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Both Reversible Self-Association and Structural Changes Underpin Molecular Viscoelasticity of mAb Solutions

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The role of antibody structure (conformation) in solution rheology is probed. It is demonstrated here that pH-dependent changes in the tertiary structure of 2 mAb solutions lead to viscoelasticity and not merely a shear viscosity (η) increase. Steady shear flow curves on mAb solutions are reported over broad pH ($3.0 \leq \text{pH} \leq 8.7$) and concentration ($2 \text{ mg/mL} \leq c \leq 120 \text{ mg/mL}$) ranges to comprehensively characterize their rheology. Results are interpreted using size exclusion chromatography, differential scanning calorimetry, analytical ultracentrifugation, near-UV circular dichroism, and dynamic light scattering. Changes in tertiary structure with concentration lead to elastic yield stress and increased solution viscosity in solution of "mAb1." These findings are supported by dynamic light scattering and differential scanning calorimetry, which show increased hydrodynamic radius of mAb1 at low pH and a reduced melting temperature T_m, respectively. Conversely, another molecule at 120 mg/mL solution concentration is a strong viscoelastic gel due to perturbed tertiary structure (seen in circular dichroism) at pH 3.0, but the same molecule responds as a viscous liquid due to reversible self-association at pH 7.4 (verified by analytical ultracentrifugation). Both protein–protein interactions and structural perturbations govern pH-dependent viscoelasticity of mAb solutions.

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Introduction

mAbs are ubiquitously applied in the treatment of a plethora of diseases, ranging from autoimmune disorders to cancers.[1-6] They are polyampholytic macromolecules as they contain both acidic and basic functional groups. They are typically formulated at high concentrations to meet patient weight–based dosing and efficacy requirements demanded by pharmacokinetics and potency. A major challenge in the development of protein therapeutics is high solution viscosity, which is common in high-concentration mAb solutions. In particular, viscosity can compromise unit operations, such as protein purification, formulation, and fill or finish, affect storage stability, and make facile dose delivery to patients a challenge. Intrinsic molecular properties like reversible self-association (RSA) and exogenous factors, such as pH, temperature, and excipient concentration can significantly affect mAb stability and

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solution rheology. Additionally, common pH-dependent processing steps and agitation-induced stresses imparted by pumping and transportation can alter tertiary structure, thereby increasing aggregation propensity and viscoelasticity[3,5-7] due to attractions between hydrophobic amino acid residues.

Numerous literature studies have nicely elucidated the interrelationship between intermolecular interactions and viscosity for self-associating antibody solutions. It has been proposed that the high viscosity of concentrated (>100 mg/mL) mAb solutions is primarily due to the formation of self-associated equilibrium clusters.[8-13] Several studies[12-16] of the pH dependence of viscosity of self-associating antibody solutions used measurements at 10 MHz frequency to quantify the influence of self-association on viscoelasticity. Strong correlations between the increased apparent molecular weight of aggregates in high-concentration mAb solutions and high viscosity lend credence to the notion that self-associated clusters contribute to increased viscosity. Indeed, several recent studies of the relationship between cluster size and solution viscosity confirm a general physical picture that the high viscosity of self-associating mAb formulations is primarily due to an increase in the size of self-associated clusters, which increases the microscopic friction coefficient, ζ.[17-19] ζ is related to the self-diffusion coefficient, D, through the fluctuation−dissipation theorem derived by Einstein: $D = k_BT/\zeta$; k_B and T denote Boltzmann's constant and absolute temperature, respectively. As ζ increases, D decreases, and hence matrix viscosity, η, increases because D and η are inversely related through the generalized Stokes−Einstein−Sutherland equation.

Given the broad range of timescales associated with formulation development and bioprocessing of therapeutic protein formulations, spanning seconds to months, rheological tests must be carefully chosen to gauge the relationship between thermodynamic and kinetic stability and viscoelasticity of high-concentration liquid formulations. The timescales associated with stability of biotherapeutics typically span minutes to several years (shelf-life stability). Steady shear rheology can provide insights into viscoelasticity related to simple shear of drug substance and product in pumps[20] and delivery devices. Low shear rate ($\dot{\gamma}$)/frequency (ω) viscosity is particularly useful as an indicator of stability against aggregation in protein formulations,[21] whereas high shear measurements are representative of subcutaneous delivery of 1 mL of drug in 10 s through a regular wall 27G needle (inner diameter = 290 μm), which gives an apparent wall $\dot{\gamma} = 1.6 \times 10^5\ s^{-1}$).

