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► **To cite this version:**

Ilaria Mosca, Kévin Pounot, Christian Beck, Louise Colin, Olga Matsarskaia, et al.. Biophysical Determinants for the Viscosity of Concentrated Monoclonal Antibody Solutions. *Molecular Pharmaceutics*, 2023, 10.1021/acs.molpharmaceut.3c00440 . hal-04187078

HAL Id: hal-04187078

<https://hal.science/hal-04187078v1>

Submitted on 2 Sep 2023

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Biophysical determinants for the viscosity of concentrated monoclonal antibody solutions

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Abstract

Monoclonal antibodies (mAbs) are particularly relevant for therapeutics due to their high specificity and versatility, and mAb-based drugs are hence used to treat numerous diseases. The increased patient compliance of self-administration motivates the formulation of products for subcutaneous (SC) administration. The associated challenge is to formulate highly concentrated antibody solutions to achieve a significant therapeutic effect, while limiting their viscosity and preserving their physicochemical stability. Protein-protein interactions (PPIs) are in fact the root cause of several potential problems concerning the stability, manufacturability, and delivery of a drug product. The understanding of macroscopic viscosity requires an in-depth knowledge on protein diffusion, PPIs, and self-association/aggregation. Here, we study the self-diffusion of different mAbs of the IgG1 subtype in aqueous solution as a function of the concentration and temperature by quasi-elastic neutron scattering (QENS). QENS allows us to probe the short-time self-diffusion of the molecules and therefore to determine the hydrodynamic mAb cluster size and to gain information on the internal mAb dynamics. Small-angle neutron scattering (SANS) is jointly employed to probe structural details and to understand the nature and intensity of PPIs. Complementary information is provided by molecular dynamics (MD) simulations and viscometry, thus obtaining a comprehensive picture of mAb diffusion.

Keywords: monoclonal antibodies, self-association, self-diffusion, quasi-elastic neutron scattering, small angle neutron scattering, MD simulations

1 Introduction

Since the development of the hybridoma technology by Köhler and Milstein [1], monoclonal antibodies (mAbs) made their way to therapeutic applications with 10 mAbs approved by the Food and Drug Administration (FDA) in 2001 [2] to 100 mAbs in 2021 [3], and 153 in 2022 (among which 59 were approved for cancer therapy) [4]. The transition from mouse antibodies, to chimeric and eventually human

antibodies [2] has led to a drastically reduced immune reaction, and has driven the recent increase in the number of mAbs in use or under development. The actual and potential clinical applications of mAbs cover a wide range of diseases [5], including cancers [6], infectious and autoimmune diseases (such as rheumatoid arthritis and Crohn's disease) [7, 8] and multiple sclerosis [9]. The most employed type of mAb for therapeutic use is immunoglobulin G (IgG), which is a Y-shaped molecule of ≈ 150 kDa. The

four main types of IgGs - IgG1, IgG2, IgG3 and IgG4 - show very similar structures and amino acid sequences (90% of identity). They differ in their relative abundance in the serum, their affinity with different types of antigens and receptors and their half-life [10]. IgGs consist of four polypeptide chains, two heavy chains of ≈ 50 kDa each and two light chains of ≈ 25 kDa each, crosslinked by disulfide bonds. Heavy chains contain a variable domain (VH) and three constant domains (CH1, CH2 and CH3), with a flexible

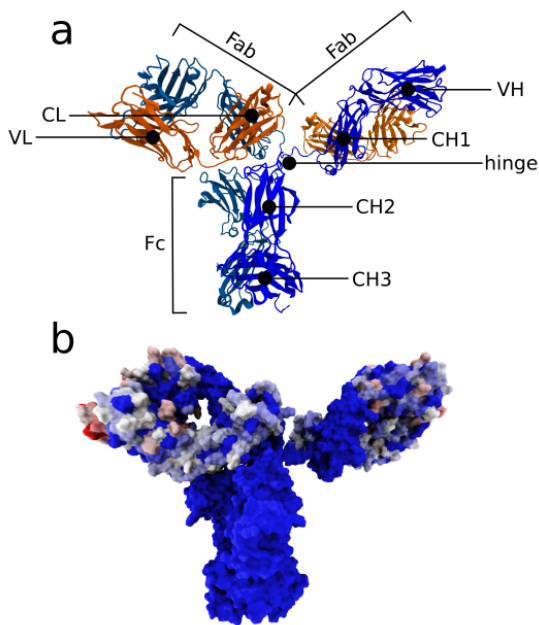


Fig. 1. Structure of the mAbs and sequence similarity between the mAbs. **(a)** The structure of mAb1 was used to represent the mAb using VMD [11]. The different regions of the mAb are annotated on the structure represented using the ‘NewCartoon’ style. The heavy chains are represented in shades of blue and the light chains in shades of orange. **(b)** The sequences of the mAb variants were aligned using the VMD ‘MultiSeq’ module [12] and the sequence similarity obtained with the BLOSUM100 matrix is represented with a colour range from blue for high similarity to red for low similarity. The different mAbs under study show almost identical structures in the Fc region, while they differ in the extremal parts of the Fab regions (CDR), namely the ones involved in the binding with the specific antigen.

hinge region between CH1 and CH2; light chains contain a variable domain (VL) and a constant domain (CL) [13], [14]. The segments CH1, VH, CL and VL form the “fragment antigen binding” (Fab) region, while the CH2 and CH3 domains form the “fragment crystallizable” (Fc) region (Fig. 1).

While polyclonal antibodies are usually produced by several different plasma cell lineages and bind to multiple epitopes, monoclonal antibodies result from the cloning of a unique cell and thus bind only a single epitope [15]. This feature gives mAbs high specificity and high affinity due to the complementarity determining region (CDR) within the Fab, whose shape complements the one of the corresponding antigen. The Fc region of mAbs instead allows to reduce side-effects and toxicity compared to polyclonal antibody solutions [5]. In addition, their half-life can extend to a month after injection thanks to the binding to the neonatal Fc receptor. However, their size prevents them from entering cells and crossing the blood-brain barrier. In addition, oral administration results in rapid degradation of the mAbs and limited bioavailability [16], thus limiting their administration to parenteral routes, namely intravenous (IV), intramuscular (IM) or subcutaneous (SC) injections. Some diseases require life long treatment; if the drugs are administered via IV, patients need to see a doctor or go to a hospital to receive their medication, which can compromise patient compliance. For this reason, mAb delivery via SC injection has recently become a market interest in pharmaceuticals, since no skilled personnel is required for drug administration, thus enabling patients to benefit from self-administration and more flexibility, and also ensuring lower healthcare costs [17].

The amount of mAb required to ensure a significant therapeutic effect (typically hundreds of mg) and the typically low injection volume in subcutaneous administration (0.5-2 mL) [18]) imply the use of highly concentrated mAb solutions, which can exhibit high viscosities [18]. This potentially affects the stability, manufacturability, and delivery of these biopharmaceuticals [19, 20]. The dynamic viscosity of highly concentrated mAb formulations can easily exceed the tolerance threshold for injectability (~ 15 -

20 mPa·s) [21] and render their administration difficult or impossible for patients and some injection devices due to the high forces required [22, 17]. Extensive research aiming to minimize the viscosity of highly concentrated mAb solutions is ongoing [18], along with several studies on the optimization of physico-chemical solution stability and manufacturability [23, 24].

The presence of reversible self-association was proposed as the main mechanism determining viscosity changes in the works by Kanai et al. [25] and Liu et al. [26]. Self-association and hence viscosity of highly concentrated mAb solutions can be influenced by multiple parameters, including concentration, pH, ionic strength [26], or specific interactions, like charge-dipole or dipole-dipole, between domains of monomers [27, 28]. Charged molecules such as NaCl or Arginine-HCl can shield protein charges, thus reducing protein-protein-interactions and thereby viscosity [29]. Although electrostatic interactions seem to dominate in driving reversible self-association, hydrophobic interactions also play a key role [30] and therefore have impact on the solution viscosity. Arginine can also shield antibody hydrophobic interactions [31], enhancing its viscosity-reducing function. Other excipients with analogous effects are caffeine [32], hydrophobic salts [33], or amino acid derivatives [34], but they are not yet employed in commercialized drug products. Addition of viscosity-reducing pharmaceutical excipients to mAb formulations is indeed a common practice and also the subject of continuous research [35, 36, 37].

More recent work involving experiments and coarse-grained simulations paved the way to viscosity predictors based on amino acid sequence and structural properties of the Fv region [38, 39]. Apgar and coworkers applied the same approach optimizing the charge distribution and hydrophobicity in the variable region (VH and VL) to successfully reduce the viscosity of a mAb solution while preserving its stability and its affinity for the antigen [40].

Schmitt et al. recently presented a predictive modeling approach for viscosity employing artificial neural networks and based on both experimental and simulation-derived parameters and viscosity data

from 27 highly concentrated mAbs [41], showing that sequence-based optimization of mAb properties is a powerful method for rational mAb design. In addition, recent studies have successfully investigated the link between viscosity and cluster formation using small-angle X-ray scattering, microrheology and coarse-grained simulations and used cluster theory to predict structure factors of highly concentrated mAb solutions [42, 43]. The same systems have also been investigated using again SAXS, viscometry, static and dynamic light scattering, in combination with other analytical techniques [44] and also coarse-grained modeling [45]. In general, computational approaches, such as coarse-grained and atomistic modeling, hydrodynamic calculations and machine-learning based methods, seem to successfully predict viscosity, inter-molecular interactions, aggregation and physical instabilities in highly concentrated mAb solutions [46, 47, 48, 49, 50].

