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Micro- and macro-viscosity relations in high concentration antibody solutions

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Abstract

Molecular crowding in highly concentrated monoclonal antibody (mAb) solutions results in significant increases in viscosity, which complicates fill-finish steps and patient administration by subcutaneous injection. As viscosity measurements for optimization of the mAb formulation require significant amounts of material not always available in early development, fluorescence correlation spectroscopy (FCS) is evaluated as a potential ultra-low volume technique for viscosity measurement of high concentration protein solutions assuming the Generalised Stokes Einstein relation (GSE) remains valid. Using like-charge fluorescent tracers of different sizes, FCS provided measurements of microviscosities which were compared to the macroviscosity. After parametrising the protein concentration dependence of the viscosity by the exponential coefficient (k) of a simple exponential model, FCS derived k -values of like-size tracer to the crowder followed the same ordering as the macroviscosity derived k -values with respect to solvent conditions. Furthermore, k and the diffusion-derived protein-protein interaction parameter, k_D , are linked, and, attractive conditions for mAbs result in a stronger concentration dependence of the viscosity. For tracers and crowders of like-size, a key result is negative deviations from the GSE relation are observed in presence of strong attractive interactions between crowder molecules. These data demonstrate that FCS has application to the screening of high concentration mAb solutions for formulation selection.

Keywords: fluorescence correlation spectroscopy, viscosity, protein solutions, antibodies, molecular crowding, Generalised Stokes Einstein relation

Introduction

The viscosity of high concentration protein solutions (> 100 g/L) is governed by weak protein-protein interactions and transient conformational changes [1, 2] which are dependent on environmental parameters such as the solution dielectric constant, ionic strength and pH [3, 4]. Apart from traditional rheological techniques, microviscosities can be characterized from measurements of the long-time self-diffusion or the mean square displacement of tracers and applying the generalised Stokes Einstein (GSE) relation [5]. This was first demonstrated by Muramatsu and Minton, who studied the diffusion of various fluorescently labelled tracer proteins in solutions crowded with globular proteins via measurements of boundary spreading [6], showing that the fractional reduction of the diffusion of the tracer increased with increasing size of tracer species and with decreasing size of crowding protein. Additional techniques commonly used for measuring tracer diffusion include pulsed-field-gradient NMR [5, 7, 8] or dynamic light scattering (DLS) with micron sized tracer particles[9].

FCS is an ideal tool to study the long-time translational self-diffusion of tracer particles in monoclonal antibody (mAb) solutions because fluorescence fluctuations are measured in a microscope focal volume of less than one femtolitre, which, in turn, requires very small protein sample volumes [8, 10-12]. The long-time self-diffusion coefficient derived from FCS measurements relates to a solution microviscosity, η' , according to the generalized Stokes–Einstein (GSE) relation [5].

$$D = \frac{k_B T}{6\pi\eta' R_H} \quad (1)$$

where $k_B T$ denotes the thermal energy, R_H is the hydrodynamic radius of the fluorescently labelled tracer. Being able to use the GSE requires knowing how to relate the microviscosity η' to the macroviscosity η [13], [14] [15]. It is common to define a retardation factor, r' in terms of the relative diffusion coefficient D_0/D (or η'/η_0), and a macroscopic retardation factor r , which is equal to the relative viscosity η/η_0 . The GSE is applicable when the retardation factors are equal to each other, while negative deviations correspond to when the GSE underpredicts the macroscopic viscosity ($r' < r$), and positive deviations occur when GSE overpredicts the slowing down of motion or when $r' > r$.

When the GSE is applicable ($r' = r$), the microviscosity is equal to the macroviscosity: this case is observed when using tracer particles with sizes much larger than the crowder protein, [16] but a key question is the applicability of the GSE when the tracer particle size is similar to that of the crowder. This issue has been addressed by different groups using fluorescence correlation spectroscopy (FCS) to measure the diffusion of a range of tracers in polymeric crowders [17-19]. Holyst et al. measured the self-diffusion of rhodamine 6G, lysozyme, apoferritin and silica spheres in concentrated polyethylene glycol (PEG) solutions (molecular weights 6000, 12000, 20000 g/mol at concentrations 0-50 %v/v). They found the motion of nanoscopic probes in PEG solutions could be related to the macroviscosity of aqueous PEG solutions [17] using a semi-empirical model; following analyses they suggested that a probe needed to have four times the hydrodynamic radius of PEG to feel the macroviscosity [18]. Lavalette et al. [19] measured the rotational and translational self-diffusion of proteins (GFP, BSA, α 2-macroglobulin, earthworm haemoglobin and integral earthworm haemoglobin) in various dextran crowders (from 6kDa to 2,000kDa). They described the variation of the diffusion ratio as a function of the relative macroviscosity by including an exponent (q) related to the ratio of tracer size over the crowder size. In an apparent conflict with Muramatsu and Minton [6], they suggested translation and rotation of proteins were accelerated in dilute macromolecular crowders compared to their value in solvents of the same viscosity. In other studies, when using colloidal crowding agents, the GSE is applicable for describing long-time self-diffusion coefficients when the tracer and crowder particles have the same size [20, 21]. However, it still remains unclear how size difference between the crowder and tracer impacts protein solutions. Muramatsu and Minton [6] measured the diffusion coefficients for four types of tracer proteins with sizes ranging between 17 and 150 kDa each in solutions with four different crowder proteins with sizes between 13 kDa and 150 kDa. Analogous to polymers crowders, the retardation of a tracer protein decreased with increasing size of the crowder protein. However, Wang et al. [16] found that polymers do not mimic the behaviour of protein crowders when using chymotrypsin inhibitor 2 (CI2) as a tracer protein. Negative deviations to the GSE occur when using synthetic polymers as crowders, which is expected because the polymer molecular weights are ten times as large as CI2. However, in concentrated solutions of lysozyme, CI2 motion is retarded more than expected from the macroscopic viscosity, while

