Cluster formation and the link to viscosity in antibody solutions

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Abstract

Monoclonal antibody solutions are set to become a major therapeutic tool in the years to come, capable of targeting various diseases by clever designing their antigen binding site. However, the formulation of stable solutions suitable for patient self-administration typically presents challenges, as a result of the increase in viscosity
that often occurs at high concentrations. Here, we establish a link between the microscopic molecular details and the resulting properties of an antibody solution through the characterization of clusters, which arise in the presence of self-associating antibodies. In particular, we find that experimental small-angle X-ray scattering data can be interpreted by means of analytical models previously exploited for the study of polymeric and colloidal objects, based on the presence of such clusters. The latter are determined by theoretical calculations and computer simulations of a coarse-grained minimal model, in which antibodies are treated as Y-shaped colloidal molecules and attractive domains are designed as patches. Using the predicted cluster size distributions, we are able to determine how the concentration-dependent increase in viscosity is indeed originated by the presence of small and medium-sized clusters. Our findings provide new insights on the aggregation of monoclonal antibodies, which can be exploited for guiding the formulation of stable and effective antibody solutions.

Introduction

Monoclonal antibodies (mAbs) have moved into the focus of pharmaceutical industry as a major platform for potential drug candidates. However, successful mAb applications that allow for facile home administration require stable and low viscosity high concentration formulations with concentrations of the order of 100 g/l or more, which are often difficult to achieve. In fact, mAbs are prone to exhibit reversible self-association at high concentrations that result in enhanced viscosity, which creates the need for an advanced predictive understanding of the stability and viscosity of concentrated protein solutions.

There is considerable evidence in the literature that the formation of (equilibrium) transient clusters can strongly influence the relative viscosity, and for example results in a dynamic arrest through a so-called cluster glass transition, as long as the life time of the transient bonds between proteins or colloids is long enough. The presence of such clusters strongly influences the zero shear viscosity $\eta_0$ of concentrated solutions, resulting in an
arrest transition at lower concentrations when compared to a purely monomeric solution.\textsuperscript{9} There are in fact a number of studies where increased viscosity in concentrated solutions of mAbs is linked to cluster formation.\textsuperscript{12–20} These have made attempts to characterize cluster formation in mAb solutions, and to interpret antibody solution properties through analogies with colloids or polymers. In particular, scattering techniques were used to investigate protein interactions and self-association.\textsuperscript{13,17,21–25} However, we are far from having any predictive understanding and a generally accepted methodology and/or theoretical framework to detect antibody cluster formation.

The complexity of the system, formed by anisotropic and flexible large molecules that interact through a number of different intermolecular forces, makes a theoretical treatment capable of providing a quantitative link between the molecular structure, the resulting intermolecular interactions and the various structural and dynamic quantities obtained in experiments a real challenge. In particular at high concentrations this requires different coarse graining strategies that allow us to incorporate crucial molecular information into colloid-like models that are then amenable to computer simulations as well as to analytical calculations.

Here, we explore the application of small-angle X-ray scattering (SAXS) experiments and investigate whether such experiments are able to provide us with a typical "fingerprint" for the presence of small equilibrium clusters. We focus on a well-characterized models system of a humanized IgG4 against trinitrophenyl, which was found to exhibit an increased viscosity at high concentrations.\textsuperscript{26} We have previously reported on a detailed experimental and theoretical investigation of key structural and dynamic properties, such as the apparent weight-average molecular weight $\langle M_w \rangle_{\text{app}}$ obtained by static light scattering (SLS), the apparent z-average hydrodynamic radius $\langle R_h \rangle_{\text{app}}$ measured by dynamic light scattering (DLS) and the relative zero shear viscosity $\eta_r = \eta_0/\eta_s$, where $\eta_0$ is the zero shear viscosity of the antibody solution and $\eta_s$ the solvent viscosity, obtained by microrheology.\textsuperscript{18} There, we could establish a theoretical framework for a coarse-grained approach where we explicitly consid-
ered the anisotropy of both the shape and the interactions of the antibody molecules that allowed us to successfully reproduce the experimental data and thus obtain a quantitative prediction for the cluster size distribution as a function of antibody concentration. However, the presence of self-assembled clusters was derived from macro- and mesoscopic experimental quantities only.

In this work we extend our approach to SAXS, since this technique provides high resolution structural data down to the molecular scale. We thus derive an analytical model for the expected scattering signal from antibody clusters as a function of the cluster size or aggregation number $s$. We test this model against the results from novel, coarse-grained computer simulations of an improved model of Y-shaped patchy molecules that is built from calculations of the electrostatic properties of the considered mAbs. Using the predictions for the cluster size distribution as a function of concentration, we then make a final comparison of our model calculations with the experimental data from SAXS. The available data is indeed well reproduced by the model, providing for the first time a clear experimental signature of the presence of small clusters in antibody solutions that exhibit enhanced values for the reduced viscosity at higher concentrations.

**Methods**

**Experimental sample preparation**

In this study we have used a humanized IgG4 antibody against trinitrophenyl (TNP). The antibody was manufactured by Novo Nordisk A/S and purified using Protein A chromatography, and subsequently concentrated and buffer exchanged into a 10mM Histidine 10mM NaCl pH 6.5 buffer at a concentration of 100mg/mL. From this stock solution, samples were prepared by concentrating and buffer exchanging into either 20mM Histidine 10mM NaCl pH 6.5 or 20mM Histidine 50mM NaCl pH 6.5 buffers using Amicon spin filters with a molecular weight cutoff at 100kD (Merck, Germany). The two different solvents thus have a total ionic strength corresponding to either
17 mM or 57 mM NaCl, respectively. Samples with decreasing concentration were then prepared from the concentrated sample by dilution, determining the antibody concentration using UV/Vis absorption at 280 nm and an extinction coefficient of \( e_{280nm}^{1\%,1cm} = 2.234 \). The determination of the concentration was repeated three times in order to access the uncertainty.

**Light scattering measurements**

SLS experiments were performed using a 3D-LS Spectrometer (LS Instruments AG, Switzerland) with a 632 nm laser, recording DLS and SLS data simultaneously. The measurements were conducted at 90° scattering angle. Before measurement, the samples were transferred to pre-cleaned 5 mm NMR tubes and centrifuged at 3000 g and 25 °C for 15 min, to remove any large particles and to equilibrate temperature. Directly after centrifugation, the samples were placed in the temperature equilibrated sample vat and the measurement was started after 5 minutes to allow for thermal equilibration. Additional low concentration SLS measurements were done using a HELIOS DAWN multi-angle light scattering instrument (Wyatt Technology Corporation, CA, USA), connected to a concentration gradient pump. The instruments were calibrated to absolute scale using toluene (with a Rayleigh ratio of 1.37 \( \cdot 10^{-5} \) cm\(^{-1} \) at 25° C and \( \lambda = 632.8 \) nm) in the case of the 3D-LS Spectrometer, and toluene and a secondary protein standard with a known molecular mass for the HELIOS DAWN, allowing for direct comparison of the two data sets.

From the SLS experiments, the apparent weight average molecular weight \( \langle M \rangle_{w,app} \) of the antibodies in solution was calculated using

\[
\langle M \rangle_{w,app} = \frac{R(90)}{KC}
\]

where \( R(90) \) is the absolute excess scattering intensity or excess Rayleigh ratio measured at a scattering angle of 90°, \( K = 4\pi^2 n^2 (dn/dC)^2 / N_A \lambda_0^4 \), \( n \) is the refractive index of the solution, \( dn/dC = 0.192 \) L/g is the refractive index increment of the antibodies, \( \lambda_0 \) is the vacuum wavelength of the laser, and \( C \) is the antibody concentration in milligrams per milliliter. The excess Rayleigh ratio \( R(90) \) is obtained from the measured scattering of the protein solution, \( I(90) \), of the solvent \( I_s(90) \) and of the reference standard \( I_{ref}(90) \) using \( R(90) = [(I(90) - I_s(90))/I_{ref}(90)] R_{ref}(n/n_{ref})^2 \),
where $R_{\text{ref}}$ is the Rayleigh ratio of the reference solvent, and $n$ and $n_{\text{ref}}$ are the index of refraction of the solution and the reference solvent, respectively. Note that, due to the small size of the antibody molecules and of the antibody clusters, there is no measurable angular dependence in the scattering intensity, and we can directly use the intensity values measured at a scattering angle of 90° instead of the corresponding values extrapolated to $\theta = 0$.