We elucidate in this article the connection among macromolecular conformation, protein−protein interactions, and rheology of solutions of an antibody that weakly self-associates at pH 7.4 (mAb1) and also for a strongly reversibly self-associating antibody (mAb2). We characterize intermolecular interactions using dynamic light scattering (DLS) and conformational changes using differential scanning calorimetry (DSC) and near-UV circular dichroism (nUV CD). We find that tertiary structure changes profoundly with concentration and pH for non−self-associating mAb1. Conformational changes profoundly affect steady shear solution rheology. Conversely, the conformation of the self-associating mAb (mAb2) is only perturbed at pH 3.0, whereas its conformation remains unchanged at pH = 5.0, pH = 6.0, and pH = 7.4; DLS in dilute solutions reveals that intermolecular attractions are strongest at pH 7.4, where RSA is manifest. These concentrated mAb solutions display viscoelasticity that run the gamut from viscoelastic liquid behavior to viscoelastic solid behavior. These phenomena occur over a broad range of timescales, thus underscoring the critical need to study solution rheology over a suitably broad range of shear rates/frequencies. Our results of the role of antibody structure in determining solution rheology agree qualitatively with results reported by Cheng et al.[22]

Materials and Methods

Monoclonal Antibodies

mAb1 and mAb2 are recombinant humanized monoclonal IgG1 and IgG2 antibodies, respectively, which are expressed in Chinese hamster ovary (CHO) cells and purified by chromatographic methods such as ion-exchange chromatography and protein A chromatography for removing host cell proteins; mAb1 comprises 2 heavy chains of approximately 49.5 kDa each and 2 light chains that are 23.1 kDa each, for a total molar mass of ~145 kDa; mAb2 consists of 2 identical heavy chains and 2 identical light chains giving a total molar mass of 147 kDa. The isoelectric points (pI) of mAb1 and mAb2, determined by capillary isoelectric focusing, are 8.65 and 8.3, respectively. The purification procedure is described in detail in the Supplementary Material.

Tangential Flow Filtration and Formulation

Tangential flow filtration (TFF) was performed on mAb1 and mAb2 using a PureTech TFF unit (SciLog, Madison, WI). The starting concentration was 100 mg/mL for mAb1 in a histidine buffer at pH 6.0 and 5 mg/mL in an acetate buffer for mAb2. Pellicon 3 TFF membranes (Millipore, Billerica, MA) with surface areas of 88 cm^2 were fastened in a membrane holder (Sartorius, Göttingen, Germany) and exchanged into 20 mM ionic strength (I) buffers in H_2O. Constant volume diafiltration, where new buffer is added to the protein solution at the same rate as volume is removed through the TFF membrane, was used to achieve exchange exceeding 99% into the new buffer.

The buffers comprised sodium citrate/citric acid at pH 3.0, sodium acetate/acetic acid at pH 4.0 and pH 5.0, respectively, histidine hydrochloride at pH 6.0, mono/disodium phosphate at pH 7.4, and Bicine at pH 8.7. After diafiltration, volume was again reduced in the retentate, thus concentrating the mAb to >120 g/L during each run. The protein solution was then drained from the TFF system into a separate container. Yield was maximized by recirculating a small volume of fresh buffer through the system for ~5 min and subsequently draining this solution into the same container as the concentrated product. The stock solutions were subsequently filtered through 0.22 μm filters made of poly(ethersulfone) membranes (Thermo Scientific, Billerica, MA) and were then gravimetrically diluted to the final concentrations (2-120 mg/mL). Protein concentration was measured using absorbance at wavelength, λ = 280 nm (A_{280}) on an Agilent 8453 UV-visible spectrophotometer with λ = 1.42 mg/cm-mL for mAb1 and 1.67 mg/cm-mL for mAb2. All solutions were stored between 2°C and 8°C until use.

Size Exclusion Chromatography

Size exclusion chromatography (SEC) was performed on 10 mg/mL sample of mAb; 250-μg samples were injected into a UV-detector−equipped Agilent 1100 series HP-SEC, which was loaded with a Tosoh G3000WXL column of 25 nm average pore size. The HP-SEC mobile phase contains 100 mM anhydrous dibasic sodium phosphate (JT Baker catalog # 5062-05), 100 mM sodium sulfate (JT Baker catalog # 3898-05), and 50 μM sodium azide (Sigma-Aldrich, St. Louis, MO) at pH 6.8, titrated with 1 N hydrochloric acid (Sigma-Aldrich).

SEC−Multiangle Light Scattering

SEC was performed on mAb2 using a TSK gel G3000 SW 7.8 mm × 30 cm, 5 μm (TOSOH Bioscience) column connected to an

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