Controlling the viscosities of antibody solutions through control of their binding sites

Miha Kastelic*, Ken A. Dillb,1, Yu.V. Kalyuzhnyc, and Vojko Vlachya

aFaculty of Chemistry and Chemical Technology, University of Ljubljana, Večna pot 113, 1000 Ljubljana, Slovenia; bLauffer Center for Physical and Quantitative Biology and Departments of Physics and Chemistry, Stony Brook University, Stony Brook, NY 11794; cInstitute for Condensed Matter Physics, Svidnuitska 1, 79011 Lviv, Ukraine

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We model the aggregation properties of antibody solutions. For commercial biotech, it is desirable to formulate antibody solutions to have low viscosities. We go beyond previous colloid theories in treating protein–protein self–association of molecules that are antibody–shaped and flexible and have spatially specific binding sites. We consider interactions either through Fab–Fab or Fab–Fc binding. We adapt Wertheim’s theory of strongly interacting particles. We compute the cluster-size distributions, viscosities, second virial coefficients and Huggins coefficients, vs. antibody concentration. We find that the aggregation properties of concentrated solutions can be anticipated from simpler–to–measure dilute solutions. A principal finding is that aggregation is controllable, in principle, through modifying the antibody itself, and not just the solution it is dissolved in. In particular: (i) Monospecific antibodies having 2 identical Fab arms can form linear chains, with intermediate viscosities. (ii) Bispecific antibodies having different Fab arms can, in some cases, only dimerize, having low viscosities. (iii) Arm–to–Fc binding allows for 3 binding partners, leading to networks, and very high viscosities.

Significance Statement

Antibodies are natural molecules of the immune system. They are also tailored by the biotechnology industry to serve as biological drugs. When antibodies are used as drugs, they are needed in high concentrations, leading to liquid solutions that have unfavorably high viscosities. A goal is to find ways to reduce the viscosities of antibody solutions. Here, we develop a theoretical model for understanding how the viscosities of antibody solutions depend on the nature of sticky sites on the molecules. One key conclusion is that bispecific antibodies could be designed to have low viscosities, whereas regular (2 identical arms) molecules have intermediate viscosities, and 3–armed molecules (where the Fab and Fc fragments are all sticky), have high viscosities.

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Please declare any conflict of interest here.

* To whom correspondence should be addressed. E-mail: dill@lauffercenter.org

Ab aggregation.

We have recently found that an approach based on the Wertheim theory of solutions can satisfactorily handle orientation-dependent and short–ranged interactions between spheres, giving good predictions of the phase behaviors in simpler protein solutions (32–34). However, antibodies are more complex than simpler proteins. Most similar in spirit to the present work is the elegant theory of Schmit et al. (35). Schmit et al use a binding–polynomial formulation to compute the clustering of featureless 2–arm particles that can link together into chains of different lengths. Then, they compute the viscosities of the few–particle clusters by using long–chain polymer entanglement theory.

Our approach here is different than those above, in the following respects. First, we develop a structure–based theory. While simple proteins can often be approximated as spheres or featureless particles, antibodies, in contrast, are big, flexible and Y–shaped, and have interaction sites at particular locations on the Y. Second, we are able to treat a broader range of situations than just 2–arm binding. For example, of recent interest are synthetic bispecific antibodies (bsAbs), where each arm of the Y can bind to a different epitope, or with a different affinity (36). BsAbs are attractive for cancer immunotherapies, where one arm binds to the tumor cell, while the other arm binds to a natural killer T cell, bringing the killer cell close enough to destroy the tumor cell (37, 38). More than 20 bispecific antibodies are now in clinical development, and two (catumaxomab and blinatumomab) are currently on the market (38). The present model is able to explore the aggregation properties of both monospecific and bsAbs, as well as situations in which the Fc (fragment crystalizable) fragment is sticky. Third, we relate the viscosities to clusters using traditional solution theories of Einstein, Huggins, and Sudduth (39–41), rather than as entangled chains, since anti-
body clusters appear too small to be treatable as long–chain polymers.