**Microrheology**

The zero shear viscosity $\eta_0$ of the antibody solutions relative to that of the pure buffer, denoted as the relative viscosity $\eta_r = \eta_0/\eta_s$, was obtained using DLS-based tracer microrheology. Sterically stabilized (pegylated) latex particles were mixed with protein samples to a concentration of 0.01 % v/v using vortexing and transferred to 5 mm NMR tubes.

The sterically stabilized particles were prepared by cross-linking 0.75 kDa amine-PEG (polyethylene glycol) (Rapp Polymere, 12750-2) to carboxylate stabilized polystyrene (PS) particles (ThermoFischer Scientific, C37483) with a diameter of 1.0 $\mu$m using EDC (N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide) (Sigma Aldrich, 39391) as described in detail in Ref. 27

DLS measurements were performed on a 3D-LS Spectrometer (LS Instruments AG, Switzerland) at a scattering angle of 46-50° to avoid the particle form factor minima and thus maximise the scattering contribution from the tracer particles with respect to the protein scattering. Measurements were made using modulated 3D cross correlation DLS28 to suppress all contributions from multiple scattering that occur, in the attempt to achieve conditions where the total scattering intensity is dominated by the contribution from the tracer particles. Samples were either prepared individually or diluted from more concentrated samples using a particle dispersion with the same particle concentration as in the sample as the diluent. The diffusion coefficient $D$ of the particles was then extracted from the intensity autocorrelation function using a first order cumulant analysis of the relevant decay. This diffusion coefficient is compared to that of tracer particles in a protein-free solvent (buffer) resulting in a relative diffusion coefficient, $D_r = D_{\text{sample}}/D_{\text{solvent}}$. The relative viscosity $\eta_r$ is related to $D_r$ through $\eta_r = 1/D_r$, again according to the Stokes-Einstein equation, where now $R_h$ is the known and constant hydrodynamic radius of the tracer particles.27,29
SAXS measurements

Form factor

In order to avoid any concentration-induced antibody clusters and other aggregates, the SAXS form factor of the mAb was measured at the SWING beamline at synchrotron SOLEIL (Gif-sur-Yvette, France) using a combined size exclusion chromatography and SAXS setup. The setup consisted of an Agilent HPLC system composed of a BioSEC-3 300 column, an automatic sample loader and a UV/VIS detector, connected to a flow through cell located at the sample position in the SAXS instrument. The sample loading and flow was controlled by the HPLC software, whereas the SAXS measurements were initiated manually. The SAXS measurements consisted of a background measurement $100 \times 1500 \ \mu s$ exposures once a stable UV/VIS baseline signal was acquired, and a sample measurement of $150 \times 1500 \ \mu s$ exposures initiated in order to cover the chromatogram. Between each exposure, a pause of $500 \ \mu s$ was automatically inserted in order to let the exposed material to flow out of the exposed volume to minimize radiation damage. The azimuthal averaging of the detector image and absolute calibration of each frame was performed by the FOXTROT software available at the beamline, which also allowed for background subtraction, calculation of $R_g$, and forward scattering. After the data treatment, the scattered intensity was given in absolute units as a function of the scattering vector from $q = 0.00626 \ \text{Å}^{-1}$ to $q = 0.591 \ \text{Å}^{-1}$. The final scattering curve was composed by averaging the measurements around the central peak of the chromatogram. The concentration was determined using the UV/VIS absorption in the same area of the chromatogram measured by the HPLC UV/VIS detector. The time delay and peak broadening between the UV detector was determined using a protein standard.

Structure factors

The higher concentration samples used to obtain the SAXS structure factors were measured on a pinhole camera (Ganesha 300 XL, SAXSLAB) covering a $q$-range from $0.003$ to $2.5 \ \text{Å}^{-1}$. In order to calculate the structure factors from data measured on two different SAXS instruments, the measured intensity data needed to be converted to the same scale and at the same scattering vectors. Common $q$ values were obtained by interpolating the measured intensities of the pinhole
camera at the $q$ values of the SOLEIL data. In order to bring the samples measured on the pinhole camera to absolute scale, low concentration samples were measured, and a scaling factor maximizing the overlap between the measurements from the pinhole camera and the SOLEIL data at $q > 0.04 \, \text{Å}^{-1}$ was found. Using the interpolated intensities and the scaling factor, the structure factors were readily obtained by dividing the concentration normalized intensities $I(q)/c$ of the dilute sample.

**Antibody model**

In order to study the antibody collective behavior, each antibody is modeled in a coarse-grained fashion using a colloid-inspired approach. In particular, it consists of 9 beads arranged in a Y-shaped symmetric colloidal molecule, where each sphere has a unit-length diameter $\sigma$. The three central beads are arranged in an equilateral triangle, and the three arms of the Y, each made of three spheres, form angles of $150^\circ$ and $60^\circ$ with each other. The geometric construction of the antibody implies that the circle tangent to the external sphere has a diameter $d_Y \approx 6.16 \sigma$. Each bead in the coarse-grained Y model is a hard sphere with infinite repulsive potential at contact and each antibody is treated as a rigid body. The specific choice of a 9-bead model is justified by matching its excluded volume interactions to that of the hard sphere model system on which the patchy hard sphere model introduced below is based on, i.e. by calculating its excluded volume for different densities and by comparing it to the theoretical Carnahan-Starling prediction for hard spheres. This aspect is discussed in-depth in the Supplementary Information file.

To account in a primitive fashion for the electrostatic-driven aggregation of the antibodies, the extremities of the three arms are decorated with patches of size $0.2633 \sigma$, one of type A on the tail and two of type B in the upper arms of the Y. This patch width allows to match in the simulations the bond probability $p$ determined from Wertheim theory, as described in more detail in a later section, although slightly exceeding the one-bond-per-patch condition that is assumed within the theory. However, we verified that the overall number of double bonds in simulations never exceeds a small percentage of the total for all considered mAb concentrations, thus allowing it to be safely ignored. Bonds are allowed to occur only between A and B type patches and are modeled with an attractive square well potential of depth $\epsilon_0$, which sets the energy scale. AA and BB interactions are not considered. The comparison between the radius of gyration of the experimental antibody
and the Y-shaped model allows us to convert simulation units into real ones: being the former $R_{g}^{m,Ab} = 4.7$ nm and the latter $R_{g}^{hY} = 1.7297\sigma$, we obtain the size of each bead in the model as $\sigma = 2.72$ nm.

Monte Carlo simulations

We run Monte Carlo (MC) simulations with $N = 1000$ Y-shaped antibodies. We start by preparing ten independent random configurations at each number density $\rho = N/V$, with $V$ the volume of the cubic simulation box. Then, we perform simulations at the desired temperature $T$ and average the results over the different configurations in order to improve statistics particularly at small scattering vectors. To perform simulations and analytical calculations at the same concentration as in experiments, we consider that the mass of a mAb molecule is 150 kDa. Therefore, at a weight concentration of 1 mg/ml, we have $4.098 \times 10^{15}$ particles/ml. With $\sigma^3 = 20.06$ nm$^3$, we obtain $1 \text{ ml} = 10^{21}$ nm$^3 = 4.098 \times 10^{19} \sigma^3$. In this way, a weight concentration of 1 mg/ml or a particle number density of $4.098 \times 10^{15}$ particles/ml can be rewritten as $8.229 \times 10^{-5}$ particles/$\sigma^3$.