Polyclonal antibody solutions have also been widely investigated with SAXS [51], photo-correlation spectroscopy (XPCS) [52], quasielastic neutron spectroscopy (QENS) and neutron spin-echo (NSE) [53], establishing a quite robust framework for the research on monoclonal antibody solutions. In this area, neutron scattering techniques have already been successfully used [54] to fully understand the link between macroscopic and microscopic phenomena. For example, neutron reflectometry has been widely employed to study adsorption of mAbs on hydrophobic surfaces, which typically leads to protein aggregation and degradation [55, 56]. Neutron spin-echo and small-angle neutron scattering experiments have identified the formation of dynamic clusters of proteins (including antibodies) in concentrated solutions [57, 58].

In this context, the self-association or cluster formation of mAbs in aqueous solution can be reliably obtained via the self-diffusion of these clusters measured by high-resolution quasi-elastic neutron spectroscopy (QENS) probing the spatially incoherent scattering and, thus, ensemble-averaged single-particle self-correlation of the mAb hydrogen ^1H atoms. This self-diffusion unambiguously informs on the hydrodynamic size of the clusters via the Stokes-Einstein relation. In concentrated solutions of protonated (^1H)

proteins in D₂O (²H), the signal from the proteins dominates over the solvent signal in the neutron scattering experiment.

Here, we comprehensively study a set of 5 different mAbs in terms of this cluster size which is accessed via their self-diffusion at different temperatures and concentrations in solution, the associated macroscopic viscosity of these solutions, and, for selected samples, their solution structure and protein-protein interactions by small-angle neutron scattering (SANS). Moreover, we perform fully atomistic molecular dynamics (MD) simulations to identify possible determinants for the experimentally observed behaviour in the sequence of the mAbs. Simultaneously, we also obtain information on the internal diffusive dynamics of these mAbs on the level of protein backbone and side-chain fluctuations from both simulations and QENS spectra. Thus, we associate the macroscopic properties with several microscopic properties in the pursuit to enhance the understanding required for rational design of high-concentration, low-viscosity mAb formulations. Resulting from the energy resolution of the QENS experiment, the diffusion of the proteins is observed during the coherence time of a few nanoseconds. On this time scale, protein-protein collisions can be neglected, and the observed center-of-mass diffusion corresponds to the so-called short-time diffusion, which is governed by hydrodynamic and electrostatic interactions [59].

2 Materials and methods

2.1 Sample preparation

We employed five different monoclonal antibodies of IgG1 isotype with κ and λ light chains, with molecular weights (MW) ranging from approximately 145 to 148 kDa and different isoelectric points (pI) ranging from 7.2 to 8.4 (see table S1 in Supporting Information). In this work, we denote these antibodies mAb1, mAb9, mAb12, mAb16 and mAb24, consistent with previous work on the same molecules [41]. All mAbs were manufactured in-house at Lonza AG/Ltd. Double gene vectors containing the heavy and light chains were transfected into CHOK1SV GS-KO cells [60]

from Lonza Biologics (Slough, UK) and cultured under selection conditions as stable pooled cultures. Clarified supernatant was obtained by centrifugation followed by filter sterilization using 0.22 μ m filters Stericup[®] Quick Release from Merck/MilliporeSigma (Darmstadt, DE) and Protein A chromatography was used for mAb purification. SE-HPLC (size exclusion-high-performance liquid chromatography) showed that all mAbs were > 96% monomers, with small amounts of aggregates and fragments. All proteins were concentrated to final concentration of 10 mg/mL (nominal) and buffer exchanged into the formulation buffer by tangential flow filtration. A 20 mM histidine-HCl buffer at pH 6.0 was employed as for most (> 80%) formulations of highly concentrated approved mAb drug products [61]. All mAb solutions were then frozen in aliquots, stored at -80°C and slowly thawed prior to use.

For QENS and SANS samples, molecules were buffer-exchanged into 20 mM Histidine in pure D₂O at pD 6.0, using 3 mL dialysis cassettes containing cellulose membranes with 30 kDa nominal molecular weight cutoff from ThermoFisher Scientific (Waltham, MA, USA). Two baths of at least 2 hours were performed to obtain a dilution factor of at least 10^4 of residual H₂O in the samples. We remark that exchanging the buffer from H₂O to D₂O is an essential step in neutron experimental techniques, because it enables to measure proteins in their aqueous environment while minimizing the scattering signal from the surrounding solvent. Subsequently, the samples were concentrated using 15 mL 30 kDa Amicon[®]-Ultra concentrators from Merck/MilliporeSigma until the volume needed for the QENS experiment ($\sim 800 \mu\text{L}$) was reached. Final concentrations of the samples were determined via UV-Vis spectroscopy on dilution series of each mAb using a Jasco V-630 Spectrophotometer. For each dilution step, absorbance curves were collected and their peak values at 280 nm were plotted against the sample reciprocal dilution factor. The data obtained were therefore fitted using the Lambert-Beer law, which links the absorbance A of the sample to its concentration c via $A = \varepsilon c \ell$, with ℓ being the optical path-length, i.e., the thickness of the cuvette, and ε the sam-

ple extinction coefficient. The determined concentrations were (76.53 ± 2.59) mg/mL for mAb1, (54.44 ± 1.82) , (64.34 ± 4.60) and (80.12 ± 3.65) mg/mL for mAb9, (76.12 ± 3.15) mg/mL for mAb12, (80.44 ± 4.79) mg/mL for mAb16 and (45.38 ± 1.52) mg/mL for mAb24.

For reference, samples of polyclonal IgG solutions were prepared employing lyophilized powder of γ -globulin from bovine serum ($\geq 95\%$ purity, essentially salt-free), purchased from Merck/MilliporeSigma (Darmstadt, DE) and directly dissolved in a 20mM Histidine-HCl D₂O buffer at pD 6.0. The desired concentrations for these samples were reached and verified via UV-VIS using a Nanodrop One[©] Spectrophotometer from ThermoScientific, obtaining three samples at $c_p = 60, 140, 180$ mg/mL, respectively.

2.2 Quasi-elastic neutron backscattering spectroscopy

Quasi-elastic neutron scattering (QENS) accesses molecular dynamics on time and length scales commensurate with the motions of individual proteins and their domains and side chains.

The experiments were performed at the spectrometer IN16B at the Institut Max von Laue - Paul Langevin (ILL), Grenoble, France. This instrument provides an energy resolution of $0.8 \mu\text{eV}$ and an energy transfer range of $\pm 30 \mu\text{eV}$, allowing the investigation of motions on a timescale of approximately 100 ps to 10 ns [62]. IN16B was used with Si(111) monochromator and analyser crystals, corresponding to an elastic wavelength of 6.27 \AA . A linear motor Doppler drive carrying the monochromator was used to define the energy transfer. The samples were put into double-walled cylindrical aluminum cans with a 0.15 mm gap and an outer diameter of 23 mm, sealed with indium wire, and mounted in a standard cryofurnace for temperature control during the data acquisition. The QENS signal was integrated for 4 hours for each sample at each temperature. The QENS data are curated under DOI 10.5291/ILL-DATA.8-04-908 [63]. The data were processed with Mantid [64] applying standard reduction including monitor normalization and empty can subtraction, and subsequently fitted

using python employing *scipy.optimize.curve_fit* [65]. The observable in neutron spectroscopy is the dynamic structure factor $S(q, \omega)$ depending on the energy transfer $\hbar\omega$ and momentum transfer $\hbar q$. IN16B spans a q -range from 0.2 to 1.9 \AA^{-1} , corresponding to lengths from 3 to 30 \AA . S is the sum of the scattering contributions from the aqueous (D₂O) solution and the antibodies. The latter consists of the contributions from the global and internal diffusion of the proteins. The superposition of these contributions is convoluted with the spectrometer resolution function $R(q, \omega)$ obtained from a Vanadium calibration measurement. The experimental data were fitted by [66]

$$S(q, \omega) = R(q, \omega) \otimes \left\{ \beta(q) \left[A_0(q) \mathcal{L}(\gamma(q), \omega) + (1 - A_0(q)) \mathcal{L}(\gamma(q) + \Gamma(q), \omega) \right] + \beta_{D_2O}(q) \mathcal{L}(\gamma_{D_2O}(q), \omega) \right\}, \quad (1)$$

where $R(q, \omega)$ is the resolution function, $\beta(q)$ and $A_0(q)$ are q -dependent scalars, where the latter is identified with the elastic incoherent structure factor (EISF). The parameters $\beta_{D_2O}(q)$ and $\gamma_{D_2O}(q)$ were fixed based on measurements of the pure solvent, accounting for the solvent volume excluded by the proteins in $\beta_{D_2O}(q)$. Each contribution to $S(q, \omega)$ accounts for a diffusive motion and, thus, is a Lorentzian function $\mathcal{L}(\Gamma, \omega) = \Gamma / [\pi(\omega^2 + \Gamma^2)]$, whose width provides the associated relaxation rate. $\mathcal{L}(\gamma(q), \omega)$ is the Lorentzian connected to the self-diffusion of the protein center of mass, $\mathcal{L}(\gamma(q) + \Gamma(q), \omega)$ describes the internal diffusive motions and $\mathcal{L}(\gamma_{D_2O}(q), \omega)$ is the signal from the deuterated buffer solution. Samples at $c_p \leq 50$ mg/mL were fitted accounting for just two Lorentzian functions (center of mass and solvent diffusion), due to their lower signal potentially causing overfitting if using Eq. 1. All samples were prepared in D₂O solutions to reduce the signal from the solvent relative to the protein signal, due to the large difference of the incoherent neutron scattering cross sections of hydrogen ¹H and deuterium ²H.

A two-step approach was used for fitting. First, by q -wise fits, scalar fit parameters were fitted indepen-

dently for each q . From this procedure we observe that the center-of-mass dynamics follows a Fickian-type diffusion as expected and found previously [66], meaning that the width γ of the center of mass Lorentzian is

$$\gamma(q) = D q^2 \quad (2)$$

where D is the apparent global diffusion coefficient. From a physical point of view, this means that the center of mass exhibits continuous diffusion.

