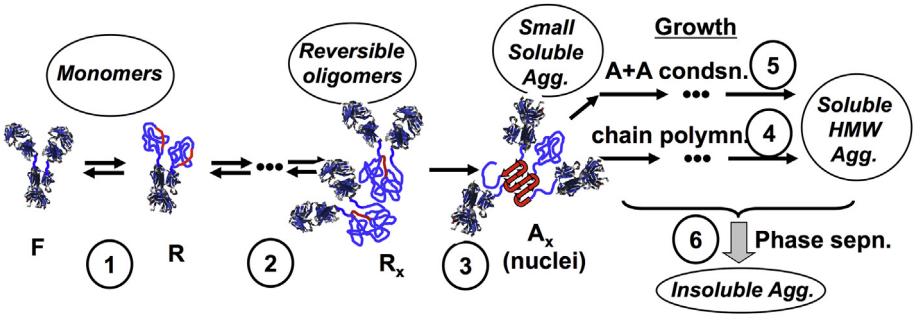

Fig. 1. Schematic depiction of multiple stages in non-native aggregation of monoclonal antibodies in solution: (1) partial unfolding to reveal aggregation-prone "hot spots" (red); (2) weak, reversible self-association, prior to (3) structural rearrangement to form effectively irreversible nuclei that then have the potential to grow via (4) monomer addition (termed chain polymerization) or (5) aggregate-aggregate coalescence (association polymerization) and ultimately (6) aggregate phase separation. Adapted from Ref. [6,23].