1 the GSE is applicable in crowded solutions with bovine serum albumin (BSA) or ovalbumin,
2 even though BSA and ovalbumin are larger in size than Cl2.
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4 Another key factor is direct tracer interactions with the crowder molecules that impede or
5 retard the tracer motion. The qualitative difference in diffusivity of Cl2 was attributed to
6 specific interactions of Cl2 with protein crowders that do not exist with inert polymers,
7 which was also evident from measurements of rotational diffusion coefficients that are
8 more sensitive to tracer-crowder association. Positive deviations in the GSE have also been
9 observed in mixtures of labelled apomyoglobin with either human serum albumin (HSA) or
10 ribonuclease-A (RNase-A) [22]. The observed behaviour followed predictions based on
11 Muramatsu and Minton's model [6]. Attractive electrostatic interactions between the
12 crowder proteins (HSA or RNase) and the tracer (apomyoglobin) could not be ruled out as
13 a cause of the GSE positive deviations [22]. A similar conclusion was reached in a study
14 using mixtures of the Src-homology 3 (SH3) domain with BSA [5]. SH3 was chosen as a
15 model protein because the protein binds to arginine and proline rich motifs that exist on the
16 surface of BSA. The binding between SH3 and BSA was confirmed by isothermal titration
17 calorimetry, which provided direct evidence that strong tracer-crowder associations cause
18 significant tracer retardation. In contrast, when tracer and crowder are the same protein,
19 the GSE is applicable for describing self-diffusion coefficients. In addition to solutions
20 containing SH3 or BSA, the agreement has been observed for α B-crystallin and lysozyme
21 when using a labelled tracer of the same crowder protein [8]. Out of these proteins, hard
22 sphere models capture the thermodynamic and transport properties only for α B-crystallin.
23 For BSA and lysozyme, fitting to structure factor profiles indicates there is weak protein self-
24 association, which is also apparent from rotational diffusion coefficient measurements.
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26 More recently, Hung et al. measured the self-diffusion of a labelled mAb in a solution
27 crowded by the unlabelled mAb using FCS across a range of solution conditions [23]. The
28 protein-protein interactions were tuned from weakly attractive to hard-sphere like by using
29 solubility enhancing excipients such as arginine chloride or imidazole [24]. In contrast to
30 previous protein studies, the tracer protein is retarded less than expected based on the
31 macroscopic viscosity, where the negative deviations to the GSE become more significant as
32 the protein-protein attractions are mitigated. To explain this phenomenon, self-association
33 between tracers was suggested to increase in more viscous formulations i.e. in 250mM NaCl
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solutions as opposed to 0.1M imidazole solutions; however, this is in stark contrast to Roos et al. paper [8]. After implementing the Holyst model [18], Hung et al. found a correlation between Holyst's interaction parameter and the relative viscosity or the strength of the short range attractive protein-protein interactions derived from SAXS data using the Yukawa model. Finally, it should also be noted that the diffusion of a tracer particle in a concentrated protein solution may be anomalous as often suggested for transport in cells or in polymers [11] although caution should be taken as the origin of the anomalous behaviour may have a number of origins not related to the macromolecular transport itself such as change of refraction index.

Here, we move away from the recent Hung et al. work as FCS measurements are carried out to determine relative microviscosities using tracer molecules of different sizes in solutions with two different mAbs or BSA and compared against rheometry measurements. Previous studies suggest mAbs exhibit negative deviations to the GSE, while the GSE is applicable to globular proteins where the tracer and crowder are the same protein. It is not clear if the difference in behaviour between mAbs and globular proteins arises due to the differences in shape and size or due to effects of transient interactions between the protein molecules. In the present study, measurements are carried out over a range of solution conditions chosen to tune the protein self-association properties. We show that the FCS experiments demonstrate a clear effect of tracer size and observe a negative deviation from the GSE with the mAb solutions. However, in contrast to Hung et al. [23], we find GSE is applicable to mAb solutions in the limiting case of weakly attractive interactions between proteins, but negative deviations arise when there exists strong association between crowder proteins.

Materials and Methods

Materials

Two mAbs termed COE-03 (human IgGk, MW 144.8 kDa, pI = 8.44) and COE-19 (human IgG1 λ , MW 148 kDa, pI = 7.4) were kindly provided by Astra Zeneca (Cambridge, UK). Protein-protein interaction measurements for COE-03 have been reported in Roberts et al. [25, 26]. In Singh et al. [27] results are provided for COE-03 and for COE-19 (COE-03 is mAb1 and COE-19 is mAb2 in that study).