We present the results for 3 different situations: (1) Monospecific Natural mAbs, where the two Fab arms bind equally, and there is no binding to Fc. (2) Bispecific Synthetic Abs, where each Fab arm binds differently, and there is no binding to Fc. And (3) Arms–to–Fc: Fab arms are identical, and either one of them can bind to Fc. Schematic illustration of clustering described above is shown in Fig. 1.

Methods

Wertheim’s theory of strongly interacting particles (42–44) affords us an opportunity to treat molecules having shapes as complex as antibodies. We model antibody solution in two steps. First, we start with a multi–component mixture of hard spheres that have different specific sticky interactions. Those spheres self–assemble into Y–shaped molecules, as if they were covalently linked; see Fig. 2. Each Y is assembled as an identical collection of 7 hard spheres, each sphere having diameter σ = 3 nm, and each sphere being decorated by different attractive short–ranged sites, located on the surface. Second, those Y–shaped molecules then form a one–component fluid of molecules, interacting via short–range attractive potentials from their unsatisfied sticky sites; see Fig. 2b. The sticky sites are labelled A (green), B (blue), C (red), D (orange) and E (black). Only sites of the same color can bond to each other. For example, spheres of type 1 assemble at the center of the molecule; it has 3 C sites, contacting other sphere types 2, 3, and 4. In the first stage of incipient assembly, type D–D and E–E bonds form to create the Y–shaped antibody molecules.

If the number density of hard spheres of type i (i = 1…7) is ρi, then the number ρ of Ab molecules is also equal to ρ1.

The mathematical details of the model are given in SI, see Eqs. (S1)–(S4). Here is a brief overview. Each type of interaction – say between A and B – is expressed by an energy εAB, where positive values indicate attraction, and the range of interaction ω. Then, we construct the free energy of the system, see Eqs. (S5)–(S12) of SI, which allows calculation of thermodynamic properties. Next, we compute the distributions of cluster sizes, i.e. the populations of Y–shaped molecules as single molecules, as 2–molecule clusters, as 3–molecule clusters, etc. Then, as described below, we can compute the viscosities of the antibody solutions, which are functions of the populations of the different antibody clusters.

Solution viscosity depends on the antibody cluster–size distribution. Standard theories give the viscosity, η, of a solution as a polynomial function of the mass concentration γ of the solute (mg of protein per mL of solution) (40, 45):

\[
\eta/\eta_0 = 1 + [\eta]_3 \gamma + k_H [\eta]_5 \gamma^2 + O(\gamma^3). \tag{1}
\]

We give this equation in order to define quantities the quantities that will be useful throughout this paper. Here, η0 is the viscosity of solvent, [η] is the intrinsic viscosity, and k_H is the Huggins constant (40, 45). Higher terms are of order γ^3. On the other hand, we note that this polynomial expansion, Eq. (1), is not itself sufficient to account for systems of associating particles, such as antibody solutions. To model antibody solutions, we must establish the connection between the antibody cluster size and solution viscosity. For this, we assume that an increase of the mass concentration of the cluster with Ab molecules dγn, contributes to the relative increase of viscosity dη/η as

\[
\frac{d\eta}{\eta} = \sum_{n=1}^{\infty} f(n) d\gamma_n. \tag{2}
\]

The one–component version of Eq. (2), which was originally proposed by Sudduth (41), is generalized here to account for the presence of clusters (n > 1). For f(n) we adopt the form

\[
f(n) = c n^d. \tag{3}
\]

where c and d are adjustable parameters to fit the data. The functional form is suggested by the experimental observations (35, 46), indicating a strong increase of solution viscosity for mAb concentrations above 90 mg/mL. Note that f(n) depends solely on the number of molecules involved, n, and not on their spatial distribution in the cluster. Increments of dγn depend on the total mass concentration of antibodies, γ, and the weight fraction distribution P(n, γ), which is the mass–weighted probability of finding an antibody molecule as a part of n–mer (i.e. a cluster containing n antibody molecules), as

\[
\gamma_n = \gamma P(n, \gamma). \tag{4}
\]
Integration of Eq. (2) yields an expression for \( \eta \) of the form
\[
\ln \left( \frac{\eta}{\eta_0} \right) = \sum_{\gamma=1}^{\infty} \gamma f(n) P(n, \gamma).
\]