Results and discussion

Apparent aggregation number and relative viscosity

Figure 1 summarizes the concentration dependence of the key structural and dynamic quantities, namely the apparent aggregation number $\langle N_{\text{app}} \rangle$ and the relative viscosity $\eta_r$. Here $\langle N_{\text{app}} \rangle = \langle M_w \rangle_{\text{app}}/M_1$, where $\langle M_w \rangle_{\text{app}}$ is the apparent weight-average molecular weight measured by SLS and $M_1$ the molecular weight of the individual mAb, and $\eta_r = \eta_0/\eta_s$, where $\eta_0$ is the zero shear viscosity of the mAb solution and $\eta_s$ is the solvent viscosity. The data shown in Fig. 1 is for two different ionic strengths of the solvent corresponding to 17 mM and 57 mM NaCl. Both $\langle N_{\text{app}} \rangle$ and $\eta_r$ exhibit a behavior frequently found for proteins undergoing the formation of equilibrium clusters with a cluster size that increases with increasing concentration $c$. For $\langle N_{\text{app}} \rangle$ this results in a non-monotonic concentration dependence with an initial weak increase due to the concentration-dependent cluster growth, followed by a strong
decrease at higher values of $c$ due to the contributions from excluded volume interactions between clusters that become dominant at high concentrations. In contrast, $\eta_r$ increases with increasing concentration and appears to diverge at a concentration of around 200-300 mg/ml, where the solution undergoes an arrest transition.

In general, increasing the ionic strength in mAb solutions results in an enhanced propensity for self-assembly and cluster formation since stabilising charges on mAbs are screened, resulting in a reduced electrostatic repulsion and thus colloidal stability. However, in our case we observe an opposite behavior, with the addition of salt actually reducing self-assembly, as evident from both experimental quantities. As discussed below in more detail, this counterintuitive behavior occurs in our system because of the electrostatic attraction at work, which is effectively screened by the addition of a large amount of salt.

A similar pattern can also be observed from the results of the small-angle X-ray scattering (SAXS) experiments summarised in Fig. 2 which shows the concentration-normalized scattering data $I(q)/c$ as a function of the magnitude of the scattering vector $q$ for different mAb concentrations $c$ at both ionic strengths. We see the same non-monotonic $c$-dependence

Figure 1: SLS and microrheology data. (a) Experimental $\langle N_{app} \rangle$ as a function of $c$ for 10 mM NaCl (blue) and 50 mM (red) NaCl, respectively. Also shown is a comparison with calculations based on a sticky hard sphere cluster model as the solid lines. (b) Experimental $\eta_r$ as a function of $c$ for 10 mM NaCl (blue) and 50 mM (red) NaCl, respectively. Also shown is a comparison with calculations based on a sticky hard sphere cluster model as the solid blue and red lines, and the theoretical predictions for monomers only where the relative viscosity is either given by the MCT prediction (power law, dotted black line), or by the Quemada relationship for hard spheres (dashed black line).
Figure 2: SAXS data for different concentrations and ionic strength. Experimental $I(q)$ as a function of $q$ for (a) 10 mM and (b) 50 mM NaCl. Insets show the corresponding structure factors $S(q)$ for both solvents.

of the forward scattering as already observed for the SLS data shown in Fig. 1(a), while the high-$q$ data appears completely independent of concentration, indicating that the solution structure of the individual mAbs does not change with increasing concentration. Moreover, the initial increase of the forward intensity appears more pronounced at low ionic strength, while the data at higher concentrations and high ionic strength indicate a much more repulsive behavior characterised by a significant decrease of the data at low $q$-values. This is further illustrated with plots of the effective static structure factors $S(q)$ for all data sets (insets of Fig. 2), which describe the influence of structural correlations only without the additional contributions from the monomer solution structure that is identical for all concentrations.

Coarse-grained colloid models

We have previously pointed out the importance of electrostatic interactions for the solution behavior of this mAb, and subsequently conducted a detailed study of the electrostatic isosurface of a single antibody molecule in the considered buffer solution. The resulting charge distribution is illustrated in Fig. 3(a), which clearly shows that the considered mAbs have an overall positively charged surface on the two arms (FAB domains) and a largely negative charge on the tail (FC domain). Assuming that the main driving mechanism for
mAbs aggregation has to be an electrostatically driven attractive arm-to-tail interaction, we were then able to construct a coarse-graining strategy based on a patchy colloid model that was capable to quantitatively reproduce the experimental findings for the lower ionic strength data set described in our earlier work.\cite{18} The approach is illustrated in Fig. 3(b-c), where we include the 9-bead model used for computer simulations (Fig. 3(b)) and the patchy hard sphere model required for the analytical/numerical analysis (Fig. 3(c)). Further modeling details are provided in Methods.

Here, we first focus on the analysis of the experimental SLS data using a combination of Wertheim theory (WT) for patchy particles and hyperbranched polymer theory (hpt) that allows us to calculate the concentration dependence of the cluster size distribution compatible with the SLS data, and investigate whether the ionic strength dependence observed is also compatible with this approach. In Wertheim theory,\cite{32,33} which is a thermodynamic perturbation theory, the YAB molecule is represented as an effective patchy sphere, illustrated in Fig. 3(c), with a hard sphere diameter $\sigma_{HS}$. The free energy $F$ of a system of $N$

Figure 3: Design of the patchy model of mAbs. (a) Isosurfaces of the -1 (red) and +1 $k_BT$ (blue) electrostatic potential at pH 6.5 with 10 mM NaCl, indicating an overall positive charge for the arms (FAB domains) and a largely negative charge for the tail (FC domain).\cite{18} (b) Simulation snapshot of the YAB model: 9 hard spheres each of diameter $\sigma$ are constrained to a rigid Y shape, constituting a single mAb molecule. Each molecule is decorated with one A patch on the tail (red) and two B (blue) patches, one on each arm, mimicking the negative and positive charges respectively. Only AB attractive interactions are considered mimicking the arm-to-tail electrostatic interactions. In transparency, we also report the atomistic representation of the antibody. (c) Representation of the YAB model as an effective patchy hard sphere of diameter $\sigma_{HS}$ as in the Wertheim theory.
patchy spheres in a volume $V$, with number density $\rho = N/V$, is calculated as the sum of the free energies of a hard sphere (HS) reference term $F_{HS}$ plus a bonding term $F_b$. For the reference term $F_{HS}$ we use the Carnahan-Starling (CS) free energy of an equivalent HS system, that has to be determined according to the nature of the molecule. For non-spherical molecules, the HS reference system effective diameter $\sigma_{HS}$ is not known and needs to take into account correctly the excluded volume of the particles, which is established from the direct comparison to the experimental SLS data. The bonding free energy $F_b$ per particle of our 3-patch YAB model is then calculated as a function of the strength of the attraction $\epsilon_0$ and the bond probability $p_B$ as described in detail in Refs.\textsuperscript{18,35}

We can now perform a direct comparison between the analytical results for the YAB patchy model from WT and the experimental results for the apparent aggregation number $\langle N_{app} \rangle$ shown in Fig. 1(a). To this aim, we calculate the isothermal compressibility $\kappa_T = -\frac{1}{V} \left( \frac{\partial V}{\partial P} \right)_T = \frac{1}{V} \left( \frac{\partial^2 V}{\partial^2 P} \right)_T$ for our YAB model as a function of concentration, where $P$ is the pressure and $V$ is the volume, by simply differentiating twice the analytic free energy $F$ with respect to volume.\textsuperscript{36} Since $\kappa_T$ is related to $\langle N_{app} \rangle$ through

$$ N_{app} = S(0) = \rho k_B T \kappa_T, \quad (2) $$

where $\rho$ is the number density of particles, Eq. \textsuperscript{S2} provides the link for the comparison between WT and the experimental results from SLS. By appropriately converting analytical and experimental results as described in Methods, for the samples with 10 mM NaCl added we obtain good agreement between the experimentally measured $S(0)$ and the Wertheim calculation for $\sigma_{HS} = 2.95\sigma$ and a temperature $T = 0.11$, which corresponds to a strength of the attraction between AB patches of $\epsilon_0 = 9.09 \ k_B T$. For the data at higher ionic strength, the attraction between the oppositely charged ends is slightly reduced due to the stronger screening, and we obtain best agreement for a temperature $T = 0.114$, which corresponds to a strength of the attraction between AB patches of $\epsilon_0 = 8.77 \ k_B T$.