1 ATTO-Rho6G, BSA-Alexa Fluor™ 488 (BSA-AF), mouse IgG1 isotype control labelled with
2 Alexa Fluor™ 488 (IgG-AF) and mouse IgM isotype - custom labelled with Alexa Fluor™ 488
3 by Thermo Fisher- (IgM-AF) were acquired from Thermo Fisher Scientific (Leicestershire,
4 UK). 5-Tetramethylrhodamine labelled RRADDSDDDDD peptide (single letter amino acid
5 code, termed hereafter 'TMR peptide') was obtained from Cambridge Bioscience
6 (Cambridge, UK). Rhodamine Green™ was acquired from Invitrogen (Paisley, UK). Note that
7 all tracers were chosen to have a charge of the same sign as the molecular crowder.
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10 Lab-Tek Nunc® eight-well chamber slides, scintillation vials, toluene and sucrose were
11 obtained from Fisher Scientific Ltd (Leicestershire, UK). BSA, buffer components and salts
12 (acetic acid, sodium acetate, Tris, Tris.HCl, sucrose, histidine, sodium thiocyanate, and
13 sodium chloride) were acquired at analytical grade from Sigma Aldrich (Dorset, UK). All
14 buffers (Table 1) and solutions were prepared with Millipore de-ionised water (18 MΩ.cm)
15 and pre-filtered prior to experiments.
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18 mAbs COE-03 and COE-19 were dialysed with 20 kDa molecular weight cut off (MWCO)
19 Slide-A-Lyzer cassettes (Slide-A-Lyzer G2 cassette, 10,000 MWCO, Thermo Fisher Scientific,
20 Leicestershire, UK, following the manufacturer's instructions) at 4°C from stock pH 6.0
21 buffer (25mM Histidine, 205mM sucrose) to pH 5.0 buffer (25 mM ionic strength (IS) sodium
22 acetate) for COE-03 or directly into the target buffer for COE-19. mAb was concentrated
23 with a 30 kDa MWCO ultrafiltration membrane in a nitrogen pressurised Amicon stirred cell
24 at 4°C. The mAb solution was finally dialysed at 4°C overnight, with a typical exchange factor
25 of 1000×, into the target experimental conditions described in Table 1. Protein preparations
26 were filtered with 0.2 or 0.45 µm syringe or centrifuge filters where possible. To monitor
27 protein self-buffering, concentrated protein solution pH was measured with a semi-micro
28 pH probe. In instances of pH shift from protein self-buffering where dialysis failed to correct
29 this, buffer was reformulated to match and dilute the protein. Protein concentration was
30 measured by UV absorption at 280 nm (Nanodrop 2000, Thermo Fisher Scientific,
31 Leicestershire, UK) following a solution equilibration period of hours for reliable
32 measurement (COE-03, $\epsilon = 1.435 \text{ mg/ml}^{-1}\text{cm}^{-1}$, pI 8.44; COE-19, $\epsilon = 1.78 \text{ mg/ml}^{-1}\text{cm}^{-1}$, pI 7.8).
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35 BSA was first dissolved in buffer pH 7.0 (8 mM sodium acetate, 145 mM NaCl and 0.05 g/L
36 PS80) then dialysed with 20 kDa molecular weight cut off (MWCO) Slide-A-Lyzer cassettes
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following the manufacturer's instructions) at 4°C from stock pH 7.0 (8 mM sodium acetate, 145 mM NaCl and 0.05 g/L PS80) to pH 7.0 buffer (8 mM sodium acetate, 145 mM NaCl and 0.05 g/L PS80 with excipients i.e. sucrose (250mM) or Arg (250mM), Glu (250mM) or Arg-HCl (250mM)). BSA was concentrated with a 30 kDa MWCO ultrafiltration membrane in a nitrogen pressurised Amicon stirred cell at 4°C. The pH of the solutions was adjusted using sodium acetate (for acetate buffer) or hydrochloric acid (for TRIS and histidine buffers). Protein concentration was measured by UV absorption at 280 nm (Nanodrop 2000, Thermo Fisher Scientific, Leicestershire, UK) following a solution equilibration period of hours for reliable measurement ($\epsilon = 0.667 \text{ mg/ml}^{-1}\text{cm}^{-1}$).

Fluorescence Correlation Spectroscopy (FCS)

The experimental approach has been described elsewhere [28]. Briefly, the 488nm Argon laser line of a Zeiss Confocor2 LSM 510 META (Zeiss, Jena, Germany) and a 40x/1.2NA water-immersion objective was utilised. The laser beam waist, ω_0 , was determined by measuring Rhodamine Green™ experimental diffusion time, τ_D , ($33 \pm 5 \mu\text{s}$) then by using Equation 2 and a published diffusion coefficient of $2.8 \times 10^{-6} \text{ cm}^2/\text{s}$ [29]:

$$\omega_0^2 = 4D\tau_D \quad (2)$$

where τ_D is the diffusion time, ω_0 the laser beam waist and D is the diffusion coefficient.

Samples were loaded in duplicate into the corner of Lab-Tek Nunc eight-well chamber slides (Fisher Scientific, Leicestershire, UK) and duplicate measurements collected. A constant volume of fluorescently labelled tracer was added to each mAb or BSA solution in order to have a constant number of fluorescent particles (in the region of 5 and 10 particles per focal volume depending on the experimental condition); care was taken so that the maximum tracer : protein ratio would be at the maximum 1:500 to avoid nucleation [30]. The 40x/1.2W NA collar was adjusted manually in order to minimize the change of the aforementioned number of particles per focal volume as described by Goins et al. [14]. Diffusion measurements were performed at least 40 times and the acquisition time was varied between 5 s and 60 s per run depending on the molecular crowder's concentration.

The correlation function $G(\tau)$ of the temporally changing fluorescence signal $I(t)$ for a single species is defined as:

$$G(\tau) = \frac{\langle \delta I(t) \delta I(t+\tau) \rangle}{\langle I(t) \rangle^2} \quad (3)$$

For a three dimensional diffusion of a single species with triplet state correction $G(\tau)$ is given by

$$G(\tau) = \left(1 - T + T \exp(-\tau/\tau_T)\right) G_D(\tau) \quad (4)$$

where T is the triplet fraction and τ_T is the triplet time

A single-exponential model was applied to fit the autocorrelation curve, $G_D(\tau)$:

$$G_D(\tau) = \frac{1}{N} \left(\frac{1}{1+(\tau/\tau_D)} \cdot \sqrt{\frac{1}{1+S^2(\tau/\tau_D)}} \right) \quad (5)$$

where N is the number of particles and S is the structure parameter.

In complex media, diffusion is often described as being anomalous in which the mean square displacement, MSD , follows the following expression:

$$MSD^2(t) = 6\Gamma t^\alpha \quad (6)$$

Transforming Eq (2) to account for anomalous diffusion results in:

$$G_D(\tau) = \frac{1}{N} \left(\frac{1}{1+(\tau/\tau_D)^\alpha} \cdot \sqrt{\frac{1}{1+S^2(\tau/\tau_D)^\alpha}} \right) \quad (7)$$

When the anomalous coefficient, α , is <1 it is termed subdiffusion; when it is >1 , it is termed superdiffusion. The autocorrelation functions $G(\tau)$ were fitted using either the nonlinear least squares method of the fitting program OriginPro2015 (OriginLab, Northampton, MA) or the MEMFCS software QuickFit 3 [31].