In addition to \( P(n, \gamma) \), we also defined the number fraction distribution, \( H(n, \gamma) \), which defines the average cluster size, \( \langle n \rangle \), as (45)
\[
\langle n \rangle = \sum_{n=1}^{\infty} nH(n, \gamma).
\]

\( P(n, \gamma) \) and \( H(n, \gamma) \) are both normalized and related to each other through \( \langle n \rangle P(n, \gamma) = nH(n, \gamma) \). To obtain these functions, we use the Wertheim theory of strongly associating liquids (42–44), as described in detail in the SI.

Results and Discussion

Viscosity increases as power law of the antibody concentration. First, we computed solution viscosities for case (1) of monospecific antibodies (details of case (1) are given in Section “Modeling monospecific antibodies” of SI), so we set \( \varepsilon_{AA} = \varepsilon_{AB} = \varepsilon_{BB} \). We fit the model calculations to the experimental data of Schmit et al (35) for the antiestreptavidin IgG1 monoclonal antibody, as functions of concentrations of protein, added NaCl, pH, and temperature of the solution.

The mass concentration of solution is \( \gamma = M_2/\rho N_A \), where \( N_A \) is Avogadro’s number and \( M_2 = 142,000 \text{g/mol} \) is the molar mass of the antiestreptavidin IgG1. Other parameters are obtained as follows: (i) We assume range of interaction \( a \) equals the hydrogen bond length (32), so \( a = 0.18 \text{nm} \) for all the experimental conditions studied here. (ii) We obtain the energy depths \( \varepsilon_{AA} \) and constants \( c \) and \( d \) in Eq. (3) by fitting to the experimental data (35). Note that interaction energies define the fractions of the particles which do not bond via sites A, B, and C in Eqs. (S8)–(S12). This is the only information we need for further calculations. The resulting values of the parameters \( c \) and \( d \) are \( c = 0.01205 \text{mL/mg} \) and \( d = 0.3762 \), while \( \varepsilon_{AA} \) is taken to depend on solution conditions (\( \rho \) and salt concentration but not temperature). \( \varepsilon_{AA} \) ranges from 32.8 to 37.9 kJ/mol, where larger values mean stronger attraction. Viscosity data for pure solvent, \( \eta_0 \), are included in fit. Overall, we use 18 parameters to fit 96 experimental curves. Details of fitting procedure are given in Section “Monospecific antibodies: Extraction of \( \varepsilon_{AA} \) from experimental data” of SI. The results, Fig. 3 and Fig. S1 of SI, show that viscosity increases sharply with protein concentration. And, with a few exceptions, the solution viscosities increase more rapidly with higher salt (NaCl) content and at higher pH values.

Using Eq. (1) and the fit shown by the lines in Fig. 3 and Fig. S1, we obtained a value for the intrinsic viscosity, \( [\eta] = (12.05 \pm 0.05) \text{cm}^2/\text{g} \) for all solution conditions. The value is close to the range from 7.0 to 11.5 cm²/g, observed in experimental studies on human, bovine, and pig IgG dissolved in aqueous solutions (47). These values are higher than for globular proteins, where \( [\eta] \) ranges from 2.5 to 5.0 cm²/g, reflecting the sizes and shapes of the antibodies.

The model gives a microscopic explanation for the dramatic increases in viscosity. We define the fractional contribution of \( n \)-mers to the viscosity as
\[
\xi(n, \gamma) = \frac{\gamma f(n) P(n, \gamma)}{\ln(\eta/\eta_0)} = \frac{f(n) P(n, \gamma)}{\sum_{n=1}^{\infty} f(n) P(n, \gamma)}.
\]