While Wertheim theory provides us with a method capable of calculating the osmotic
compressibility or apparent aggregation number that can be compared with the SLS data, it does not allow us to calculate other experimental quantities such as those obtained by microrheology or SAXS. To this aim, we need the distribution $n(s)$ of clusters of size $s$ as a function of concentration $c$. We therefore use the fact that our YAB model belongs to a class of so-called hyperbranched polymers\textsuperscript{37} which allows us to calculate the full cluster size distribution at each concentration and solvent condition using hyperbranched polymer theory (hpt) as was previously described in Ref.\textsuperscript{18} In hpt terminology, a YAB molecule corresponds to a functionality type $AB_{f-1}$ with functionality $f = 3$, for which the bond probability $p$ of Wertheim theory is the fraction of bonded $B$ groups and $(f-1)p$ the fraction of bonded $A$ groups. For hyperbranched polymers there is one non-bonded $A$ group for each cluster, therefore the average number of monomers per cluster is the reciprocal of the fraction of unreacted $A$ groups. Hence, the only input needed to evaluate $n(s)$ is the bond probability $p$, which we directly get from Wertheim theory. In the YAB model, calling $p$ $(2p)$ the fraction of $B$ ($A$) patchy sites, the cluster size distribution in the framework of hyperbranched polymer theory is finally given as

$$n(s) = \frac{(2s)!}{s!(s+1)!} p^{s-1} (1 - p)^{s+1}. \tag{3}$$

Therefore, $n(s)$ is the probability of finding clusters of size $s$ for a system with bond probability $p$. The corresponding cluster size distributions obtained with the parameters from the Wertheim analysis are shown in Fig. 4(a) for four different concentrations and both ionic strengths. We see that the resulting cluster size distributions are very broad, resulting in significantly different values for different weighted averages as pointed out already earlier.\textsuperscript{18} This is important when considering results from different methods such as SLS, DLS or rheology. The corresponding results for the weight-average aggregation number $\langle s \rangle_w = \sum n(s)s^2/\sum n(s)s$ as a function of concentration are also shown in Fig. 4(b).
Figure 4: (a) Cluster size distribution \( n(s) \) as a function of the cluster size \( s \) for different mAb concentrations based on the parameters from a Wertheim analysis of the SLS data for 10 mM (solid lines) and 50 mM (dashed lines) NaCl. (b) Weight average aggregation number \( \langle s \rangle_w \) as a function of concentration for the parameters from the Wertheim analysis for 10 mM (blue line) and 50 mM (red line) NaCl.

Predicting SAXS data for self-assembling mAbs

Having theoretical descriptions for the concentration dependence of both \( n(s) \) and \( \langle s \rangle_w \), we now make an attempt to reproduce the experimental data. The task is to develop analytical models that allow us to calculate scattering intensities and structure factors of mAb solutions that undergo self-association into concentration-dependent transient clusters. The scattering intensity measured in a SAXS experiment can be described by:

\[
\frac{1}{cK} \frac{d\sigma}{d\Omega}(q) = sP_c(q)S^{\text{eff}}(q),
\]

where \( c \) is the weight concentration, \( K \) is a contrast term that primarily reflects the excess electron density between mAb and solvent, \( M_1 \) is the molar mass of an individual mAb (i.e., a monomer), \( d\sigma(q)/d\Omega \) is the normalized \( q \)-dependent scattering intensity, \( s = \langle s \rangle_w \) is the weight-average aggregation number of the self-assembled clusters, \( P_c(q) \) is the intensity-weighted average form factor of the clusters and \( S^{\text{eff}}(q) \) is the effective structure factor of the cluster fluid.

In order to reproduce the measured scattering intensity for the mAb solutions, there are thus two tasks, namely (i) to calculate the cluster form factor \( P_c(q) \) as a function of the
aggregation number $s$, and (ii) to find an appropriate model and expression for the effective structure factor $S^{eff}(q)$ for the cluster fluid at the different concentrations.

Here we follow two approaches, both based on a coarse grained view of the mAb clusters as schematically drawn in Figure 5. Clusters are either modeled as assembled from patchy hard Ys formed by 9 spheres (see Fig. 3 and Methods) or then, after a further coarse-graining step, as made up of spheres with radius $R_1$. The form factor of a cluster formed by $s$ monomers can then be directly calculated using

$$P_c(q) = \frac{1}{s^2} P_1(q) \sum_i \sum_j \langle \frac{\sin(qr_{ij})}{qr_{ij}} \rangle,$$

where $P_1(q)$ is the form factor of the monomer ($s = 1$), and $r_{ij}$ is the center-center distance between monomer $i$ and $j$, and the brackets $\langle \cdots \rangle$ denote a conformational average of a distance pair $ij$. For the calculation of $P_c(q)$ we then either use the actually measured form factor of the mAb, or strictly adhere to the cluster of Ys or spheres model and use the form factor of a Y or a sphere with radius $R_1$. The term $\frac{1}{s^2} \sum_i \sum_j \langle \frac{\sin(qr_{ij})}{qr_{ij}} \rangle$ in Eq. 5 then

![Figure 5](image)

Figure 5: Schematic view of the coarse grained cluster model used to calculate the cluster form factor $P_c(q)$. Shown are examples of mAb clusters with $s = 12, 7$ and 4, from simulations of rigid Ys, where each mAb monomer is modelled as a rigid Y consisting of 9 spheres (see Figure 3(b)), and the further coarse grained cluster where each mAb monomer is modelled as a sphere.
corresponds to $\frac{1}{s} S_c(q)$, where $S_c(q)$ is the structure factor of the cluster, with a normalization that yields $S_c(0) = s$ and $S_c(\infty) = 1$. Using this notation, the normalized scattering intensity $I_c(q)$ from a single cluster can be written as $I_c(q) = M_1 P_1(q) S_c(q) = M_1 s P_c(q)$.

In the next step, we thus develop a model that provides us with an explicit description of $S_c(q)$. This can either come from simulations, or from an analytical model that yields $S_c(q)$ as a function of $s$.

**Using computer simulations of a colloid-inspired antibody model**

In order to obtain a microscopic description of the antibody assembly and thus the structure factor for single clusters, we run Monte Carlo simulations of an ensemble of antibodies,
treated via the 9-bead model depicted in Fig. 3(b). To allow the antibodies to self-assemble, the temperature is fixed to $T = 0.11$, as determined from Wertheim theory. More details on such simulations and on the conversion between real and simulation units are provided in Methods.

We first study the cluster size distribution $n(s)$ as a function of the cluster size $s$, which is reported in Fig. 6(a, b) for two concentrations $c = 61.7$ and 147 mg/ml, respectively. Together, we also plot the corresponding theoretical predictions from hpt. As expected, we find that the clusters are rather polydisperse in size, reaching $s > 20$ at the highest concentration. Representative simulation snapshots are shown in Fig. 5 for $s = 12, 7$ and 4. We note that larger clusters are also found, but they are beyond our numerical accuracy, since their number is $< 0.1\%$ of the total. The agreement between numerical data and theoretical predictions is overall good for both concentrations, thus confirming that the employed model does follow hyperbranched polymer theory. Small deviations at large $s$ are due to the minimal presence of multiple bonds for the same patch, which are not taken into account in the theoretical treatment. However, their contribution is negligible for both concentrations and implying that we can use the theoretical prediction to evaluate $n(s)$ at all concentrations.