Viscosity measurements

Vilastic. The Vilastic-3 Viscoelasticity Analyser (Vilastic Scientific, Inc., Austin, TX) was used. In Vilastic-3 experiments, the sample fills up a cylindrical measurement tube (radius $R = 0.05$ cm and length $L = 6.4$ cm) and is forced to flow at a given oscillatory shear rate $\dot{\gamma}(t) = \dot{\gamma}_0 \sin(\omega t)$ with frequency ω by a coupling fluid. A sensor measures the pressure drop $\Delta P(t)$ between the two ends of tube. A momentum balance relates $\Delta P(t)$ to the in-phase and out-of-phase components of the stress with the wall shear rate, $\tau'(\omega) = \omega \eta'(\dot{\gamma}_0)$ and $\tau''(\omega) = \omega \eta''(\dot{\gamma}_0)$. $\eta'(\dot{\gamma}_0)$ and $\eta''(\dot{\gamma}_0)$ are the dynamic viscosity and “elasticity” respectively [32]. The dynamic viscosity of the IgG solutions was measured at a frequency of 1 Hz over a range of

shear rates $\dot{\gamma}_0 = 1 - 100 \text{ s}^{-1}$ at 25°C. 2 ml sample cups were used in order to load the samples, and the protein buffer was systematically chosen as the coupling fluid. Measurements were performed 100 times and the output was averaged and repeated in triplicate for each sample.

Rheochip. Shear viscosity measurements were performed using the in-house RheoChip platform. The RheoChip is a microfluidic rheometry device made of polymethyl methacrylate (PMMA) using soft lithographic methods to create three internal rectangular channels of widths $w_c = 800, 200$ and $100 \mu\text{m}$ respectively and nominal height $h = 50 \mu\text{m}$. Channels are oxygen plasma treated to make them hydrophilic and protein adhesion resistant. All experiments are performed using the $200 \mu\text{m}$ channel unless otherwise specified. The channel contains a pressure tap, at a distance from the inlet sufficient for one-dimensional, Poiseuille-like, laminar flow to be achieved. A second pressure tap is located 30mm further along the channel. These pressure taps are each fitted with pressure detection unit consisting of two identical linear strain gauge pressure transducers, enabling the differential pressure drop across the length of channel between the taps to be measured. The RheoChip is calibrated using deionised water with known viscosity corresponding to the measured temperature during calibration. A calibration constant, f , is established for each chip, where

$$f = \frac{1}{\left(\frac{\Delta P}{Q}\right)_{Avg.}} \quad (8)$$

Where ΔP is the differential pressure, Q is the imposed flowrate and the Avg subscript reflects average over multiple shear rates. The RheoChip is filled with the solvent of the sample and allowed to reach steady state prior to measurement. A 1 mL glass syringe filled with protein sample is secured in a Nexus 6000 syringe pump (Chemyx, TX, USA) and connected to the RheoChip apparatus, ensuring there are no air bubbles in the system. The sample is pumped into the chip in a cycle of flowrates from 10 mL/hr to 2 mL/hr in increments of 2 mL/hr, each time allowing sufficient time for steady state flow to be achieved. The pressure drop across the channel at each flow rate is detected and relayed to a data acquisition platform consisting of National Instruments CompacDAQ 9172 chassis, a National Instruments 9237 data acquisition modulus and LabView software (National Instruments).

For each imposed flow rate Q , the shear viscosity at the given shear rate $\dot{\gamma} = \frac{2Q}{w_c h^2}$ is

$$\eta(\dot{\gamma})_{sample} = f \cdot \eta_{H_2O} \left(\frac{\Delta P}{Q} \right)_{sample} \quad (9)$$

where f and η_{H_2O} are the chip calibration constant and the viscosity of deionised water (respectively) at calibration temperature.

k_D measurements- Dynamic light scattering

A Wyatt miniDAWN TREOS with an online QELS module was used for dynamic light scattering measurements (Wyatt Technology, Santa Barbara, CA). The instrument uses a GaAs laser at a wavelength of 658nm at 90°. Measurements were made using a flow cell, with samples delivered using the Wyatt Calypso. The Calypso is a pumping and mixing system which uses three syringe pumps which can be programmed to generate precise concentration step gradients. The flow from each syringe pump is filtered through an inline 0.1 µm membrane. Before entry to the flow cell, the individual streams are mixed by a static mixer.

In each experiment, the protein concentration was varied in equal step changes between 0.4 and 4 mg/ml. All measurements were performed at 25°C. Data analysis was performed using the Wyatt Technology ASTRA Software (version 6.1). The QELS instrument determines the hydrodynamic radius (R_h) by measuring fluctuations in the light scattering intensity due to Brownian motion. The hydrodynamic radius is related to the diffusion coefficient using the Stokes-Einstein equation (see Equation (1)). Linear plots of the mutual diffusion coefficient (D_m) obtained from a cumulants analysis method were plotted against concentration and the interaction parameter was calculated using the following relation

$$D_m = D_0(1 + k_D c) \quad (10)$$

where D_0 is the diffusion coefficient at infinite dilution and c is the protein concentration.

Values of k_D , which reflect effects of protein-protein interactions on the mutual diffusion coefficient, contain both thermodynamic and hydrodynamic contributions. k_D values greater than the excluded volume term indicate the net protein-protein interaction is repulsive, while lesser values indicate attractive protein-protein interactions. The excluded

volume term is given by $k_D^{\text{ex}} = (k_v^{\text{ex}} - k_H^{\text{ex}})V_p$, where V_p is the protein volume in units of inverse protein concentration. k_v^{ex} and k_H^{ex} are the contributions from the thermodynamic and hydrodynamic terms, respectively. For spheres, $k_v^{\text{ex}} = 8$ and $k_H^{\text{ex}} = 6.55$. While proteins have anisotropic shapes, it has been shown that the thermodynamic term can be approximated by setting the protein volume equal to that of a sphere with the same hydrodynamic radius as the protein. If a similar assumption is made for the hydrodynamic term, for the mAbs, $k_D^{\text{ex}} \approx 3.8$ mL/g, which is based on a measured hydrodynamic radius R_H equal to 5.3 nm [26, 27]. For BSA, using a value of $R_H = 3.7$ nm [33] gives $k_D^{\text{ex}} \approx 3.0$ mL/g.