Second, global fits were performed by imposing the q -dependence of some parameters to render the fit more robust and include knowledge on the systems from the q -wise fits, as established in previous works [66, 67, 68, 69]. One approach was to impose a Fickian center of mass diffusion (Eq. 2) without imposing any q -dependence of the internal diffusion. A q -dependence on the initial guess for the internal dynamics parameters was, however, included, by following the so-called jump-diffusion model [70]

$$\Gamma(q) = \frac{D_{\text{int}} q^2}{1 + D_{\text{int}} \tau q^2}, \quad (3)$$

where D_{int} and τ respectively represent the diffusion coefficient related to the internal dynamics and the average residence time in the state of oscillatory motions. A prior knowledge from the q -wise fit was also exploited in the EISF $A_0(q)$, which was parameterised in the global fits as [67, 71, 72]

$$A_0(q) = p_0 + (1-p_0) [p_1 A_{3\text{jump}}(q) + (1-p_1) A_{\text{sphere}}(q)] \quad (4)$$

i.e., by a superposition of a component accounting for orientational jumps among three sites equally distributed on a circle and placed at distance a one from another,

$$A_{3\text{jump}}(q) = \frac{1}{3[1 + 2j_0(qa)]}, \quad (5)$$

and a contribution from diffusion confined in a spherical volume with radius R ,

$$A_{\text{sphere}}(q) = \left| \frac{3j_1(qR)}{qR} \right|^2, \quad (6)$$

where $j_0 = \sin(x)/x$ and $j_1(x) = \sin(x)/x^2 - \cos(x)/x$ denote the spherical Bessel functions of the zeroth

and first order, respectively. p_0 (Eq. 4) is the fraction of hydrogen atoms that appear immobile on the timescale explored by the instrument (elastic contribution), while $(1-p_0)$ is the fraction of mobile H atoms. In this picture, the coefficients p_1 and $(1-p_1)$ respectively represent the fraction of H atoms undergoing jump-diffusion among three sites and diffusion confined in a sphere. The first class of H atoms accounts for methyl groups, the reorientations of which are described by Eq. 5 with the fixed jump distance $a = 1.715 \text{ \AA}$. In this interpretation, H atoms jump between three sites at an angular distance of 120° . The second class of H atoms, namely those undergoing diffusion inside a sphere (Eq. 6), accounts for the protein backbone, with R being a free fit parameter. The q -dependent scalar $\beta(q)$ in Eq. 1 accounts for the thermal Debye-Waller factor due to vibrational motions of hydrogen atoms:

$$\beta(q) \propto \exp\left(-\frac{1}{3} \langle r^2 \rangle q^2\right), \quad (7)$$

with $\langle r^2 \rangle$ being the corresponding mean square displacement (MSD).

2.3 Small-angle neutron scattering (SANS)

Small-angle neutron scattering (SANS) was performed on a subset of the antibodies, namely mAb9, mAb12 and mAb24, in order to determine their time-averaged structural and thermodynamical properties as a function of temperature and protein type. The SANS experiments were carried out on D11 [73] at the ILL. MAb samples at 80 mg/mL each were filled into 1 mm round quartz cuvettes (Hellma, Mülheim, Germany) and placed onto a copper sample holder. A q range from $0.006 - 0.7 \text{ \AA}^{-1}$ was covered by two sample-to-detector distances (16 and 1.7 m) with respective collimation lengths of 16.5 and 2.5 m. A wavelength of 4.6 \AA with a full width-half maximum (FWHM) wavelength spread of 9 % was used. Scattered neutrons were detected using a multitube ^3He gas detector with a pixel size of $4 \times 8 \text{ mm}^2$. Raw data were saved in the .nxs (NeXuS) format [74, 75]. Data reduction was performed using Mantid [64]. All data were corrected for empty cell scattering, transmis-

sion (by measurements performed using beam attenuators) and electronic noise (by measuring a $^{10}\text{B}_4\text{C}$ absorber). Calibration to absolute scale was performed using attenuated direct beam measurements. Scattering of the solvent (20 mM His-HCl deuterated buffer at pD 6.0) was subtracted from sample scattering. The SANS data are curated under DOI 10.5291/ILL-DATA.8-04-923 [76].

2.4 Viscometry

All mAb samples were characterised by viscometry as reported in Schmitt et al. [41]. They were measured at $T = 25^\circ\text{C}$ ($\sim 298\text{ K}$) in their original 20 mM His-HCl aqueous buffer at pH 6.0. In addition, a subset of the antibodies (mAb9, mAb12, mAb16 and polyclonal IgG) was measured in the corresponding deuterated buffer at the same nominal concentrations at 7, 22 and 37°C (280, 295 and 310 K), to collect complementary information on viscosity for the conditions studied in neutron experiments. The procedure used for preparation is the same as the one described for QENS and SANS samples in 2.1, with dilutions from 180 to 30 mg/mL.

The employed apparatus was a Rheosense VROC[®] Initium rheometer (San Ramon, CA, USA) equipped with a B05 chip and operating using VROC[®] (Viscometer/Rheometer-on-a-Chip) technology [77] (<https://www.rheosense.com/technology>). A medical grade viscosity standard from Paragon Scientific (ISO 17025 & 17034), reporting a dynamic viscosity of 9.994 mPas and a density of 1.1567 g/mL at 25°C , was used for the system suitability test (SST). The protocol consisted in measuring the dynamic viscosity of each sample ten times at 25°C whilst applying an automatic shear rate, which is determined by the instrument software in order to induce a pressure inside the chip targeting the 50% of the full instrumental range [41].

2.5 Sample characterization by DLS, SLS, and HIC

Dynamic light scattering (DLS), static light scattering (SLS) and hydrophobic interaction column (HIC) measurements were carried out by Schmitt et al. [41]

and these data were used as input for the simulations.

2.6 MD simulations

Single-molecule simulations and analysis. The pdb files for the different mAbs available internally at Lonza were employed to run single-molecule simulations using NAMD [78] with the CHARMM36 force field [79], [80]. For each antibody, the starting condition was to place a single monomer in a box with explicit water (TIP3 model [81]) using the solvate plugin of VMD [11] with a padding distance of 25 Å. The system was neutralized (every charge having a counter-charge) using Na^+ and Cl^- ions to avoid electrostatic artifacts during the simulation using the autoionize plugin of VMD [11]. The pressure was maintained at 1 atm using the Nosé-Hoover Langevin piston algorithm [82], [83] and the temperature controlled using Langevin dynamics. All bonds were constrained with the SHAKE algorithm [83]. The integration of the equation of motions was performed using the Verlet-I/r-RESPA algorithm [84], [85] with integration time of 2, 2 and 4 fs for the short-range bonded and non-bonded forces and long-range forces, respectively. The electrostatic interactions were computed using the Ewald summation method [86], [87] with a smooth switching function between 12 and 14 Å. The five systems, mAb1, mAb9, mAb12, mAb16 and mAb24, were equilibrated at 300 K in the NVT (N = number of atoms, V = volume, T = temperature) ensemble for 2 ns to remove any bad contacts that may occur during model building. The NPT (P = pressure) ensemble was then used for 60 ns equilibration at 300 K. The production runs were performed for 120 ns in the NVT ensemble to match the experimental conditions where V and T are fixed. The features (MSD, angles between the lobes) were computed using the block average method [88]. Briefly, the simulation trajectory is divided into blocks of increasing size. For each block at a given size, the observable and its standard deviation between the blocks are computed. The standard deviation is expected to reach a plateau value when the sampling is sufficient for the computed observable [88] (Figure S4). The minimum block size is defined when the standard deviation reaches the plateau and that

block size is used for calculating the variable average and standard deviation. Using this block average method, the large scale motions of the lobes are computed by taking the scalar product of the normalized position vectors shown in Figure S4 to extract the angles. Subsequently, for different time origins, the deviation of the angle from the initial value is computed. The result is averaged over the blocks and the standard deviation between the blocks is extracted. The mean-square displacement (MSD) is obtained as

$$MSD = \langle u^2 \rangle = \frac{1}{N} \sum_i^N \langle \|\vec{r}_i(t_0 + \Delta t) - \vec{r}_i(t_0)\|^2 \rangle, \quad (8)$$

where $\vec{r}_i(t)$ is the position vector of atom i at time t and the angular brackets denote the average over multiple time origins.

Feature extraction from the structure and the sequence. To obtain the charge at pH 6, a protein structure file in the .pqr format was generated using *pdb2pqr* [89] along with *propKa* [90] to assign the expected protonation states. The charge of the different protein domains is then extracted by summing the charge of the atoms pertaining to a given domain. The other features, namely isoelectric point (pI), grand average of hydropathy (GRAVY) index and aromaticity were obtained using the BioPython [91] package.

Simulations at high concentration, computed SANS profiles. The system was constructed by randomly placing 6 monomers into a simulation box using the VMD [11] tcl script. The preparation of the system and the simulations were performed using a modified GROMACS [92, 93] package (GROMACS-SWAXS [94]) designed for interfacing with experimental small-angle scattering data. As above, the CHARMM36 force field was used, the proteins were solvated using TIP3P water and the system was neutralized with NaCl using GROMACS command-line tools. The 10 ns equilibration and the 200 ns simulation runs were performed using the same algorithms cited above. The computed SANS curves were obtained by averaging 100 frames on the last 20 ns of simulation for mAb9, mAb12 and mAb24. An envelope at a distance of 0.7 nm from the protein surface

was used. Further analysis of protein contacts network and hydrogen bonds was conducted using the MDAnalysis package [95, 96]. Protein network graphs were generated by finding the minimum distance between each pair of monomers for each frame in the simulation. Subsequently, all pairs for which the minimum distance was lower or equal to 5 Å were registered as interacting for the frame concerned. Among these interacting pairs, only the ones whose interactions were lasting for more than 1 ns were kept and used to produce the protein network graphs 10. The number of times a given pair is involved in an interaction throughout the simulation was used as a weighing factor to draw the graphs, where a higher number of times is represented by a thicker black line. Graphs were generated and clustering coefficients obtained using the NetworkX Python package [97].