From simulations, we can then assess the shape of single clusters. To this aim, we calculate the average radius of gyration $R_g$ for clusters of the same size $s$, that is reported in Fig. 6(c) as a function of size for the same concentrations as in panels a and b. From this plot, we can extract information on the clusters fractal dimension $d_F$, since $R_g \sim s^{1/d_F}$. We identify two different regimes, for clusters smaller and bigger than $s \approx 10$: in the former range, we find $d_F \approx 1.5$, while for larger clusters a fractal dimension $d_F \approx 2.5$ is compatible with the data. For each cluster size, we also calculate the corresponding cluster structure factors $S_c^s(q)$ as,

$$S_c^s(q) = \frac{1}{N_c^s} \left\langle \sum_{n=1}^{N_c^s} \sum_{i_b,j_b=1}^{9} e^{-iq(r_{i_b,n}-r_{j_b,n})} \right\rangle,$$

where $N_c^s$ is the number of clusters of size $s$, $r_{i_b,n}$ and $r_{j_b,n}$ are the coordinates of each bead.
of the \( n \)-th cluster and the average is taken over all clusters of size \( s \) in the whole simulation trajectory. The structure factors for clusters of different size are reported in Fig. 7 for \( c = 61.7 \) mg/ml. With increasing \( s \), the first peak of \( S_s^c(q) \) becomes more pronounced, accompanied by an increase of its signal at low wavenumbers. In addition, simulations allow us also to calculate the total structure factor of the system, defined as,

\[
S_{\text{tot}}(q) = \frac{1}{P(q)} \left\langle \sum_{s=1}^{S} \sum_{n=1}^{N_s} e^{-i\mathbf{q} \cdot (\mathbf{r}_{i_b,Y} - \mathbf{r}_{j_b,Y})} \right\rangle
\]

\[
= \frac{1}{P(q)} \frac{1}{S} \sum_{s=1}^{S} \frac{1}{N_c} \left\langle \sum_{n=1}^{N_s} \sum_{i_b,j_b=1}^{9} e^{-i\mathbf{q} \cdot (\mathbf{r}_{i_b,n} - \mathbf{r}_{j_b,n})} \right\rangle
\]

\[
= \frac{1}{P(q)} \sum_{s=1}^{S} S_s^c(q)
\]

where \( N \) is the number of bead-based antibodies in the system, \( \mathbf{r}_{i_b,Y} \) and \( \mathbf{r}_{j_b,Y} \) are the coordinates of each bead in each antibody and \( P(q) \) is the form factor of the 9-bead model. The total structure factor calculated in this way is also shown in Fig. 7 and, strikingly, it shows very little oscillations and almost no peaks, except for a slight increase at small \( q \). These calculations will be compared in later sections with analytical calculations and

Figure 7: Cluster structure factors \( S_s^c(q) \) for \( s = 2, 4, 6, 8, 10, 12 \) and total structure factor \( S_{\text{tot}}(q) \) obtained from computer simulations for \( c = 61.7 \) mg/ml. Data are shown in simulation units (where \( \sigma \) is the bead size).
experimental results to provide a comprehensive description of the solution structure.

**Using polymer theory to calculate the cluster form factor**

To develop an analytical model for $S_c(q)$, given the relatively open structure of the clusters found in simulations, we first use a simple polymer model, where we assume that the conformational average of the internal distances $r_{ij}$ is given by a freely jointed chain (fjc) model.\cite{37,39,40} In this model for the conformation of a polymer chain of size $s$, we assume that the chain consists of $s$ monomers linked by $s - 1$ bonds of length $b$ that are able to point in any direction independently of each other, i.e. with no correlation between the direction of different bonds. The average radius of gyration of such a chain is thus given by a scaling law of the form $\langle R_g \rangle \sim s^{1/2}$.\cite{37,39,40} The conformations described by the fjc model would thus be compatible with a fractal cluster structure with $d_F = 2.0$, that is intermediate between the fractal dimensions found in our simulations for small and large cluster sizes. This implies that in Eq. \ref{eq:5}

\[
\langle \sin(qr_{ij})/qr_{ij} \rangle = \left( \frac{\sin(qb)}{qb} \right)^{|j-1|}
\]

where $b$ is the distance between two spheres, i.e. the sphere diameter $2R_1$ in our model. Evaluating the double sum finally results in the following expression for the cluster form factor:

\[
P_c(q) = \frac{2P_1(q)}{s^2} \left[ \frac{s}{1 - \sin(qb)/qb} - \frac{s}{2} \frac{1 - (\sin(qb)/qb)^s \sin(qb)}{(1 - \sin(qb)/qb)^2 qb} \right]
\]

An example of the resulting cluster intensity $sP_c(q)$ using Eq. \ref{eq:9} is shown in Fig. \ref{fig:8}(a) for a mAb cluster with $s = 10$ and $b = 12$ nm. This value of $b$ was chosen based on the actual geometrical dimensions of the mAb and corresponds approximately to the diagonal distance between the positively and negatively charged ends, i.e. to the expected bond length in our model. The chosen normalization allows us to directly compare the cluster form factor $sP_c(q)$ with the measured monomer form factor $P_1(q)$. We see that at low $q$-values
the overall scattering pattern is dominated by the overall cluster size. At higher $q$-values, $P_c(q)$ approaches the monomer form factor, modulated however with the local correlations between individual monomer beads in the cluster expressed by the cluster structure factor $S_c(q)$, shown in Fig. 8(b).

**Using colloid cluster theory to calculate the cluster form factor**

We also develop a second coarse-grained model that is instead based on colloid theory. Here we start from the cluster structure factor $S_c(q)$ of a fractal colloid cluster (fc) given by the double sum in Eq. 5, which we rewrite according to

$$S_c(q) = \frac{1}{s} \sum_i \sum_j \left\langle \frac{\sin(qr_{ij})}{qr_{ij}} \right\rangle = 1 + \frac{1}{s} \sum_i \sum_{j \neq i} \left\langle \frac{\sin(qr_{ij})}{qr_{ij}} \right\rangle$$

(10)

where the last term in Eq. 10 is the cross term between the individual monomers in the cluster and the large embedding sphere with radius $R_c$. This term can be rewritten as

$$S_c(q) = 1 + (s - 1)P_L(q)$$

(11)

**Figure 8:** (a) Normalized intensity $sP_c(q)$ for a mAb cluster with aggregation number $s = 10$ using the freely jointed chain model (Eq. 9 and $b = 12$ nm, black dashed line), and a fractal cluster model (Eqs. 12 and 13 and a hard sphere structure factor with $R_1 = 6$ nm) for different values of the internal volume fraction ($A_\phi = 1.0, 0.9, 0.8, 0.7$ and $0.6$ (blue lines)), and the experimentally measured mAb form factor $P_1(q)$ obtained at a concentration of 4.9 mg/ml (circles). (b) The corresponding cluster structure factors $S_c(q)$ for the same models.
where $P_L(q)$ is the form factor of the embedding sphere (or cluster). Eq. 11 does not take into account correlations between monomers within the cluster, which can for example be considered by introducing a hard sphere structure factor $S_{HS}(q, \phi_{int})$, where the internal volume fraction of a cluster of radius $R_c$ is given by $\phi_{int} = s(R_1/R_c)^3$

\[
S_c(q) = S_{HS}(q, \phi_{int}) + (s - S_{HS}(q, \phi_{int}))P_L(q)
\]  

(12)

In a final step, we need to select appropriate models for $S_{HS}(q, \phi_{int})$ and $P_L(q)$. For $S_{HS}(q, \phi_{int})$ we can for example use liquid state theory and the corresponding structure factor for hard spheres given by the Percus-Yevick (PY) expression.\footnote{34} For $P_L(q)$ we choose the Fisher-Burford expression that has been used to describe the scattering intensity of fractal clusters with fractal dimension $d_F$;\footnote{42,43}

\[
P_L(q) = \left(1 + \frac{2R_g^2q^2}{3d_F}\right)^{-d_F/2}.
\]  

(13)

In order to calculate the cluster structure and form factors for this model, we also need to determine the radius of gyration $R_g$ and the internal volume fraction $\phi_{int}$. To be internally consistent, we have used the common relationship for the radius of gyration of a fractal cluster $R_g^c$ given by

\[
R_g^c = R_1 \left(\frac{s}{k}\right)^{1/d_F}
\]  

(14)

where $R_1$ is the monomer size and $k$ a constant that depends on the fractal dimension $d_F$.

The internal volume fraction $\phi_{int}$ is thus given by

\[
\phi_{int} = A_\phi s \left(\frac{R_1}{R_g^c}\right)^3
\]  

(15)

where the parameter $A_\phi$ corrects for the fact that the monomers in the fractal cluster are treated as spheres with size $R_1$, whereas their effective hard sphere radius and thus their
excluded volume is smaller than $R_1$ due to the Y-shape of the mAb.