Results and Discussion

Determination of protein-protein interactions

Prior to measuring the microviscosity for a number of labelled tracers, protein-protein interactions were categorised as attractive or repulsive using the interaction parameter, k_D . The values of k_D shown in Table 2 for COE-03 were taken from two previous studies [25, 26], while those for COE-19 and for BSA were measured here. The solution conditions for COE-03 were chosen such that the protein-protein interactions range from attractive to repulsive. The strongest attraction occurs in solutions at pH 9 followed by pH 5 with 250 mM NaSCN, while there is a net protein-protein repulsion due to electrostatic double-layer forces in solutions at pH 5 and to a lesser extent at pH 6.5.

Adding 250 mM NaCl to the solution at pH 5 screens the electrostatic interactions and leads to a weak protein-protein attraction. The k_D values for COE-19 reflect a much stronger self-association than observed for COE-03 under any solution condition [27]. On the other hand, the values of k_D for BSA under all solution conditions investigated here are slightly greater than the excluded volume term indicating a weak net protein-protein repulsion. The results are consistent with the finding that human serum albumin, the human variant of BSA, does not exhibit any self-association in solutions at neutral pH values [34].

Validation of the FCS method

The potential of FCS to determine the relative macroscopic viscosity of sucrose ($R_h \sim 0.8$ nm) solutions (0.1-50% w/w) was confirmed by comparing the ratio of the diffusion times

obtained by FCS for a small molecule tracer (ATTO-Rho6G, $R_h \sim 0.6$ nm) and a large molecule tracer (IgG-AF, $R_h \sim 5.5$ nm) with the relative macroscopic viscosity measured using a rheometer Vilastic-3 (Figure 1). Excellent agreement between the relative macroviscosity and the ratio of the diffusion times or relative microviscosity is observed, with no effect of tracer size, confirming that the GSE relation (Eq. (1)) holds as $D\eta = D_0\eta_0$ for tracers much larger than the crowding molecule [16].

The diffusion of different fluorescently labelled tracers e.g. IgG-AF and ATTO-Rho6G was measured in mAb solutions. Figure 2 presents a typical example of the variation in the normalised autocorrelation curve for IgG-AF in the pH 5 solution with increasing concentration of COE-03. A clear increase of the diffusion time of IgG-AF is observed with increasing COE-03 concentrations, indicating retardation occurs during passive transport of IgG-AF in mAb solutions of increasing concentration.

Setting concentration limits of the study

All data were fitted with Equation (7) to check for anomalous diffusion, which has been related to molecular crowding [35], associated with the shape of the molecular crowder (in that case BSA and IgG) [10], linked to refractive index mismatch in single focus FCS [36], transient binding between crowder and tracer [37], and observed using FCS for high concentration protein solutions [11]. The diffusion was considered anomalous when, based on literature [14, 38, 39], the anomalous coefficient, α , was found to be less than 0.8 and a clear concentration dependence towards strong anomalous behaviour (i.e. less than 0.7) was observed although strictly speaking, $\alpha < 1$ indicates anomalous diffusion. For most experimental conditions (see Figure S1), there is no definitive pattern due to the uncertainty in the parameter estimation. However, for solutions of COE-03 at pH 9 there is a significant reduction in α at the highest protein concentrations (i.e. 195 g/L). At this concentration, the behaviour was considered anomalous and the corresponding measurements with IgG-AF and Atto-Rho6G were not considered further in this work: the concentration limit for this condition was fixed at 150 g/L. The maximum concentration of COE-19 at pH 5 (without any NaCl) was limited to 115 mg/mL as higher concentration samples became too viscous to handle.

Effect of tracer size on the microviscosity

The influence of the tracer size on the ratio of diffusion times (or relative microviscosity) in protein solutions is assessed (Figure 3) and compared to the relative macroviscosities observed for solutions containing mAbs or BSA. In solutions crowded with BSA (Figure 3a), the effect of tracer size (TMR peptide vs BSA-AF) is not apparent in solutions up to BSA concentrations of 200 g/L due to the limited change in viscosity. The effect of excipient type (e.g. sucrose versus arginine salts) on the diffusion time of either tracer was negligible compared to the effects of BSA concentration or tracer size, particularly for BSA concentrations ≥ 150 g/L (Figure S2). The relative BSA-AF microviscosity appears to closely match the variation of the relative macroviscosity.

In mAb solutions (COE-03 and COE19 solutions) and in contrast with BSA, FCS derived microviscosities were strongly affected by tracer size (Figure 3 b and c). The 2 to 3 fold increase in retardation of the small molecule tracer, ATTO-Rho6G, when increasing concentration from 1 to 160 mg/ml was unaffected by solution conditions (Figure S2). In comparison, the microviscosities obtained under the same solution condition for IgG-AF and IgM-AF increased by a factor of between 10 and 20, and, IgM-AF is usually more retarded than IgG-AF. This visible size effect may be partially related to the more favourable ratio of sizes between ATTO-Rho6G and mAbs compared to TMR-peptide and BSA. For COE-19 solution concentrations ≥ 50 g/L at pH 5, diffusion of IgG-AF became less retarded upon addition of 250 mM NaCl; high ionic strength dominated IgG-AF diffusion time over buffer pH, with data for pH 5 250 mM NaCl, pH 6.5 250 mM NaCl and pH 8 250 mM NaCl overlapping. In the case of COE-03, at concentrations < 150 g/L, the effect of high ionic strength or increasing buffer pH had little effect on IgG-AF diffusion times (Fig S2).