3 Results and discussion

3.1 Viscometry

The viscosity was analysed depending on antibody concentration c_p and volume fraction φ (Fig. 2). φ is calculated from the protein concentration c_p as $\varphi = c_p V_s$, assuming the specific volume $V_s = 0.739$ mL/g [67]. An increase in the viscosity of the solutions at increasing antibody concentration is observed. This increase follows an exponential trend and shows large differences between the mAbs. In addition, some of them exceed 15-20 mPa·s already at relatively low protein concentration. Data obtained were fitted using the heuristic model

$$\eta_r(\varphi) = \frac{\eta}{\eta_0} = 1 + \exp(a\varphi + b\varphi^2), \quad (9)$$

with η_0 representing the solvent viscosity, and a and b being scalar fit parameters. Data collected from the mAbs in their original H₂O buffer are shown in Fig. 2; the viscosity of the solvent alone was $\eta_0(\text{H}_2\text{O}) = 0.92$ mPas. Results for the deuterated solutions are reported in the Supporting Information. The data show a strong dependence of the viscosity η on the type of mAb, although their structures only differ in the Fab and CDR regions. This observation suggests the microscopic origins of this phenomenon

and underlines the importance of investigating the interactions between mAb molecules.

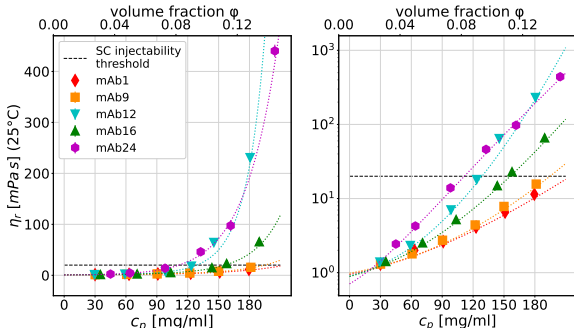


Fig. 2. Linear (left) and logarithmic (right) plots showing the relative viscosity $\eta_r = \eta/\eta_0$ (symbols) of aqueous (H_2O) solutions of different mAbs of the IgG1 subtype produced and characterised at Lonza, in 20 mM Histidine-HCl buffer at pH 6.0, at $T = 25^\circ\text{C}$ (≈ 298 K), versus mAb concentration c_p (lower x-axis) and volume fraction φ (upper x-axis), from dynamic viscosity measurements using a Rheosense VROC[®] Initium rheometer. Dotted lines are fits to the data using Eq. (9). The black dashed line represents the typically defined viscosity threshold for syringeability ($\sim 15\text{-}20$ mPa·s used in this work as guidance value, but in practical terms depending on volume, syringe, needle, acceptable injection force). The strong dependence of the viscosity on the mAbs, in spite of them only differing in the CDR (Fig. 1), underlines the need of understanding its microscopic origins.

3.2 Dynamic and static light scattering

Parameters obtained via light scattering measurements can give indications on the interactions between mAbs in solution. In particular, the second virial coefficient A_2 can be obtained from static light scattering (SLS) and diffusion-interaction parameter k_D from dynamic light scattering (DLS). In Table 1, we report the values determined previously by Schmitt et al. [41]. mAb1 and mAb9 are characterized by larger positive values of A_2 , which are

pointing towards electrostatic repulsion and might explain the relatively low viscosity. In contrast to mAb1 and mAb9, mAb12 shows a still positive but slightly smaller A_2 , indicating the presence of weaker repulsive forces between its monomers. However, mAb12 undergoes a significant increase in the viscosity at high concentrations compared to mAb1 and mAb9. On the other hand, mAb16 and mAb24 feature negative A_2 values, suggesting the presence of attractive interactions between monomers and justifying their viscosity increase at increasing mAb volume fraction. Concerning the diffusion-interaction parameter k_D , $k_D > 0$ indicates net repulsive interactions, whereas $k_D < 0$ is a signature of net attractive interactions between mAb monomers [27]. Again, while mAb1 and mAb9 show almost equal and largely positive k_D values, mAb12 has a weakly positive k_D , meaning that mAb1 and mAb9 feature way more repulsive PPIs and lower viscosities than mAb12. An opposite behaviour is observed for mAb16 and mAb24, which are characterized by negative k_D and therefore experience attractive PPIs and higher viscosities. Moreover, the trend of increasing viscosity at increasing mAb concentration is more pronounced for mAb24, having $A_2 = -0.72$ mol mL/g² and a largely negative k_D (Table 1).

mAb	$A_2 \times 10^{-4}$ (mol mL/g ²)	k_D (mL/g)
mAb1	1.96	19.40
mAb9	1.10	18.50
mAb12	0.44	1.37
mAb16	-0.11	-6.17
mAb24	-0.72	-21.10

Table 1: Second virial coefficients A_2 determined by SLS and diffusion-interaction parameters k_D from DLS obtained for the five mAbs by Schmitt et al. [41].

3.3 Quasi-elastic neutron spectroscopy

QENS spectra from different mAb solutions at comparable protein concentration c_p at the same temperature and same momentum transfer q show significant differences (Fig. 3) already visible without modelling.

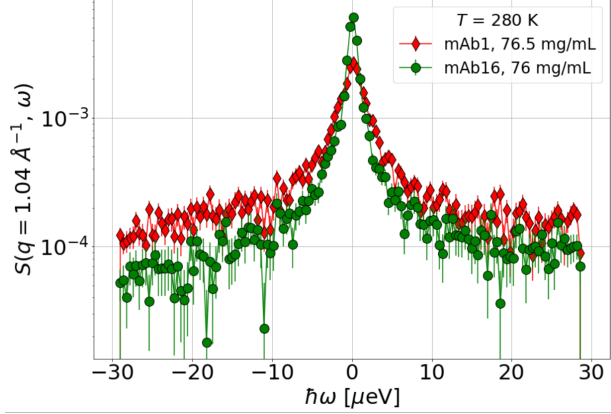


Fig. 3. Model-free comparison of QENS spectra at $q = 1.04 \text{ \AA}^{-1}$ for $T = 280 \text{ K}$ from two mAbs (mAb1 and mAb16) in D_2O solutions with 20 mM His-HCl, at nearly the same protein concentration ($\sim 76 \text{ mg/mL}$). Despite the identical conditions, the two spectra show significant differences in intensity and width.

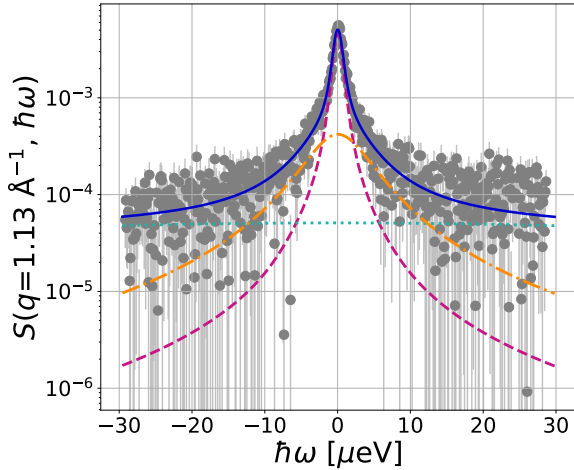


Fig. 4. QENS spectrum (symbols) obtained from mAb16 at $c_p = (80.44 \pm 4.79) \text{ mg/mL}$ at $T = 280 \text{ K}$ with different dynamic contributions (lines) at $q = 1.13 \text{ \AA}^{-1}$. The blue solid line represents the fit consisting of the different dynamical contributions in Eq. 1: global (magenta dashed), internal (orange dash-dotted) and solvent diffusion (cyan dotted line).

Global self-diffusion varies substantially among the mAbs. Due to the dominant nuclear incoherent scattering from the proteins, the obtained dynamic structure factor represents the ensemble-

averaged single-particle self-dynamics. From the narrowest Lorentzian contribution in the QENS spectra the observable apparent global self-diffusion coefficients $D = D(D_r, D_t)$ were obtained, which contain the contributions from both rotational D_r and translational D_t diffusion. $D = D(\varphi)$ is sensitive to the crowding effects mediated by hydrodynamic and electrostatic interactions, but also to the presence of clusters, which can be transient [69]. The comparison with existing models from colloid theory gives hints on the factors affecting short-time diffusion and ultimately, viscosity.

Diffusion of antibody solutions as soft colloid suspensions.

As stated above, proteins in solution experience both translational center-of-mass diffusion and rotational diffusion. The measured dynamic structure factor contains both contributions which together account for the global dynamics and result in the observable D . The width $\gamma(q)$ of the first Lorentzian contribution in Eq. 2 is associated with this apparent diffusion coefficient D . To interpret the experimental D in terms of colloid physics, a theoretical $D^{\text{theo}}(\varphi_t) = D^{\text{theo}}(D_r^{\text{theo}}(\varphi_t), D_t^{\text{theo}}(\varphi_t))$ was calculated as established in Ref. [66], based on an analytical expression for this implicit function and on the protein hydrogen radial density distribution $\rho(r)$ calculated from their pdb structures (see Supporting Information). An interpretation of the φ -dependence of D in terms of colloid physics is only possible by simplifying the protein shape [98, 99, 100]. Due to the observation time of our backscattering experiment of a few nanoseconds, resulting from its energy resolution, we access the diffusion in the so-called short-time limit, where protein-protein collisions can be neglected and hydrodynamic as well as electrostatic interactions prevail.