We again use $s = 10$, and choose $d_F = 2.5$ in agreement with the computer simulation results for larger clusters, which in turn yields $k = 0.71$. The resulting cluster structure factor does depend on the value of $R_1$, as this determines both the low-$q$ behavior through the overall cluster size $R_y^c$ as well as the position of the nearest neighbor correlation peak roughly given by $q^* \approx 2\pi/2R_1$ (note that the internal volume fraction is independent of the choice of $R_1$, since it only depends on the ratio $R_1/R_c$). Moreover, the internal correlation peak will also depend on the internal volume fraction due to the concentration dependence of the structure factor of hard spheres calculated for example via the Percus-Yevick (PY) expression.\(^{34}\)

The results for both $sP_{c,fc}(q)$ and $S_{c,fc}(q)$ are also shown in Fig. 8 for different values of $A_\phi$, together with the results of the fjc model. Due to the similar $R_y^c$, the low-$q$ part almost overlap for both models. In fact, $R_y^c = 2R_1(s/6)^{1/2} = 15.5$ nm for the fjc model\(^{27}\) and $R_y^c = 17.4$ nm for the fc model. Since both models have a slightly different asymptotic slope given by $1/d_F$, with $d_F = 2$ for the fjc and $d_F = 2.5$ for the fc model, this then results in a very similar initial $q$-dependence that would be difficult to distinguish in real experimental data. However, at higher $q$-values, differences become much larger. Nearest neighbor correlations for the fc model are strongly dependent on the internal volume fraction, which becomes highlighted when looking at $S_{c,fc}(q)$ for different values of $A_\phi$. For the fjc model, longer range correlations persist due to the underlying linear chain structure with a well defined bond length, while these decay more quickly for the fc model.

**Comparison between fjc and fc models, and computer simulations**

We can now compare the cluster structure factors $S_c(q)$ obtained by the fjc model with those calculated from the computer simulations using Eq. 6. The individual $S_c(q)$’s are plotted as $S_c(qd)$ as a function of $qd$, i.e. normalized by the effective distance $d$ between different mAbs in the clusters given either by the bead size $d = b = 12nm$ or the diagonal distance between
the oppositely charged patches given by \( d = 5.8\sigma \), respectively. In order to test the absence of concentration effects on \( S_c(q) \), we compare the data for different concentrations. This is illustrated with the results from simulations at three different concentrations corresponding to \( c = 61.7\) mg/ml, 102.2 mg/ml and 147.3 mg/ml, respectively. As shown in Fig. 9(a), the three different \( S_c(q) \)'s obtained for \( s = 10 \) overlap within the statistical errors, indicating that the average structure of the clusters formed are independent of concentration for a given value of the cluster size \( s \). The agreement between the simulation results and those obtained from the fjc model are also very good. Fig. 9(a) furthermore illustrates that for sufficiently large cluster sizes the internal structure described by \( S_c(qd) \) becomes independent on \( s \) except for low \( qd \)-values, where \( S_c(qd) \) approaches \( s \).

For small cluster sizes the internal structure however starts to strongly depend on \( s \) as illustrated in Fig. 9(b) for \( s = 2, 3 \) and 4, respectively. Here we also see that for these small cluster sizes the results from simulations and the fjc model completely overlap, the two approaches are now identical within error bars.

While the model based on fractal colloidal clusters is obviously not suitable for small cluster sizes where a fractal description is not adequate, it does however agree quite well with the computer simulations and the fjc model at sufficiently large values of \( s \), as shown in Fig. 9(c) for \( s = 15 \).

**Solution structure factor**

In order to calculate the total normalized scattering intensity for a cluster fluid as described by Eq. 4, we finally also need a model for the effective structure factor \( S(q)^{eff} \) of the cluster fluid. Given the very broad size distribution of the self-assembled antibody clusters at higher concentrations as predicted by either hyperbranched polymer theory (hpt) or our coarse grained simulations, we do expect very weak structural correlations even at the nearest neighbor distance, similar to what one finds for example for polymer solutions. We therefore use a so-called random phase approximation (RPA), where the structure factor is given by
Figure 9: (a) Comparison of the normalized cluster structure factors \( S_c(qd) \) for \( s = 10 \) obtained from computer simulations for a hard 9 bead Y model at three different concentrations corresponding to \( c = 61.7 \), 102.2 and 147.3 mg/ml (blue symbols), respectively. Also shown are the results for \( s = 15 \) from simulation at \( c = 147.3 \) mg/ml (red triangles), and from the fjc model for \( s = 10 \) (blue line) and \( s = 15 \) (red line). (b) Comparison of the cluster structure factors \( S_c(qd) \) obtained from computer simulations for a hard 9 bead Y model at \( c = 61.7 \) mg/ml and \( s = 2 \) (red circles), \( s = 3 \) (blue squares) and \( s = 4 \) (green triangles), together with those calculated with the fjc model for the same cluster sizes (\( s = 2 \) (red line), \( s = 3 \) (blue line), \( s = 4 \) (green line)), respectively. (c) Comparison of the cluster structure factors \( S_c(qd) \) for \( s = 15 \) obtained from computer simulations for a hard 9 bead Y model at \( c = 147.3 \) mg/ml (red circles), the fjc (red solid line) and the fc models (Eqs. 12 and 13 and a hard sphere structure factor with \( R_1 = 6 \) nm for \( A_\phi = 1.0 \)) (red dashed line), respectively.

\[
S(q)_{\text{eff}} = \frac{1}{1 + \frac{1-S(0)}{S(0)} P_c(q)}
\]  

(16)

In this way, the total normalized scattering intensity given by Eq. 4 can then be rewritten as

\[
\frac{1}{cK M_1 d\Omega} \frac{d\sigma}{d\Omega}(q) = \frac{sP_c(q)}{1 + \frac{1-S(0)}{S(0)} P_c(q)}
\]  

(17)

In a next step, we need to calculate \( s \) and \( S(0) \) as a function of concentration based on our previously established approach using a combination of Wertheim theory and hpt. Here, we use the obtained bond probability versus concentration relationship and perform a next coarse graining procedure where we treat the clusters as the new hard or sticky colloids. We thus first make use of the cluster size distributions and the weight average aggregation number \( \langle s \rangle_w \) previously calculated at all concentrations with hpt (see Fig. 4). Assuming hard or sticky hard sphere-like interactions between the different clusters, we can then calculate the concentration dependence of the apparent weight average aggregation number \( N_{\text{app}} \), given by
\[ \langle N_{\text{app}} \rangle_w = \langle s \rangle_w S^{\text{eff}}(0). \] (18)

The static structure factor \( S^{\text{eff}} \) introduced here has a different definition than \( S(0) \) used previously and also shown in Fig. 1, and \( S^{\text{eff}} = S(0)/\langle s \rangle_w \) now corresponds to the effective structure factor of a solution of polydisperse spheres, reflecting the fact that the mAb clusters (and not the individual antibodies) are the new interacting objects.

We use the same conversion of the weight concentration into number densities of mAbs in units \( \sigma^{-3} \) based on \( \sigma = 2.72 \) nm, and then calculate the number densities of clusters using \( \rho_{\text{cluster}} = \rho/\langle s \rangle_n \), where \( \langle s \rangle_n = \sum n(s)s/\sum n(s) \) is the number-average aggregation number. In doing these calculations we also have to reconsider the effective hard sphere cluster volume fraction \( \phi_{HS} \). Starting point for calculating \( \phi_{HS} \) is the hard sphere volume fraction used in the Wertheim analysis \( \phi = \rho V_{hs} \). We then allow for an additional scaling parameter \( A \) and also take into account that clusters are fractal, giving\[13\]

\[ \phi_{HS} = A\phi \langle s \rangle_n^{(3-d_F)/d_F}, \] (19)

where \( d_F \) is the fractal dimension of the clusters and \( \phi \) is the nominal antibody volume fraction used in the Wertheim analysis. Given the small cluster sizes with \( \langle s \rangle_n < \langle s \rangle_w < 10 \) for all concentrations investigated (see Fig. 4(b)), we use \( d_F = 2.0 \).