High-concentration aggregation properties can be predicted from simpler low-density experiments. Exploring aggregation can be challenging because measuring properties in high-concentration solutions can be quirky, slow and technique-limited. So, it is of interest to know if antibody aggregation properties can be predicted from dilute-solution measurement. In a well-known example, this objective was achieved by George and Wilson (48), and then others (49–52), for predicting protein crystallization. George and Wilson showed a correlation between crystallization conditions (the high-concentration behavior) and the second virial coefficient, \( B_{22} \). The low-concentration, pairwise-interaction behavior. They proposed the idea of “crystallization slot”, i.e. a region of \( B_{22} \) values between \(-20 \times 10^{-4} \) and \(-80 \times 10^{-4} \text{cm}^2 \text{mol}^{-2} \) (48), where protein crystallization is most likely to occur. \( B_{22} \) is
defined through McMillan–Mayer osmotic virial equation (53),

\[ \Pi = \frac{M_2}{\gamma RT} = 1 + B_{22} + O(\gamma^2), \]

where \( \Pi \) is the osmotic pressure, \( M_2 \) the molar mass of protein and \( R \) the gas constant. \( B_{22} \) can be obtained from the Eq. (8) at low mass concentrations \( \gamma \). The idea of “crystallization slot” has been experimentally confirmed for lysozyme, BSA, ovalbumin, and other globular proteins with molar masses around 14 kDa. For larger proteins with molar mass around 140 kDa, Haas (54) and co-workers suggested for the “crystallization slot” values ranging between \(-4 \times 10^{-5}\) and \(-9 \times 10^{-5}\) cm\(^3\) mol\(^{-1}\) g\(^{-1}\). This finding is of interest for us because many antibodies (11, 12, 55), including the one studied in this work, have molar masses in this range.

First, we show that two dilute-solution properties that reflect protein–protein pair interactions – the Huggins constant, \( K_h \) and the second virial coefficient \( B_{22} \) – are closely related to each other, across a range of temperatures. Fig. 5 (upper panel) shows excellent theoretical correlation between \( K_h \) and \( B_{22} \). Note that both parameters \( B_{22} \) and \( K_h \) are valid for dilute protein solutions, in our case this is up to 90 mg/mL, where binary interactions are dominant (see also Fig. 4). On the bottom panel of Fig. 5 we show the viscosity of antistreptavidin IgG1 solutions studied at \( \gamma = 150 \) mg/mL and correlate them with corresponding \( B_{22} \) values from the upper panel (△). We supplement these data with the correlation between \( \eta \) and \( B_{22} \) measurements of Saito et al (55) for three subclasses of IgG1 antibodies at \( \gamma = 150 \) mg/mL and \( T = 20^\circ \)C; mAb–A (●), mAb–B (X), and mAb–C (△). The results indicate that \( B_{22} \) measurements, though in principle only apply to low protein concentration, contain important information about viscosity of concentrated solutions. This finding is confirmed by the recent work of Tomar et al (16), stating that large positive second osmotic coefficient is a good indicator of the low viscosity of antibody solutions up to 150 mg/mL.

**Bispecific antibodies have two different binding arms. Each arm contributes differently to viscosity.** Now, we consider the aggregation of bispecific antibodies (case (2) in Fig. 1), see also Section “Modeling bispecific antibodies” of SI for mathematical description of clustering. The two arms, A and B, are non-equivalent, so \( \varepsilon_{AA} \neq \varepsilon_{BB} \). And, we assume \( \varepsilon_{AB} = 0 \). We call this Fab–Fab’ association. We explore these properties by expressing the binding energies in terms of \( r \), the degree of asymmetry of the interactions,

\[ \varepsilon_{AA} = \varepsilon_0 (1 + r), \]

\[ \varepsilon_{BB} = \varepsilon_0 (1 - r), \]

where \( r \) takes on values from 0 (symmetric case) to 1. We set \( \varepsilon_0 \) equal to 37.8 kJ/mol, which is the value for the site-site energy extracted from Fig. 3, for the case with highest viscosity (150 mM NaCl, pH = 6.5). Notice that while \( r \) varies the total strength of interaction, \( \varepsilon_{AA} + \varepsilon_{BB} \), is kept constant. Further we assume that \( T = 10^\circ \)C, while other potential parameters remain unchanged, \( \omega = 0.18 \) nm, \( c = 0.01205 \) mM/g, and \( d = 0.3762 \).