In a strongly simplified picture, the mAbs may be approximated as colloidal hard spheres to obtain an analytical expression of the crowding dependence of the translational diffusion D_t^{theo} ,

$$D_t^{\text{theo}}(\varphi_t) = D_t(\varphi_t = 0) f(\varphi_t) = D_t(0) f(\varphi_t), \quad (10)$$

in which the theoretical reduced translational diffusion $f(\varphi_t)$ depending on the hydrodynamic volume fraction φ_t can be described by a polynomial expres-

sion [98], and $D_t(0)$ denotes the dilute limit translational diffusion coefficient [98]. The rotational diffusion can be approximated by the charged-sphere model [99]

$$D_r^{\text{theo}}(\varphi_t) = D_r(\varphi_t = 0)(1 - 1.3\varphi_t^2), \quad (11)$$

where $D_r(0)$ denotes the dilute limit rotational diffusion coefficient [99], and which holds for low volume fractions. Due to the protein hydration shell moving along with the proteins an effective hydrodynamic volume fraction φ_t was assumed [66]. φ_t is connected to the protein volume fraction φ given by the sample preparation employing a dry protein powder by

$$\varphi_t = \varphi \left(\frac{R_h}{R_{\text{dry}}} \right)^3, \quad (12)$$

such that the volume fraction is rescaled by the ratio between the hydrodynamic radius obtained from the Stokes-Einstein relation,

$$R_h = (K_b T) / (6\pi\eta_{\text{D}_2\text{O}}(T)D_t(0)), \quad (13)$$

and the bare effective antibody radius

$$R_{\text{dry}} = \sqrt[3]{3V_{\text{dry}}/4\pi}. \quad (14)$$

All parameters entering these calculations, namely $D_t(0)$, $D_r(0)$ and the bare antibody volume V_{dry} to calculate R_{dry} , were derived via HYDROPRO10 [101] employing the pdb structure for each mAb, and for the reference polyclonal IgG using the structures of murine and human immunoglobulin G [102, 14]. For consistency with the neutron data, HYDROPRO10 calculations were performed using the viscosity and solution density of D_2O [103]. Values calculated for 7, 22 and 37°C (280, 295, 310 K) can be found in Table S2. Fig. 5 depicts the summary (at $T=280, 295, 310$ K) comparing the experimental D (symbols) for the different mAbs and an average of $D^{\text{theo}} = D^{\text{theo}}(D_t^{\text{theo}}, D_r^{\text{theo}})$ for monomeric solutions of all mAbs (lines). Grey shaded areas can be uncertainties associated to the solid lines and represent the regions delimited by D^{theo} for 1IGT (lower) and mAb12 (upper limit). Dashed brown lines are the approximated dimer curves obtained by rescaling the monomer ones by the dilute limit $D^{\text{theo}}(0)$ of

the dimeric immunoglobulin IgA solution structure 2QTJ [104]. Note that $D^{\text{theo}}(0)$ was again a function of its translational and rotational components at the dilute limit $D_t(0)$ and $D_r(0)$, calculated via HYDROPRO10 [101]. Fig. 5 shows that D varies significantly among the 5 mAbs and the reference IgG. The trend in D (Fig. 5) follows the trend in η (Fig. 2). Highly viscous mAb solutions, e.g. mAb12, display a lower D compared to less viscous ones, e.g. mAb1 and mAb9. The interpretation of the observable apparent diffusion of mAb solutions by colloid physics allows to estimate the level of aggregation in these systems. Diffusion coefficients (symbols) similar to the monomer hard-sphere prediction (solid line) suggest an overall monomeric solution, whereas smaller diffusion coefficients corroborate the presence of antibody aggregates which are mainly dimers or constituted by few monomers, since the symbols are not far from the theoretical prediction of dimer diffusion (dashed lines, Fig. 5). As stressed earlier, the hard-sphere model constitutes a very simplistic approximation for the non-spherical antibodies, and the assumption on the ratio R_h/R_{dry} enters sensitively by the third power (Eq. 12).

The global diffusion also varies with temperature as expected. Diffusion coefficients increase from 280 to 310 K, meaning that attraction between proteins and self-aggregation is not favoured at high temperatures, consistently with SANS results (see Sec. 3.4); mAb clusters overall undergo dissociation when exposed from storage to body temperature (~ 7 to 37°C). Moreover, by evaluating Eq. 7, we find that vibrational mean square displacements $\langle u^2 \rangle$ of hydrogen atoms in the mAbs studied are in the range 0.2 to 1.2 Å² with the expected temperature dependence, consistent with values found for other proteins [68].

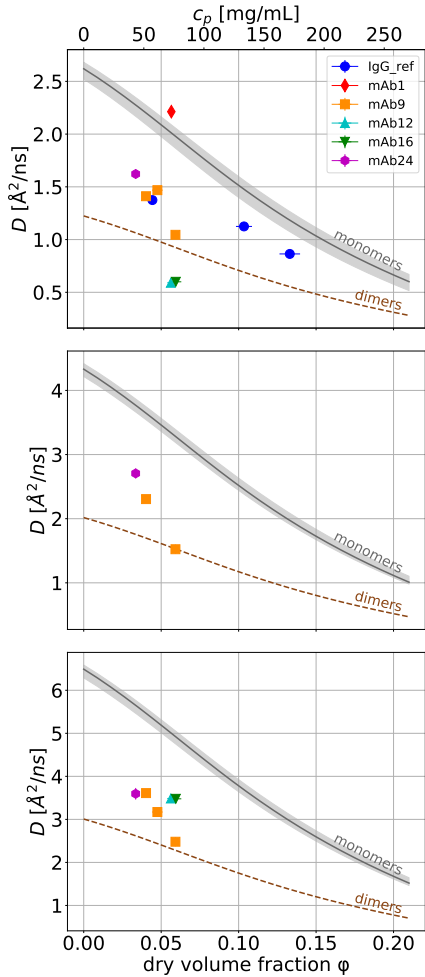


Fig. 5. Observable apparent diffusion coefficients D (symbols, obtained from global fits of QENS data) of the mAbs and polyclonal IgG *vs* protein dry volume fraction φ (lower) and concentration c_p (upper x-axis) in solution at $T = 280$ K (top), $T = 295$ K (center), $T = 310$ K (bottom). Grey solid lines denote the average value of D^{theo} for monomers of all mAbs, murine and human IgG [102], obtained using a colloid physics hard-sphere model as explained in the main text. Grey shaded areas are delimited by D^{theo} for 1IGT (lower) and mAb12 (upper limit). Dashed brown lines are the approximated dimer curves obtained by rescaling the monomer ones by the dilute limit $D^{\text{theo}}(0)$ of the dimeric immunoglobulin structure 2QTJ [104]. Symbols below the monomer lines corroborate the presence of clusters due to their larger hydrodynamic size. However, these clusters are mainly dimers or formed by few monomers and they dissociate at increasing temperature (from approximately storage to body temperature).

Internal dynamics and viscosity shows lower correlation on short time scales.

Generally, protein internal dynamics on ps to several ns timescales can relate to the capacity of the protein to swiftly change conformation and possibly adopt conformers prone to self-association [59]. Such behaviour would be in favour of high viscosities. Hence, the internal dynamics of the mAbs was investigated experimentally through the QENS spectra and computationally through the MD simulations. The diffusion coefficient associated with internal motions D_i , as obtained from QENS fits, is similar within the confidence bounds for each mAb except for mAb9, for which D_i is 2 times lower. The residence time τ is similar for all mAbs, except mAb1 and mAb9, showing slightly higher values. These ensemble-averaged values for internal dynamics obtained from QENS do not present any clear correlation with the viscosity data in Sec. 3.1.

The large scale domain motions - that is, the angle between the main lobes, Fc and Fab regions of the mAbs - were computed as described in Section 2, averaging over time blocks of 20 ns (Fig. 6). There are no significant differences between the different anti-

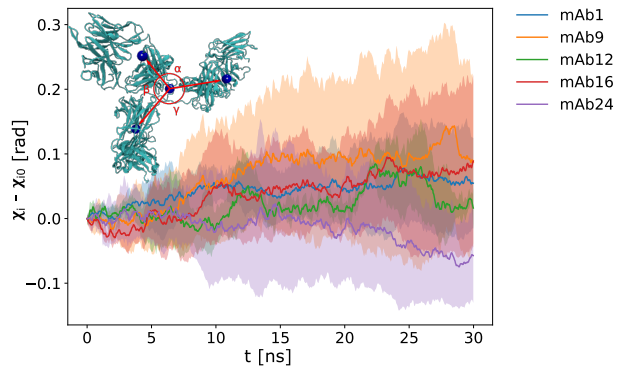


Fig. 6. Large scale domain motions observed during MD simulations of the mAbs. For each angle indicated on the cartoon structure in inset, the deviation of the angle $\chi_i = \{\alpha, \beta, \gamma\}$ from the initial value χ_{i0} was computed as indicated in Section 2 using the block average method with a block size of 20 ns. The average of the blocks is plotted using coloured solid lines and the standard deviation of the blocks using coloured shaded areas for each mAb.

-bodies within the errors, as the angles between the three main lobes of the antibody fluctuate slowly around an equilibrium value. However, the viscosity could depend on weak transient interactions that can be facilitated by fast internal dynamics in the sub-ns time domain.

To investigate fast motions, the diffusive mean-square displacement (MSD) $\langle u^2 \rangle$ was computed as explained in Section 2 for each amino acid of the different mAbs. The MSD were obtained for a time delay of 50 ps, 200 ps and 1 ns. Globally, the MSDs appear similar for all mAbs without an obvious correlation with increasing viscosity (Fig. 7(top)). Slightly more relevant differences in the MSD among the mAbs can be found in the light chains (Fig. 7(bottom)), but without a visually clear trend with increasing viscosity.