Limitations of the hard sphere protein model is revealed using tracers of different sizes

The aforementioned results are compared against the ratio of diffusion times for hard spheres (HS) to decouple the effects of crowding, attractive protein-protein interactions and anisotropic shape. The ratio of the diffusion times is calculated using the formula for describing long-time self-diffusion coefficients derived by van Blaaderen et al. [40] and used by others in similar studies [8, 24]:

$$\frac{\tau_{D,HS}}{\tau_{D,0}} = \frac{(1+1.5\phi+2\phi^2+3\phi^3)}{(1-\phi)^3} \quad (11)$$

where ϕ is the equivalent hard sphere volume fraction ($\phi = N_A V c / MW_h$), c is the protein concentration (mg/mL), N_A is Avogadro's number, V is an equivalent spherical volume of the protein, and MW_h is the hydrated molecular weight of the protein [41].

A key question is what equivalent sphere size should be used to represent a protein with an anisotropic shape. Here, we follow the work by Hung et al. [24] where the equivalent spherical mAb volumes were determined from fitting osmotic compressibility data determined by light scattering to the interacting hard sphere model derived by Minton [42]. This approach gave a value for the sphere radius equal to approximately 4.6 nm, which is used here for either mAb. BSA is represented by a sphere with radius equal to 3.4 nm, which provides the best fit of a hard sphere virial equation of state to osmotic pressure data for BSA up to protein concentrations greater than 400 g/L [34, 43].

The measured retardation factors are almost equal to or just slightly greater than the hard sphere predictions shown by the solid line in Figure 3a. The results are consistent with the study by Roos et al. [8], where the hard sphere model accurately matched the FCS-determined self-diffusion coefficients for labelled BSA in a solution crowded with BSA. In that study, the equivalent hard sphere radius was treated as an adjustable parameter by fitting a factor κ to relate the equivalent spherical volume fraction ϕ_{HS} to the actual protein volume fraction, ϕ , according to $\phi_{HS} = \kappa\phi$. For BSA, the fit value of $\kappa = 2.1$ corresponds to a sphere with radius equal to 3.4 nm, which is the same as the value used in our work. The molecular basis for why the parameter κ is greater than 1 is different in our work: Roos et al. [8] indicate the parameter is required to account for the weak protein self-association that was observed from fitting to structure factor profiles measured under ultra-low ionic strength conditions. Here, the value κ is not an adjustable parameter, but rather derived from the best fit to concentrated-solution thermodynamic data for BSA obtained in solutions at pH 7.4 and 150 mM NaCl [34, 43]. When fitting the compressibility data to a spherical model, the effective volume fraction is different from the BSA partial specific volume due to the anisotropic shape of the molecule and due to weak electrostatic repulsions [34]. Both of these factors will cause the effective spherical volume to increase.

For COE-03 and for COE-19, the ratio of diffusion times measured when using the IgG-AF probe is always greater than the predictions of the hard sphere model. The increased retardation follows the same pattern of behaviour with respect to protein-protein interactions as observed in Hung et al. [23]. In that study, the hard sphere-like behaviour was approached only under solution conditions with added excipients that weaken protein self-association. Here we find the largest deviations observed for COE-03 and for COE-19 occur under solution conditions where protein-protein attractions are the strongest.

Modelling of protein behaviours

To provide a quantitative comparison between the micro- and macro-viscosity data (Figure 3), the model of protein viscosity behaviour previously adopted by Connolly et al. [44], was used to describe the retardation of diffusion and the change of viscosity as outlined in Equations (12) and (13). Although Holyst model [18] was also considered (see Supplementary Information), Connolly's approach is simpler and provide similar information. In their work, the protein concentration dependence of the viscosity was parametrised by the exponential coefficient (k) of a simple exponential model.

$$\eta = \eta_0 e^{kc} \quad (12)$$

where k is the exponential coefficient. Assuming the Stokes Einstein relation applies:

$$\tau_D = \tau_{D,0} e^{kc} \quad (13)$$

k -values determined using Equations (12) and (13) for both mAb and BSA solutions are summarised in Table 3. Initially, the k -value was used to compare the difference between FCS and rheology data.

Model protein solutions. In BSA solutions TMR peptide derived k -values are always significantly smaller than both RheoChip and BSA-AF k -values and these are independent of the experimental condition (Tables 3 and 4). BSA-AF derived k -values indicate the micro-viscosity only results in a slightly negative deviation from the GSE in presence of sucrose; as the GSE holds for ArgGlu and ArgHCl, the limited difference between the hard sphere relative diffusion times and the experimental ones suggest BSA can be viewed as a hard sphere protein (Figure 3a and Table 3). Furthermore Roos et al. BSA data obtained in

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absence of buffer concur to our data (both FCS and RheoChip as seen Figure S3) confirming
the limited influence of co-solutes on BSA diffusional behaviour. In fact, the three proteins
they studied i.e. lysozyme, BSA and α B-crystalline (using NMR and/or FCS) followed the GSE
[8]; α -crystallin and BSA are known to behave like globular hard sphere model proteins [45-
47] or at least to a certain extent [48].

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Importance of size in mAb solutions. Overall, in mAb solutions, *k*-values for ATTO-Rho6G (a
much smaller tracer than IgG-AF) are significantly smaller than those obtained with
Rheochip, IgG-AF- or IgM-AF-FCS derived data (see Tables 3 and 4), and, insignificantly
affected by the experimental conditions, reflecting the relatively small increase in the
microviscosities observed experimentally (Figure S2). The behaviour of ATTO-Rho6G in mAb
solutions is expected as the tracer should be a similar size to or greater than the crowder for
the GSE to be applicable [16]. When the crowder becomes much larger than the tracer, the
tracer diffusion times become much less dependent on the viscosity. The dye (ATTO-Rho6G)
behaves somewhere between a tracer of the crowder's size, IgG-AF and small molecules,
such as dioxane. Roos et al showed that dioxane experienced the viscosity of a near-pure
solvent solution, because crowding proteins appeared as quasi-static objects on the time
scale of translational diffusion [5] .