Fig. 6 (upper panel) shows the predicted viscosities of bispecific antibodies as a function of both protein concentration and of the asymmetry of the arm interactions. The figure shows two points. First, it shows that when \( r = 0 \) (Fab arms are symmetrical), the viscosity reduces to standard monospecific-antibody case, increasing sharply with concentration. Second, more interestingly, it shows that when \( r = 1 \) (\( \varepsilon_{AA} = 2\varepsilon_0 \) and \( \varepsilon_{BB} = 0 \), very different arm interactions), the antibody solutions are predicted to have much lower viscosities. The interpretation is clear. Monospecific antibodies can link to each other through two linkage sites (both arms), leading to system of linear clusters, like chains of people at a party. In contrast, bispecific antibodies in this case can only link through a single arm, may lead to clusters that are not bigger than dimers. The \( P(n, \gamma) \) distribution for this case \( r = 1 \) drops from 1 to 0 for \( n = 1 \) and rises from 0 to 1 for \( n = 2 \) as soon as \( \gamma > 0 \). \( P(n, \gamma) \) distributions for intermediate values of \( r (0 < r < 1) \) are shown in the SI in Fig. S4.

Here is an implication for antibody design. Normally, for biological efficacy, it is desirable that both arms of an antibody bind to an epitope, either for monospecific or bispecific antibodies. And, normally the objective, for bispecific antibodies, is to bind well to two different epitopes. But, consider now a situation in which it would be sufficient for the biology to have an antibody that binds to only one epitope through only a single Fab arm. To explore this quantitatively, we introduce the quantity \( Q = \gamma/(\eta_0/\eta) \), which is the ratio between the concentration of antibody and its relative viscosity, with units mg of protein per mL of solution. Now
the ratio $Q(r, \gamma)/Q(r = 0, \gamma)$ gives the performance (efficacy divided by viscosity) of a bispecific antibody with asymmetry $r$ relative to a monospecific antibody with $r = 0$. When $Q(r, \gamma)/Q(r = 0, \gamma) > 1$, it means that using the second arm for viscosity control has paid off in allowing for greater concentrations, while $Q(r, \gamma)/Q(r = 0, \gamma) = 1$ implies no benefit of bispecific antibody in regard to a monospecific antibody.

In Fig. 6 (bottom panel) we investigate the effect of degree of asymmetry $r$ on $Q(r, \gamma)/Q(r = 0, \gamma)$.

![Fig. 6](image_url) # Upper panel: relative viscosity $\eta/\eta_0$ decreases sharply as the energies of the attractive sites A and B start to deviate from each other; that is for $r > 0$.

Bottom panel: normalized quality factor $Q(r, \gamma)/Q(r = 0, \gamma)$ as a function of protein concentration $\gamma$ and degree of asymmetry $r$. Asymmetry $r > 0$ increases $Q(r, \gamma)/Q(r = 0, \gamma)$ rapidly for $\gamma > 100$ mg/mL.

For $r > 0$ and for $\gamma > 40$ mg/mL we observe strong increase of the $Q(r, \gamma)/Q(r = 0, \gamma)$ ratio. At even higher $\gamma$ values, small deviations of $r$ from zero substantially increase this ratio. For example, changing $r$ from 0 to 0.2 at $\gamma = 120$ mg/mL increases the $Q(r, \gamma)/Q(r = 0, \gamma)$ ratio from unity to around 1.5, while the corresponding viscosity ($\eta/\eta_0$) decreases from 0.5 to 0.7 as seen from the upper panel in Fig. 6. Bispecific antibodies thus meet two important criteria for therapeutic applications: (i) they have low viscosity, and (ii) because higher concentrations can be used, their biological activity is enhanced. Note that in our theory the probability for the site on one arm (Fab) to be occupied does not depend on the occupancy of the other arm (Fab’). Violation of this assumption would change $Q$.