In our earlier investigations at low ionic strength, we found best agreement for a model of clusters that interact as sticky hard spheres, for which the low scattering vector limit of the effective static structure factor becomes\[13\]

\[ S_{SHS}(0) = \frac{(1 - \phi_{HS})^4}{(1 + 2\phi_{HS} - \lambda\phi_{HS})^2}, \] (20)

with

\[ \lambda = 6(1 - \tau + \tau/\phi) \left( 1 - \sqrt{1 - \frac{1 + 2/\phi}{6(1 - \tau + \tau/\phi)^2}} \right). \] (21)
where $\tau$ is the stickiness parameter that is inversely proportional to the strength of the attractive interaction.\textsuperscript{14,16} Together with the concentration dependence of $s$, obtained with Wertheim theory and hpt, we can then calculate $N_{app}$ using Eq. 18 for both ionic strengths values investigated.

The corresponding best fit results using $A = 1.4$ are shown in Figure 1. The agreement with experimental data is indeed excellent, assuming that in the coarse grained model we have an effective hard sphere volume fraction that is $\approx 40\%$ higher than for the individual mAbs in the Wertheim analysis. Given that clusters cannot overlap as much as individual antibodies do, this estimate does appear to be realistic.

A further consistency check of this additional coarse graining step can also be obtained from the microrheology data. Here we calculate the concentration dependence of the relative viscosity $\eta_r$. Theoretical work on hard sphere and attractive systems using mode coupling theory (MCT) and computer simulations predicts a power-law dependency of the reduced viscosity

$$
\eta_r = \left( \frac{\phi_g - \phi_{HS}}{\phi_g} \right)^{-\gamma}
$$

in the vicinity of the arrest transition, where $\phi_g$ is the maximum packing fraction, which depends on the polydispersity of the system and the strength of the attraction.\textsuperscript{17} The value of $\gamma$ depends on the interaction between particles, with $\gamma = 2.8$ for hard spheres, and $\gamma \geq 3$ for attractive particles.\textsuperscript{17,18} The viscosity data obtained for the mAb solutions at both ionic strengths are indeed well reproduced with a power law exponent $\gamma = 3.0$ when using $\phi_g = 0.63$ (see Fig. 1(b)), providing further support for the calculated dependence of the clusters size on concentration. This clearly indicates that it is the excluded volume interactions between the self-associating clusters that is at the origin of the strong increase of the zero shear viscosity with increasing concentration. Our simple model is capable of predicting the measured $c$ and ionic strength dependence based on SLS experiments quantitatively. In this context, it is also interesting to compare the calculations for the case of self-assembling.
antibodies with an estimate of $\eta_r$ for a hypothetical case where the mAbs do not assemble into clusters, and where $\eta_r$ is given by Eq. \ref{eq:22} but using the hard sphere volume fraction from the Wertheim analysis instead. The resulting values are also shown in Fig. \ref{fig:1}(b) for two values of the exponent $\gamma$ (3.0 and 2.0, which corresponds to the often used Quemada relation for hard spheres\cite{49}) and demonstrate the dramatic effect of cluster formation at higher mAb concentrations.

We then use the results from this analysis to calculate the full $q$-dependence of the total normalized intensity of the cluster fluid using Eq. \ref{eq:17}. However, instead of plotting the intensity, we calculate an effective measured solution structure factor $S_{\text{meas}}(q)$ where we divide the total normalized intensity with the monomer form factor, i.e.

$$S_{\text{meas}}(q) = \frac{1}{cK_M} \frac{d\sigma(q)}{d\Omega(q)} \frac{1}{P_1(q)} = \frac{S_c(q)}{1 + \frac{1-S_0(q)}{S_0(q)}} = S_c(q)S(q)^{\text{eff}}$$ \hspace{1cm} (23)

We thus compare the measured $S_{\text{meas}}(q)$ with the calculated quantity $S_c(q)S(q)^{\text{eff}}$, where $S_c(q)$ is calculated either with the fjc model (Eq. \ref{eq:9}) or the fc model (Eq. \ref{eq:12} and \ref{eq:13}), and $S(q)^{\text{eff}}$ is obtained using the RPA model (Eq. \ref{eq:16}).

A first attempt is shown in Fig. \ref{fig:10}(a) for two concentrations of 26 mg/ml and 147 mg/ml, respectively, at the lower ionic strength. For these samples, the combination of Wertheim theory and hpt predicts weight average aggregation numbers of $\langle s \rangle_w = 1.62$ for 26 mg/ml and $\langle s \rangle_w = 4.5$ for 147 mg/ml, respectively. Using the sticky hard sphere cluster model, this then results in values of $S_{\text{SHS}}(0) = 0.72$ for 26 mg/ml and $S_{\text{SHS}}(0) = 0.08$ for 147 mg/ml, respectively. For an aggregation number of 2 the fc model is obviously meaningless, and therefore we have only used the fjc model for the lowest concentration. While overall the initial low-$q$ part is reasonably well reproduced, the chosen models clearly overestimate the nearest neighbor correlations at higher $q$.

Part of the large discrepancies between measured and calculated structure factors are likely due to the fact that up to this point we have neglected polydispersity. In fact, we calculated $S_{\text{meas}}(q)$ using a monodisperse fjc or fc model, where the cluster size corresponds
Figure 10: (a) Measured effective structure factor $S_{\text{meas}}(q)$ compared with the calculated $S(q)^{\text{eff}}$ using either the fjc (solid lines) or fc (dashed line) models for the lowest and highest concentrations measured (red: 26 mg/ml, blue: 147 mg/ml). (b) Measured effective structure factor $S_{\text{meas}}(q)$ compared with the calculated $S(q)^{\text{eff}}$ for polydisperse systems based on Eq. 24 using either the fjc (solid lines) or fc (dashed line) models for the lowest and highest concentrations measured (red: 26 mg/ml, blue: 147 mg/ml). Data are for 10 mM NaCl.

to the nearest discrete value of the theoretical weight average aggregation number $s$. Since for smaller cluster sizes the internal structure depends on the aggregation number (see Fig. 9), we thus perform new calculations based on the full cluster size distributions from hpt, starting from the theoretical normalized scattered intensity of a polydisperse cluster fluid in the absence of interactions given by

$$\frac{1}{cK M_i} \frac{d\sigma}{d\Omega}(q) = \sum_s n(s) s^2 P_c(s,q) \frac{\sum_s n(s)s}{\sum_s n(s)s}$$ \hspace{1cm} (24)

where $P_c(s,q)$ is the cluster form factor of a cluster with aggregation number $s$, and $n(s)$ is the normalized cluster size distribution. In a next step we then again calculate $S_{\text{meas}}(q)$ using Eq. 23 for both models. The corresponding results are also shown in Fig. 10(b).

The agreement between the experimental data and the calculations for the fjc model is now improved, although the internal structural correlations are still overestimated, presumably due to the fact that we have completely neglected the flexibility of the individual monomers that allow for a larger variation of internal distances than assumed in the fjc model. On the other hand, the local correlation effects are even more pronounced for the
polydisperse fc model. This results from the fact that we have to use the model also for the significant amount of small clusters, where the model is not applicable and local structural correlations are thus strongly overestimated. Therefore, we drop this model in the remainder of the manuscript. Nevertheless, we would like to point out that this deficiency could easily be overcome by using a base set of internal cluster structure factors derived from computer simulations of colloidal hard sphere clusters. We also see from Fig. 10 that our model systematically slightly underestimates the low-\(q\) part of the structure factor. This is also due to the fact that we use an expression for monodisperse sticky hard spheres to calculate \(S_{SHS}(0)\) and \(S(q)^{eff}\). It is known that polydispersity not only decreases the amplitude of the nearest neighbor correlation peak but, for strongly correlated particles, it also results in an additional 'incoherent' contribution to the intensity and thus increases \(S(0)^{eff}\). Unfortunately, we have no simple analytical expression that would allow us to calculate the measured structure factor for our models in this case. However, given the many approximations made and the simple coarse-grained models used, we believe that the agreement between the experimental SAXS data and the calculated structure factors shown in Fig. 10 is quite remarkable, especially given that once we have fixed the parameters from our analysis of the SLS data there remain

![Image](image.png)

**Figure 11:** Measured effective structure factor \(S^{meas}(q)\) (symbols) compared with the calculated structure factor based on Eq. [24] using the fjc model (solid lines) for different concentrations measured. (a) 10 mM NaCl (blue lines: 26, 61.7, 102.2, 147.3 mg/ml); (b) 50 mM NaCl (red lines: 19.6, 24.3, 98.3, 149.6, 208.4 mg/ml).
no additional free parameters to be adjusted. This is further illustrated in Fig. 11, where we summarise the comparison between experimental data and calculated structure factors based on the polydisperse fjc model for both ionic strengths and all concentrations investigated. The agreement is indeed quite remarkable, and indicates that our model well captures both the concentration- and ionic strength-dependent self-assembly into small clusters as well as the structural signature of these clusters in SAXS experiments.