To better correlate with the viscosity, the dimension of the MSD vector was reduced by averaging over the main domains (VH, CH1, CH2, CH3, hinge, VL, CL) for all time delays. The resulting correlations with the viscosity are presented in Fig. 8. It appears that the viscosity is significantly correlated ($\rho_{\text{viscosity}, \theta_i} > 0.5$) with the features related to protein-protein interaction strength (virial coefficient A_2 and diffusion-interaction parameter k_D). Moreover, the charge on VL and VH regions appears to correlate with viscosity as well, suggesting that electrostatic interactions are dominant. The internal dynamics represented by the MSD presents some correlation with viscosity, but the correlation coefficients are systematically lower than 0.5 indicating that such dynamics plays a subordinate role.

To assess whether the features used to compute the correlation can provide a good predictor of viscosities, the viscosity was removed from the dataset, resulting in a reduced dataset. A principal component analysis (PCA) was performed on the covariance matrix of this reduced dataset using routines from the *scikit-learn* package [105]. The observable values from Figure 8 were then projected on the first 3 principal components to visualize how the different mAbs are separated in PCA space (Fig. 9). It appears that the use of either the full feature dataset or a reduced one where MSD are removed allows for a good separation of the mAbs according to the viscosity. However, the

MSDs alone do not provide a good separation of the mAbs, and hence do not constitute a reliable predictor for the viscosity.

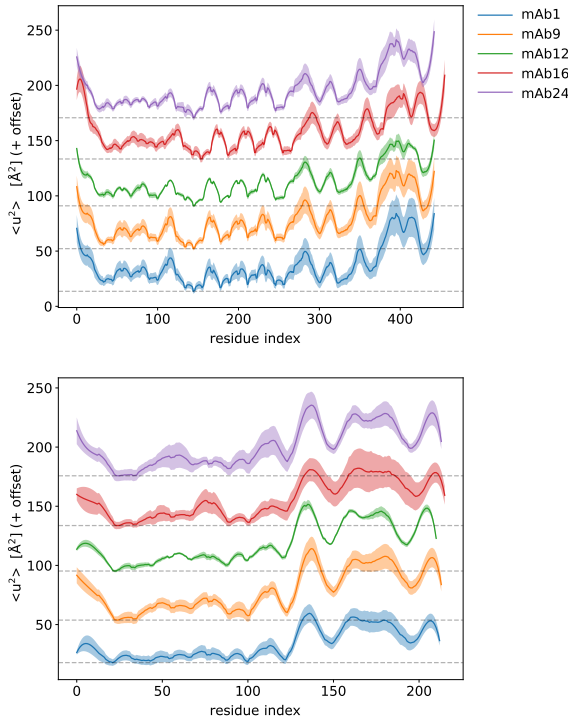


Fig. 7. Diffusive mean square displacements (MSD) $\langle u^2 \rangle$ derived from MD simulations of the 5 mAbs computed from the simulated trajectories as described in Section 2. The resulting average over the blocks of 20 ns size is plotted for each mAb using solid coloured lines and corresponding shaded areas for the standard deviations. MSD for the heavy chains (top) and for the light chains (bottom plot) with an offset on y-axis for better visualization and for increasing viscosity from bottom to top.

Viscosity strongly depends on VL and VH domain charges and hydrophobicity. To further explore the determinants of the viscosity, additional features, obtained directly from the amino-acid sequence, were computed (see Section 2). The obtained features were used to compute the statistical covariance with the viscosity. The input values used for the viscosity were collected from concentration series (30, 60, 90, 120, 150, 180 mg/mL) of the five mAbs in solution at 25°C (data in Section 3.1). The hydrophobicity and the charge, especially on the VL and VH do-

mains, are correlated with the viscosity (Fig. 8). The second virial coefficient A_2 , the diffusion-interaction parameter k_D and the isoelectric point pI present a pronounced correlation with viscosity.

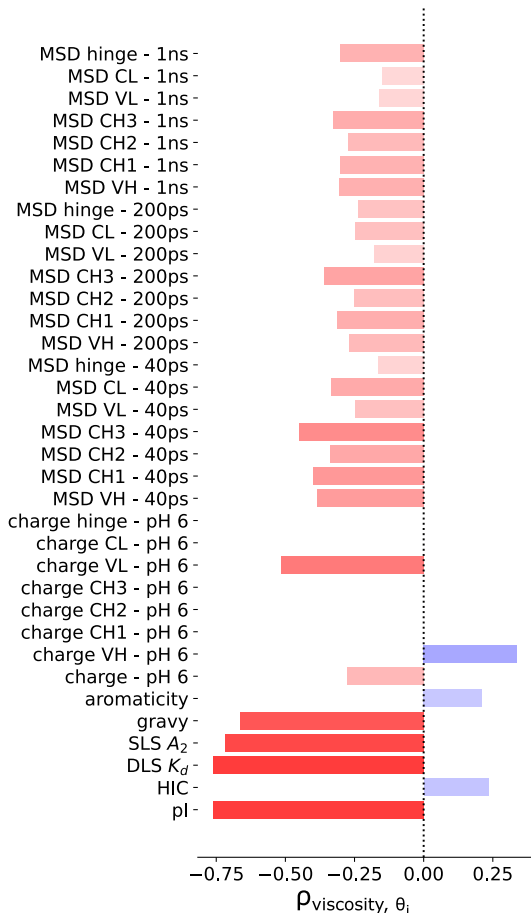


Fig. 8. Correlation of viscosity with parameters derived from protein sequence and MD simulations. The MSD as well as the features extracted from the sequence (see Sec. 2) were used to compute the statistical correlation with the viscosity $\rho_{viscosity, \theta_i}$ given by $\rho_{x,y} = \frac{\text{covariance}(x,y)}{\sqrt{\sigma(x)}\sqrt{\sigma(y)}}$, where σ is the variance and θ_i the value of the observable i . The result is shown as a vertical bar plot with the features arranged on the y-axis and the bars ranging from deep red for a correlation of -1 to deep blue for a correlation of 1. The charges are computed at pH 6 and the labels include the concerned protein domain except for ‘charge pH 6’, which is the total charge of the protein. The labels for MSD values include the concerned protein domain followed by the time delay used to compute it.

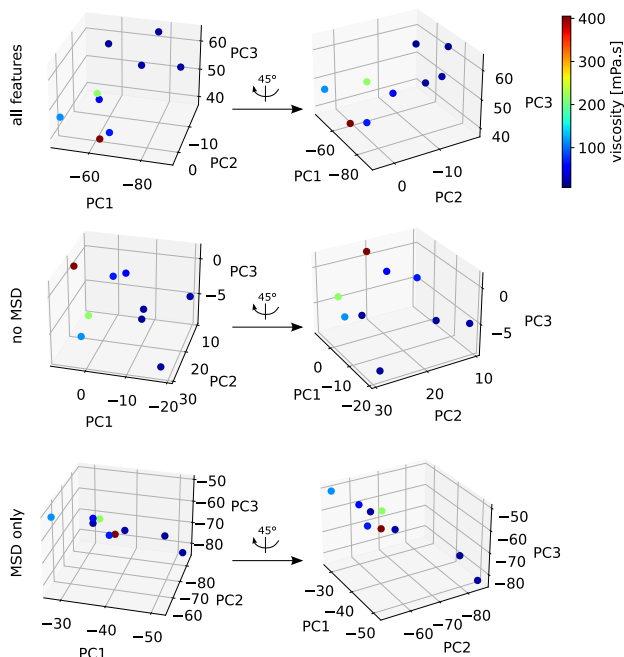


Fig. 9. Separation of mAbs using computed features. A principal components analysis (PCA) was performed on the features computed for Fig. 8 using routines from the *scikit-learn* Python package [105]. The initial observable vectors were projected on the first 3 principal components (PC) and plotted with dots whose colour corresponds to viscosity (ranging from deep blue for low η to brown for high η). Top plots: projections for a PCA using all features; middle plots: projections for a PCA on a dataset where the MSD was removed; bottom plots: projections for a PCA performed on a dataset containing MSD only.

Protein network dynamics differs between mAb9, mAb12 and mAb24. Next, we investigate the protein-protein interactions by putting 6 monomers in a simulation box. The simulations were run for 200 ns and analysed using a protein network graph computation of the hydrogen bond dynamics between individual monomers. Building a protein interaction network allows to identify protein clusters, their size and their dynamics by tracking the interaction lifetime for each pair of monomers in the simulation. The network graphs were constructed as described in Section 2 and the result is shown in Fig. 10. The graph for mAb9 shows two clusters of 4 and 2 monomers that are present for most of the trajectory

and can sometimes interact with an interaction lifetime of 1.1 ns, which crosses the backscattering observation time window. The graphs for mAb12 and mAb24 present a higher connectivity quantified by the clustering coefficient (CC) of the graphs, being 0.73 for mAb12 and 0.47 for mAb24, against a