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For COE-03 (pH 6.5) and COE-19 (pH 6.5 250mM NaCl), the variation of the IgM-AF ratio of
diffusion times matches the evolution of the relative viscosity determined using the
Rheochip indicating the GSE holds for these experimental conditions (Figure 3 b and c). This
is consistent with a previous study showing the macroviscosity is obtained using tracers
between two and four times the size of the PEG crowder [18]. However, IgM-AF
experimental diffusion times were strongly influenced by buffer conditions. For other
conditions (i.e. pH 8 250mM NaCl or pH 5 for COE-19), this close relation between
microviscosity and macroviscosity is not true as a positive deviation from the GSE is
observed [16]. This non-ideal behaviour cannot be ascribed to anomalous diffusion, since
the values for α in either condition remained between 0.9 and 1.0 (Figure S1). The positive
deviations from the GSE are likely due to interactions of the tracer with the crowder protein
as already reported for gold nanoparticles, wherein Brownian diffusion of the nanoparticles
was altered by their adsorption to BSA [49]. Rothe and al. [5] observed a positive deviation
in mixtures of BSA and SH3 which were attributed to inter protein interactions and/or

concentration dependent binding as shown by isothermal calorimetry and a potential clustering of BSA. Similarly, positive deviations from the GSE observed when using apomyoglobin as a tracer particle in solutions of either RNase A or BSA, were suggested to be related to the presence of nonspecific (e.g. electrostatic) crowder-tracer interactions [22, 50], which also provides the likely explanation for the retardation of chymotrypsin inhibitor diffusion in protein solutions [51]. Overall IgM-AF may be an unreliable tool to use to evaluate the viscosity of a solution in absence of additional information such as charge of the proteins.

IgG-AF vs Rheochip

k appears to be only affected by attractive conditions. k -values (Table 3) from FCS and RheoChip were graphed in Figure 4. A correlation between FCS and RheoChip derived data is observed although FCS derived k -values increase is limited compared to RheoChip derived data for attractive conditions. Overall, Figure 4 and Table 4 indicate: i) the ordering of the conditions according to the viscosity obtained by FCS and Rheochip is similar (i.e. k -values for COE-03 pH 9 > pH 6.5 ~ pH 5 250mM NaSCN > pH 5 ~ pH 5 250mM NaCl and for COE-19 pH 5 > pH 6.5 250mM NaCl $\geq$ pH 5 250mM NaCl $\geq$ pH 8 250mM NaCl). A key difference between COE-03 and COE-19 is that the interactions are much more attractive (see Table 2) for COE-19 except for the pH 9 conditions for COE-03. Perhaps a better way is to correlate the exponential coefficient, k , to the protein-protein diffusion interaction parameter, k_D (see equation (10)) as did Conolly et al. [44]

For our IgG-AF FCS- and RheoChip- derived data as well as the pairs (k , k_D) adapted from Conolly et al.[44], Figure 5 suggests, rather than being a linear relation between the two parameters as suggested by Conolly et al., a more complex relation exists, perhaps based on an exponential decay between k and k_D . Indeed, k -values decrease from strongly attractive conditions to repulsive conditions. It is also noticeable that this apparent relation is stronger with the RheoChip derived data than with our FCS derived data (Figure 5) suggesting self-diffusion may be less sensitive to attractive conditions than the viscosity.

Breakdown of the GSE. k -values (Tables 3 and 4) from FCS and RheoChip indicate i) for BSA, the BSA-AF relative microviscosities match the relative macroscopic viscosity for ArgGlu and ArgHCl and behave differently for sucrose ii) for COE-03, IgG-AF relative microviscosities

match the relative macroviscosity for pH 6.5, pH 5 250mM NaCl and pH 5 250mM NaSCN. The only clear difference (based on data Table 4) is at pH 9 which corresponds to the most viscous solution where there is strong reversible association. In this case a negative deviation from the GSE is observed; iii) for COE-19, in all cases FCS derived k -values are significantly lower than RheoChip derived k -values.

Thus, the GSE seems to hold for labelled BSA in BSA solutions as observed by Roos et al. [8] (see Figure S3). The negative deviations from GSE ($k_{FCS} < k_{RheoChip}$ and Figure 4) occur for solution conditions corresponding to the lowest values for the interaction parameter, k_D , indicating the diffusion of IgG-AF is slowed down in the presence of attractive protein-protein interactions (*i.e.* for COE-19, in all cases FCS derived k -values are significantly lower than RheoChip derived k -values). As such, microviscosity predictions underestimate macroviscosity where there is strong self-association between the crowder proteins. This is supported when comparing the experimental k -values to the hard-sphere derived values (Table 3) indicating greater deviations from HS behaviour for COE-19 especially when electrostatic interactions are not screened (pH 5 for COE-19). Conversely, the relative microviscosity obtained from FCS is similar to the relative macroviscosity for COE-03 under all solution conditions except for pH 9.

Negative deviations from the GSE when using a tracer protein with similar size to the crowder protein have only been reported in Hung et al. [23] for a solution containing the same mAb as a crowder and as a tracer (except the tracer was labelled). In that study, solution conditions were chosen to mitigate the amount of self-association. In contrast to our study, the negative deviations occur in the opposite limit of reducing the self-association when adding solubility enhancing excipients, while the GSE is applicable under conditions with weak self-association.