Recent study of Berteu and co–workers (56) revealed an impact of the viscosity on subcutaneous injection pain tolerance. They found that the range of viscosities from 15 to 20 mPa s were the most gentle for patients. This means that for $\eta_0 = 1.5$ mPa s the relative viscosities $\eta/\eta_0$ are between 10 and 13. These values can be reached for protein concentrations between 140 and 160 mg/mL and $r > 0.3$. In this case $Q(r, \gamma)/Q(r = 0, \gamma)$ is about 2.0 (see Fig. 6). The most often used antibody concentration is 100 mg/mL; an increase of this concentration to 140 mg/mL would make the treatment considerably more efficient. We accordingly propose for the reduction of viscosity to be induced by a small difference between the Fab fragments.

When Fc is also sticky, the viscosity increases dramatically.

Here, we consider again a situation of monospecific antibodies, in which the two arms are equally sticky. But now in addition, we allow for the possibility that the Fc site can also stick to either Fab arm. So we set $\varepsilon_{AC} = \varepsilon_{BC}$, while all the other site–site attractions are equal to zero (case (3) in Fig. 1), see also Section “Modeling the Fab–Fc association” of SI for details. The cluster–size distributions for this case are given in Fig. S5 of SI. Summarizing, we find that these solutions have very high viscosity. Fig. 7 compares this to the earlier calculations above. This 3-fold comparison is simple to interpret. (1) In arms–to–Fc binding each antibody molecule has 3 sticky arms, so it can link to 3 neighboring antibody molecules. It can form highly connected branched networks, resulting in high viscosities. (2) In monospecific binding, antibodies can, at most, link together as linear chains, leading to intermediate viscosities. (3) In bispecific binding, antibodies can, at most, link together as dimers, leading to lower viscosities. Scenarios (1), (2), and (3) are shown in Fig. 1.

![Fig. 7](image_url) # Relative viscosity $\eta/\eta_0$ as a function of antibody concentration $\gamma$ for three scenarios studies so far: (1) symmetric Fab–Fab model of antibodies (—), (2) model of bispecific antibodies at the degree of asymmetry $r = 0$ (—), and (3) model of interacting Fab–Fc terminals (—). Assiglation of energy parameters $s$ differs among scenarios, while their values remain unchanged, 37.8 kJ/mol. Other parameters are $\omega = 0.18$ nm, $c = 0.01205 \text{ mL/mg}$, and $d = 0.3762$. Calculations for $T = 10^\circ \text{C}$.

Conclusions

Monoclonal antibodies constitute a major form of modern therapeutics. These biological drugs are usually formulated and delivered as highly concentrated solutions of antibody molecules. The problem is that such solutions tend to aggregate and have high viscosities, due to protein–protein attractions. It has not previously been understood how such solution aggregation properties are encoded within the molecular energies and locations of the sticky sites on the antibody molecules. Here, we adapt the Wertheim theory of strongly associating liquids. In the present model, seven spherical particles self–assemble first into Y–shaped model antibody molecules, and...
the Y-shaped particles then further self-assemble as an essentially 1-component solution into antibody clusters. We use the data of Schmit et al. to (35) and our model to analyse viscosity measurements as functions of antibody concentration, pH, temperature and added sodium chloride concentration. We study 3 situations: (1) Monospecific: 2 identical Fab arms that are sticky. (2) Bispecific: the 2 Fab arms have different stickiness. (3) Arms-to-Fc: 2 identical Fab arms and Fc are all sticky. In limiting cases, we find that: The Arms-to-Fc case gives the highest viscosities because each molecule can link to 3 neighbors, forming dense networks. The Monospecific case has lower viscosities because each molecule can link only to 2 neighbors, leading to linear chains. The Bispecific case has the lowest viscosity since each molecule can link to only 1 neighbor. A general point here is that antibody solutions can be tailored to have different aggregation properties by tinkering with the antibody molecules themselves, rather than by current strategy of tinkering with their formulation solutions. In the spirit of previous work ([57]), the model can be extended to include ions, excipients, and/or water molecules explicitly.