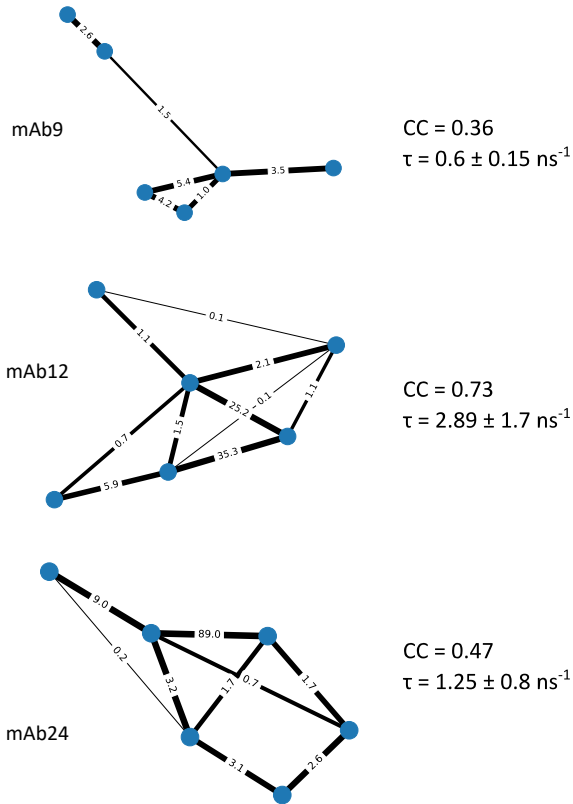


Fig. 10. Protein network graphs for mAb9, mAb12 and mAb24, produced from the simulations at high concentration. Each blue node represents a single mAb monomer and two nodes are connected by an edge when their atoms are at a minimum distance of 5 Å. The width of the edge line is proportional to the number of times a given pair of monomers are in interaction during the simulation and the number labels indicated on the edges are the average lifetime of the interaction given in ns. The clustering coefficient (CC) and the average relaxation rate (inverse lifetime) τ are shown for each protein graph. Protein network dynamics at high concentration strongly depends on the mAb.

value of 0.36 for mAb9. This result is in agreement with the slow diffusion and the marked trend to cluster of mAb12 revealed by QENS (Fig. 5), as well as with its stronger protein-protein interactions probed by SANS (Fig. 13 in the next paragraph), compared to the ones present in mAb9 and mAb24 solutions. Moreover, the average relaxation rate (obtained by computing the inverse average lifetime of graph vertices) is lowest for mAb9 and highest for mAb24, thereby demonstrating a more dynamic protein interaction network for mAbs with higher viscosities.

3.4 Small-angle neutron scattering

Small-angle neutron scattering (SANS) provides thermodynamic and structural information on the nanometer-micrometer scale. The intensity of scattered neutrons is expressed as [106, 107]

$$I(q) = \frac{d\sigma}{d\Omega}(q) = n(\Delta\rho)^2 V_{\text{part}}^2 P(q)S(q), \quad (15)$$

where n is the particle number density, $\Delta\rho$ is the difference in scattering length density between the solvent and the particles (also known as the scattering contrast), and V_{part} is the volume of a single particle [106, 107]. The term $P(q)$ is referred to as the particle form factor, determined by the protein shape [108, 107]. The structure factor $S(q)$ (details in, e.g., Refs. [109, 110, 111]) characterises the interactions between particles in solution. $S(q \rightarrow 0)$ can be expanded into a series of virial coefficients A_n [112, 113, 114]

$$S(q \rightarrow 0) = \frac{RT}{M_W} \left(\frac{\partial \Pi}{\partial c} \right)^{-1} = \frac{1}{1 + 2M_W A_2 c + \dots} \quad (16)$$

with M_W (g/mol), the molecular weight of the particles investigated.

The second virial coefficient B_2 [115]

$$B_2 = 2\pi \int_0^\infty dr r^2 [1 - e^{-\frac{u(r)}{k_B T}}] \quad (17)$$

is related to A_2 , which can be determined by SLS (see above) [41] via [116]

$$B_2 = A_2 \cdot \frac{M_W^2}{N_A}. \quad (18)$$

We employ the reduced second virial coefficient B_2^* , defined as the ratio B_2/B_2^{HS} (B_2^{HS} : second virial coefficient for hard spheres) and linked to the stickiness

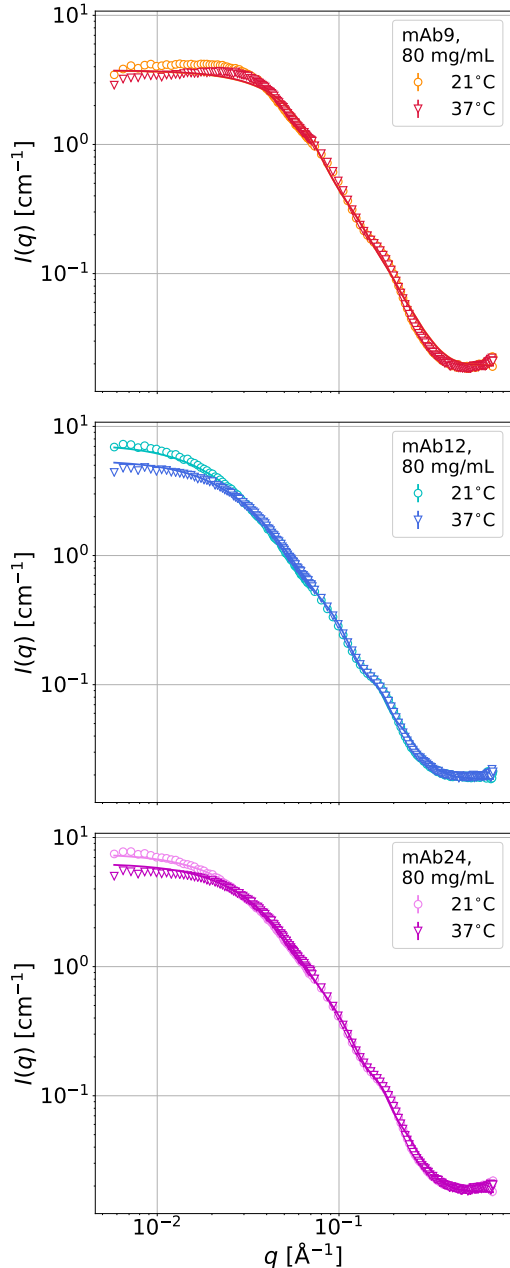


Fig. 11. Temperature dependence of mAb-mAb interactions probed using SANS. Experimental curves (empty symbols) and their corresponding fits obtained from a subset of mAbs: mAb9 (top), mAb12 (center), mAb24 (bottom) at $c_p = 80$ mg/mL in 20 mM His-HCl buffer in D_2O . A decrease of $I(q)$ at low q with increasing temperature T reveals weaker protein-protein interactions (PPIs) and a potential dissociation of aggregates.

parameter τ by

$$B_2^* = B_2/B_2^{HS} = 1 - \frac{1}{4\tau}, \quad (19)$$

where $B_2^{HS} = 2\pi\sigma^3/3$, with σ being the diameter of the hard spheres [115]. In the case of the mAb samples studied here, we employ the approach by Da Vela et al. [51] for bovine γ -globulin). The SANS data were fitted using the NIST software package for SANS analysis Igor Pro [117] by WaveMetrics, Inc. (Lake Oswego, OR, USA). The model used is a combination of an ellipsoid form factor $P(q)$ (oblate ellipsoid with axes of 7-8 Å and 58-60 Å) and a sticky hard sphere (SHS) structure factor $S(q)$ for mAb12 and mAb24. For mAb9, the SHS structure factor was not properly fitting the data at low q , so we opted for simple hard-sphere (HS) potential, also due to the repulsive feature of its $I(q)$ at low q . We note that in spite of the anisotropic shape of mAbs, an isotropic interaction potential is used here. This approach has been justified by Yearley et al. [118] and Castellanos et al. [119]. Background-corrected SANS data for mAb9, mAb12 and mAb24 with the corresponding fits are shown in Figs. 11,12, in order to better visualize antibody-type and temperature dependence of the data, respectively. Overall, a decrease of the intensity $I(q)$ in the low- q region is observed when the samples are heated up from 21°C to human-body temperature, suggesting a weaker inter-protein attraction at high temperatures and likely dissociation of microscopic aggregates formed at lower temperatures (Fig. 11). High- q features of $I(q)$ (around 0.2 \AA^{-1}) are due to the molecular shape of the mAbs, which is preserved across the temperature range studied. However, differences in the low- q region are also variant-dependent (Fig. 12), meaning that the three mAb solutions feature different PPIs. In fact, mAb9 shows less attractive PPIs than mAb12 and mAb24, which may result in a lower level of clustering and a higher diffusion coefficient, as observed through QENS (Fig. 5), along with a lower CC determined from protein network analysis (Fig. 10). For mAb12 and mAb24, based on the SHS $S(q)$ fit, the normalised second virial coefficient B_2/B_2^{HS} was determined. $B_2/B_2^{HS} < 0$ indicate overall attractive interactions between the mAb molecules, whereas

$B_2/B_2^{HS} > 0$ point towards overall repulsive interactions. The resulting B_2/B_2^{HS} are shown in Fig. 13. Lower B_2/B_2^{HS} are observed at lower temperature, which means that mAb-mAb attraction is stronger and aggregation is favoured; this result is in agreement with conclusions from QENS data (Sec. 3.3).