The full effective structure factor $S(q)^{eff}$ can also be obtained from computer simulations for the 9-bead model. The results are shown in Fig. 12. For the highest concentration the agreement between simulations and experiments is fairly good, and both the low-$q$ values as well as the full $q$-dependence of the simulated structure factor match the experimental $S(0)$ as well as the measured $S^{meas}(q)$ almost quantitatively, indicating that for the chosen parameters our simple patchy 9-bead model reproduces the cluster size distribution and the structural correlations well.

At the lower concentration of $c = 61.7$ mg/ml, however, we do observe systematic deviations. While the low-$q$ values seem to reach the correct asymptotic $S(0)$ value for this sample provided that we could extend the current $q$-range by going to a larger cell with more particles, there appears to be a systematic shift in the $q$-dependence between measured

![Figure 12: Measured effective structure factor $S^{meas}(q)$ compared with the calculated structure factor obtained from computer simulations (Eq. 7) at two concentrations at 10 mM NaCl (red: 61.7 mg/ml; blue: 147.3 mg/ml). Symbols are measured experimental values, solid lines are data from MC simulations.](image-url)
and simulated $S^{\text{meas}}(q)$. We believe that this is primarily caused by small differences in the geometrical dimensions of the real mAb and the 9-bead model already visible in Fig. 3(b). Since we use the measurable quantity $R_g$ to convert simulation units to actual dimensions in nm, this results in slight differences between the effective bond lengths in the clusters, which are given by the diagonal distance between oppositely charged groups or patches for the real bead and the 9-bead model. In turn, this likely leads to a mismatch for the $q$-dependence of the cluster structure factor $S_c(q)$, and thus for the total $S^{\text{meas}}(q)$ when plotting the results in real units of $q$ and not in dimensionless normalized units $qd$. While at lower concentrations the cluster form or structure factor dominates the total scattering intensity, and thus small systematic deviations become easily visible, at the highest concentration the total intensity and thus $S^{\text{meas}}(q)$ is dominated by cluster-cluster interactions, and these small shifts in $S_c(q)$ become less visible.

**Conclusions**

In this work, we provided a microscopic viewpoint on solutions of self-associating antibodies. By complementing multi-technique experiments with numerical simulations and analytical derivations, we thoroughly characterized the formation of clusters, which are recognized as being responsible for the increase in viscosity at high concentrations for this system. In particular, exploitation of polymer and colloid theories proves particularly effective for this purpose. In fact, we employed the freely jointed chain and the fractal colloid cluster models to derive expressions for the structure factors of clusters of various sizes at different concentration conditions. The validity of such predictions was then tested against experiments and computer simulations in which a rigid 9-bead model explicitly accounting for the anisotropic Y-shape of antibodies was used. While the freely joint chain model provides a sound description for a wide range of cluster sizes, the colloid model is best suited for cluster of intermediate and large dimensions, whose number of monomers is not less than 15 units.
The excellent agreement between the different methodologies allowed us to provide a first characterization of single clusters of mAbs. Specifically, we found that their structure is independent of the concentration of the antibody solution for a specific cluster size, and that the internal structure of clusters with few monomers is strongly dependent on their size at low scattering vectors. The solution structure factor, calculated with the fjc model, was successfully compared to that obtained by SAXS experiments, demonstrating the validity of this model for the range of concentrations and ionic strengths investigated. In this way, having been able to decipher the contribution of individual clusters, we also got information on the collective response of the polydisperse cluster fluid as a function of concentration. Thus, our theoretical approach was favourably compared to microrheology data, being able to describe the dependence of relative viscosity on mAb concentration.

Our results thus emphasize how the formation of small and medium-sized clusters is indeed critical in the concentration-dependent increase in viscosity for this type of self-assembling antibody. However, although our simplified modeling can provide important information at the qualitative level, a more faithful modeling of the molecule will have to be pursued in the future for a more quantitative study of the macroscopic response of this type of solutions. To this aim, two important aspects that will need to be investigated in depth are the internal flexibility of the antibody molecule and the effect of its inhomogenous charge distribution. In the former case, while it is known that flexibility between domains of the antibody is crucial to the immunological response, it is not yet clear how relevant this is to the assembly of antibodies with attractive domains and their resulting solution structure. At the same time, a more refined treatment of charges, beyond the patchy minimal model, which includes screening effects may lead to a more thorough understanding of the mechanisms of assembly between antibodies in solution and to the exact shape and arrangement of the clusters. The study presented here thus represents a first step for understanding the collective behavior of the solutions in terms of the individual elements that populate the system. This approach will also be instructive for other types of antibodies with different properties, with
the final aim to improve the formulation of stable, low-viscosity solutions of therapeutic monoclonal antibodies.

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Cluster formation and the link to viscosity
in antibody solutions

Supplementary Information

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Antibody excluded volume

For developing a correct theoretical and computational model for antibodies, it is appropriate to check whether its excluded volume is correct with respect to experimental evidence. To this aim, we exploit the relationship between isothermal compressibility and the static structure factor $S(q = 0)$ via the number density $\rho$.

As explained in the main text, it is possible to map the coarse-grained Y-model to an effective hard sphere. To do this, as shown in Fig. S1, we first match the experimentally determined $S(0)$ as a function of the concentration with the same curve obtained via Wertheim theory, allowing to identify the correct size of an effective hard sphere diameter $\sigma_{HS}$. Subsequently, to have a Y model that matches correctly the excluded volume, we compare the
Figure S1: Experimental and theoretical $S(0)$ ($\sigma_{HS} = 2.95\sigma, T = 0.11$) as a function of concentration.

Y-simulation results of $S(0)$ with the predictions of Carnahan-Starling obtained by using the $\sigma_{HS}$ previously determined, as reported in Fig. S2. Here we compare the previously used 6-bead\cite{18} and the newly chosen 9-bead model. We find that for the former case, the best agreement is obtained for $\sigma_{HS} = 2.7\sigma$ at low density and for $\sigma_{HS} = 2.54\sigma$ at higher concentrations. The latter value does not coincide with the one matching Wertheim theory results.

Figure S2: $S(0)$ as a function of the number density $\rho$ for 9 and 6-beads Y models. The corresponding Carnahan-Starling (CS) results are also reported.
in the presence of patches. Instead, for the 9-bead model, we find that the isothermal compressibility actually reproduces the correct behavior with the expected HS size, namely $\sigma_{HS} = 2.95\sigma$. Importantly, the 9-bead model is able to capture the hard sphere compressibility in the whole experimental concentration range, thus appearing to be a superior model than the 6-bead model. Therefore, despite a slight increase in the number of beads, in the main text we focus on this model to provide a comparison for the experimental structure factors.

Simulation snapshots

![Simulation snapshots](image)

Figure S3: Simulation snapshots of the antibody system for $c = 61.7$ and 147 mg/ml. Clusters are highlighted with different colors. Antibodies belonging to the same cluster are colored alike.

In Figure S3, we report two simulation snapshots for two different concentrations analyzed in the main text, namely $c = 61.7$ mg/ml and 147 mg/ml. Individual clusters of different sizes are reported in the main text.
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