A key question is why have negative deviations not been observed in other studies of protein solutions. Most studies on the applicability of the GSE have been carried out under very low ionic strength conditions, where interactions between crowder molecules are dominated by long-ranged electrostatic repulsions [5, 8, 16]. The only exception is the study by Zorilla et al. [22] where the GSE was tested under physiological salt conditions (150 mM NaCl, 20 mM phosphate, pH 7.4) using either HSA or RNase A as crowders. Under these

1 solution conditions, the behaviour of HSA is well represented using only excluded volume
2 interactions reflecting the absence of any self-association [34, 42]. In addition, a self-
3 interaction chromatography study of RNase A found that protein-protein interactions are
4 net repulsive, as characterized in terms of the osmotic second virial coefficient B_{22} , at pH 6.5
5 and at pH 8.0 in solutions with sodium chloride concentrations greater than 100 mM [52].
6 Hung et al. suggest the association state controls the tracer's size of tracer but not
7 crowder's; to hold this would indicate that (1) interactions between probe molecule and
8 COE-19 are weaker than interactions between probe molecule and COE-03 and (2)
9 interactions between probe molecule and COE-03 are weakest at pH 9 and no salt. In
10 contrast, our results indicate the solution conditions exhibiting negative deviations to the
11 GSE correspond to when interactions are stronger rather than weaker since all antibodies
12 have high pI values and strongest attractions usually occur under these conditions.
13 According to our previous study, we expect the values of B_{22} to be less than zero for these
14 conditions, indicating the presence of a protein-protein attraction that is greater in
15 magnitude than the excluded volume contribution to B_{22} . As such, we hypothesize that
16 negative deviations occur due to strong self-association between crowder molecules. If
17 crowder molecules bind transiently to each other, the ratio of crowder size to tracer size
18 increases. According to the Holyst model and the work by Muramatsu and Minton, an
19 increase in the size ratio causes a decrease in the tracer particle retardation factor leading
20 to the negative deviations to the GSE. The key assumption is that the interactions between
21 the tracer and crowder IgGs are less attractive than the crowder IgG self-interactions. There
22 is some evidence to suggest this is the case at least for the conditions tested with COE-19.
23 In our previous study [27], we showed that the self-interactions for COE-19 are more
24 attractive than the cross interaction between COE-03 and COE-19 over a range of pH and
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49 In the study by Hung et al. [23], the strongest self-association between crowder IgG
50 corresponded to conditions where the value of B_{22} normalized by the excluded volume term
51 is equal to -0.2. This value indicates only slightly less attractive conditions than for COE-03
52 at pH 9, where we observe the negative deviations. At this stage it is not possible to
53 hypothesize why there exists this discrepancy, in particular because the GSE applicability
54 depends sensitively on the crowder-tracer interactions. The negative deviations observed
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by Hung et al. [15] arise in solutions with solubility enhancing excipients which weaken the crowder self-association as well as any association between crowder and tracer. On the other hand, we expect our negative deviations arise when increasing the association between the crowder molecules relative to the crowder-tracer cross interaction.

Conclusion

In this work, we have considered whether Fluorescence Correlation Spectroscopy could be a suitable tool to determine protein viscosity based on the General Stokes Einstein relation. To this effect, the diffusion of fluorescent tracers of like charge to the crowder protein but with different sizes was measured in protein solutions.

Similarly to what has been observed in polymers [17] and recently in IgG solutions [53], a size effect is observed in protein solutions as shown by k -values indicating that microviscosities of tracers (AttoRho6G or TMR-peptide) smaller than the crowding protein (mAb or BSA) systematically underpredict the macroviscosity (resulting in a so-called negative deviation from the GSE). In presence of a tracer whose size is similar to the crowding protein (e.g. IgG-AF for mAbs), negative deviations from the GSE are observed in presence of strongly attractive conditions between the crowder molecules (all COE-09 experimental conditions) suggesting this behaviour may be related to the crowder's self-association. However, this breakdown of the GSE disappears in solution conditions where interactions between crowding proteins are repulsive or only weakly attractive (e.g. IgG-AF in COE-03 and BSA-AF in BSA). The experimental deviation from hard sphere ratios of diffusion times highlights the importance of attractive protein-protein interactions. This is further shown with an apparent relation between the exponential parameter, k , and the interaction parameter, k_D , indicating strong attractive interactions are more likely to affect viscosity and the GSE relation. A key question remaining is whether or not there are any interactions between tracer and crowder molecules. Indeed, for studies involving tracers, the charge of the tracer, its concentration or its size should be carefully considered to mitigate these issues as, for example, the largest tracer, IgM-AF, behaviour was less predictable in strongly attractive conditions (e.g. COE-19 at pH5) which ultimately lead to interactions between tracer and mAbs.

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Legend to Figures

Figure 1: Relative viscosity (η/η_0) of a range of sucrose concentrations (from 0.1-50% (w/w)) measured using a Vilastic-3 (squares), and inverse ratio of diffusion times, $\tau_D/\tau_{D,0}$, determined by FCS using ATTO-Rho6G (circles) and IgG-AF (triangles). Plots represent mean $\pm$ std. dev for n=3.

Figure 2: Normalised autocorrelation function (ACF) for IgG-AF (2 $\mu\text{g/mL}$) in buffer alone (pH 5) and in COE-03 solutions (5 to 125 g/L, pH 5). The average time the fluorescent probe spends in the focal volume is given by the characteristic decay time of the ACF (Equation (5)).

Figure 3: variation of the relative viscosity, η/η_0 , measured using a RheoChip and of the ratio of diffusion times, $\tau_D/\tau_{D,0}$, for tracers of increasing size: ATTO-Rho6G, TMR peptide, BSA-AF, IgG-AF and IgM-AF in crowded solutions of (a) BSA at pH 7, (b) COE-03 and (c) COE-19 (see key; all at $T = 20^\circ\text{C}$). The red line corresponds to the ratio of diffusion times based on hard sphere model.

Figure 4: correlation between k -values obtained with IgG-AF/FCS derived and RheoChip derived data (solid red line). The shaded area corresponds to 95% confidence interval) and the dashed line represents the case for which GSE is valid and equal k -values for FCS and RheoChip.

Figure 5: variation of parameter k (from model 1) as a function of the interaction parameter k_D (left). Conditions mAbs in 20mM HisOAc pH 5.5, 30mM HisCl pH 6.0 and 200mM ArgHCl pH 5.0 are adapted from Conolly et al.[44]

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