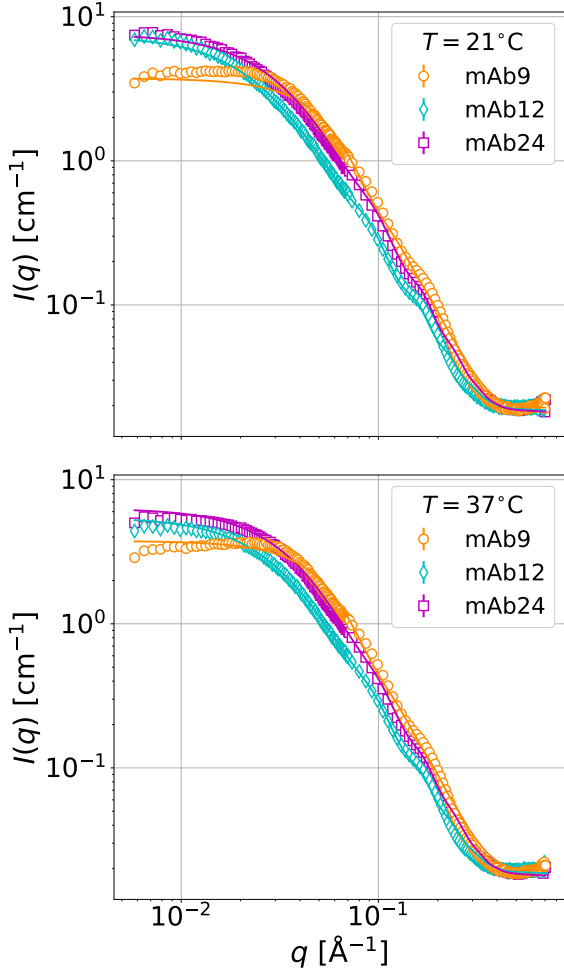


Fig. 12. Antibody dependence of protein-protein interactions probed through SANS. Comparison among SANS curves (empty symbols) and their corresponding fits of mAb9, mAb12 and mAb24 at 21°C (left) and 37°C (right). Protein concentration in all samples is 80 mg/mL in 20mM His-HCl buffer in D_2O . $I(q)$ does not show significant changes at high- q , while differences in the low- q region are visible at both 21 and 37°C and suggest the presence of different PPIs in the three mAb solutions; mAb9 shows much less attractive PPIs than mAb12 and mAb24.

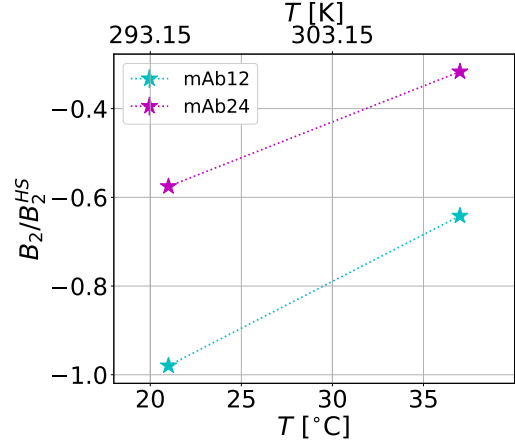


Fig. 13. Reduced second virial coefficient B_2/B_2^{HS} (symbols) determined from the SANS fits plotted against temperature T in °C (lower x-axis) and Kelvin (upper x-axis). Data from the two mAbs presented here (mAb12 and mAb24) show an increase in B_2/B_2^{HS} with increasing temperature from 21 to 37°C, meaning that attraction among antibodies decreases when the solutions experience higher temperatures. Dashed lines are guides to the eye.

For mAb12, the A_2 value mentioned above indicated a slight repulsion while the B_2/B_2^{HS} values indicate an attraction. This might be due to the usage of the sticky hard sphere model, which is incapable to capture reversible self-association or anisotropic or directional interactions. A reversible self-association would also explain the significantly reduced diffusion coefficient observed above.

4 Conclusions

The present work provides a deeper understanding of the link between microscopic dynamic properties and macroscopic viscosity of the solutions of five different monoclonal antibodies of the IgG1 subtype. By a multi-technique approach employing neutron backscattering spectroscopy, small angle neutron scattering, molecular dynamics simulations and viscometry, we find biophysical determinants for the variation in the viscosity of mAb solutions. Consistent with previous work [120, 57, 121], we identify

the formation of clusters of self-associating antibody molecules as the main mechanism responsible for the increase in viscosity at high antibody concentrations.

In particular, QENS experiments access the different dynamical contributions of the systems studied. Global and internal dynamics can be decoupled and analysed separately. The global dynamics refers to the protein center-of-mass motion, accessing the observable apparent diffusion coefficient D , which can be interpreted in terms of the physics of dense colloidal suspensions of hard spheres, within the approximations of short-time diffusion and an effective spherical shape. By approximating mAb molecules as hard spheres, D obtained from QENS are compared to the theoretical estimation $D_{\text{theo}}(\varphi)$ obtained employing the hard-sphere model. Importantly, due to the prevailing nuclear incoherent scattering from the protein hydrogen atoms, our QENS experiment probes the self-diffusion (synonymously: tracer diffusion) of the mAbs. For this reason it unambiguously provides the hydrodynamic size of the mAb assemblies unobstructed by structural features seen by coherent scattering. For most mAbs, $D < D_{\text{theo}}(\varphi)$ in agreement with a cluster picture. Moreover, most diffusion coefficients are larger than those estimated for mAb dimers, such that the QENS results support a picture of very small clusters with less than two members on average. The clusters may be transient in time, and the QENS spectra are recorded with an observation time of a few nanoseconds resulting from the $< 1\mu\text{eV}$ energy resolution, thus, not ruling out dissociation on longer times. This short observation time comes with the advantage that protein-protein collisions can be neglected, and the observed diffusion is governed by hydrodynamic and electrostatic interactions.

From the QENS analysis we can infer that all mAbs undergo self-association and cluster formation at the lowest temperature measured, 280 K ($\approx 7^\circ\text{C}$), which is a storage temperature, while short-time diffusion is enhanced at 310 K ($\approx 37^\circ\text{C}$), which is the physiological temperature. Moreover, different mAbs result in significantly different diffusion, with smaller diffusion, i.e., larger average cluster size for higher viscosity.

In addition, all mAbs measured show higher D with increasing temperature, consistent with enhanced diffusion with higher T as expected. We point out that the mAb concentrations measured by QENS in the present work are low compared to those used in previous studies on model protein solutions [59]. For this reason, the scattering signal is weak, and the accuracy of the information on the internal diffusive dynamics is limited. Nevertheless, this internal dynamics information is consistent with earlier work on γ -globulin [67] within the uncertainties. The low signal required that global fits of the spectra for all momentum transfers at once had to be used. These global fits might result in a systematic error for the global diffusion due to a possible cross-talk of the global and internal dynamics Lorentzians at large momentum transfers.

SANS was used to probe structural and thermodynamic features of mAb solutions on a subset of the mAbs studied by QENS, and the second virial coefficient was extracted to estimate the interaction among mAb molecules, revealing that protein-protein attraction decreases with decreasing viscosity and increasing temperature. This trend is consistent with the cluster formation seen in QENS. Our data set indicates that the clusters tend to dissociate with increasing temperature, the weak electrostatic interactions being outweighed at higher temperatures. As the data set is currently limited, this trend should be substantiated with further measurements in the future.

The single-molecule MD simulations provide an atomistic view of the internal dynamics, complementary to the QENS data. The result shows that the internal dynamics is not a good predictor of the viscosity. The internal dynamics was found to be similar for all mAbs, as expected based on the fact that the molecules are basically identical apart from small differences in the CDR regions of their sequences. The presence of strong correlation of viscosity with hydrophobicity and second virial coefficient indicates that protein-protein interactions and possibly protein-solvent interactions mainly drive the viscosity. The MD simulations with 6 molecules per simulation box provide some insight on the protein clus-

ter dynamics for mAb9, mAb12 and mAb24. It appears that high viscosity mAbs tend to form clusters with more monomers, but with shorter interaction lifetimes and less frequent pairs of monomers such that the protein network reorganizes faster. As a result of strong cluster dynamics, a shear stress results in inefficient momentum transfer between molecules because of short-lived interactions, in high friction between clusters, and hence in high viscosity. We note that the conclusions from the simulations are limited by the achievable sampling time. Yet, the results are convincing and appeal for further work fully dedicated to simulations of mAbs at high concentration.

We have presented an extended study of an unprecedentedly large number of different mAbs with neutron and complementary techniques, and have established incoherent high-resolution neutron backscattering spectroscopy as a new technique to study mAb solutions and to unambiguously access their average hydrodynamic cluster size. A key finding from this work is that the clusters seen on the nanosecond observation time of our neutron spectroscopy experiment consist on average of less than two members per cluster at physiological temperature (Fig. 5 bottom). This average size can be assumed to reflect a highly dynamic picture of the self-association and resulting viscosity of the mAb solutions and depends sensitively on the type of mAb. The sensitivity on the mAb type cannot be understood by internal motion, as revealed by the simulations, but rather by differences of the mAbs in specific regions near the protein surface. The simulations are consistent with this picture of highly dynamic transient protein association.

5 Associated content

Data Availability Statement Neutron data are permanently curated under DOI and available at refs [63] and [76].

Supporting Information The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.3c00440> and includes: biophysical properties of

mAbs, viscometry on deuterated antibody solutions, additional example neutron spectra, HYDROPRO10 calculations and hydrogen density distributions from pdb structures, additional QENS/SANS fit parameters and simulation results.

6 Author contribution

All authors performed experiments, contributed to data analysis and in writing the manuscript. K.P. performed simulations, L.C., K.P., C.B. prepared the samples. F.S., T.S., O.M. and C.G. designed the research and are co-proposers and supervisors of the associated InnovaXN project (innovaxn.eu).

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Journal: This manuscript has been published on July 23, 2023 as part of the Molecular Pharmaceutics virtual special issue “*Research Frontiers in Industrial Drug Delivery and Formulation Science*”.

7 Acknowledgements

This research has been supported by InnovaXN, a EU Horizon 2020 MSCA COFUND programme (innovaxn.eu, grant agreement No 847439). I.M. acknowledges an ILL PhD studentship funded by this programme. Moreover, we are grateful for support by the DFG and ANR (ANR-16-CE92-0009, ImmunoglobulinCrowding), notably in the initial phase of this project, and by the BMBF (ErUM-pro 05K19VTB and 05K22VTA). The authors acknowledge the support of the ESRF and ILL for using the platforms of the Partnership for Soft Condensed Matter (PSCM). The authors are also thankful to Trevor Forsyth, Michael Haertlein and Juliette Devos (ILL, Grenoble) for advice and for providing access to the Life Science lab facilities. We thank Jonathan Schmitt (Lonza Basel) for support with rheometry.

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