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Energetic interactions that define the model

The procedure presented in Fig. 2 of the main text, can be expressed mathematically as follows.

\[
\exp [-\beta u^{(ij)}_{\text{DD}}(z_{\text{DD}})] - 1 = \left\{ (\delta_{i1} + \delta_{ij})(1 - \delta_{ij}) \sum_{m=2}^{4} (\delta_{im} + \delta_{mj}) \right\} K_{\text{DD}}^{(ij)} \delta(r_{ij} - \sigma), \tag{S1}
\]

\[
\exp [-\beta u^{(ij)}_{\text{EE}}(z_{\text{EE}})] - 1 = \left\{ (1 - \delta_{i1})(1 - \delta_{ij})(\delta_{i(j+3)} + \delta_{i(j-3)}) \right\} K_{\text{EE}}^{(ij)} \delta(r_{ij} - \sigma), \tag{S2}
\]

where \( \beta = 1/k_B T \) and \( T \) is the absolute temperature. Here \( r_{ij} \) denotes the distance between spheres of the type \( i \) and \( j \), and \( \Omega_i, \Omega_j \) their orientations. \( z_{\text{DD}} \) and \( z_{\text{EE}} \) are the distances between sites D–D and E–E. Angular brackets \( \langle \ldots \rangle_{\Omega_i,\Omega_j} \) denote the orientational average, \( \delta(\ldots) \) is the Dirac delta function, and \( \delta_{ij} \) the Kronecker delta symbols within the curly brackets \( \{ \ldots \} \) provide rules for the intramolecular bond formations between the spheres \( i \) and \( j \) and sites D and E: 1D–D2, 1D–D3, 1D–D4, 2E–E5, 3E–E6, and 4E–E7 (six intramolecular bonds altogether). Note that each of the sites D and E can be bonded only once. Finally, the molecules modeling antibodies are formed upon enforcing the condition \( K_{\text{DD}}^{(ij)} \) and \( K_{\text{EE}}^{(ij)} \) \( \to \infty \). Once this limit is taken, no dissociation to separate spheres is possible. Within Wertheim’s thermodynamic perturbation theory (42, 43, 44) the model molecules are flexible; the only restriction is the sequence of “bonds”, Eqs. (S1) and (S2), connecting the hard spheres as shown in Fig. 2.

To account for the attractive interactions among Ab molecules, we introduce three additional short-range interactions, called A, B, and C (see Fig. 2). These sites allow intermolecular association. We can write the entire pair potential among model Ab molecules \( k \) and \( l \), \( u_{kl} \), as

\[
u_{kl}(\vec{r}_k, \vec{r}_l) = \sum_{i=1}^{7} \sum_{j=1}^{7} \left\{ u^{(ij)}_{\text{hs}}(r_{ij}) \right\} + \sum_{m=5}^{7} \sum_{n=5}^{7} \delta_{im} \delta_{jn} u^{(ij)}_{\alpha(m)\alpha(n)}(z_{\alpha(m)\alpha(n)}), \tag{S3}
\]

where primes on the summation signs label the spheres composing Ab molecules \( k \) and \( l \). Further, \( u^{(ij)}_{\text{hs}}(r_{ij}) \) is the hard–sphere potential, while the sums over \( m \) and \( n \) count the intermolecular interactions among A, B, and C. Notice that \( \alpha(5) = A, \alpha(6) = B, \) and \( \alpha(7) = C \). Similarly as above, \( z_{AA}, z_{AB}, z_{AC}, z_{BB}, z_{BC}, \) and \( z_{CC} \) designate the distances between the pairs of sites.

Interactions among sites A, B, and C in Eq. (S3), \( u^{(ij)}_{\alpha(m)\alpha(n)} \), have a form of the site–site square well potentials:

\[
\begin{align*}
u^{(ij)}_{\alpha(m)\alpha(n)}(z_{\alpha(m)\alpha(n)}) = & \begin{cases}
-m, n = 5 : & -\varepsilon_{AA} \quad \text{for} \quad z_{AA} < \omega, \\
m, n = 5 : & 0 \quad \text{for} \quad z_{AA} \geq \omega, \\
m, n = 6 : & -\varepsilon_{AB} \quad \text{for} \quad z_{AB} < \omega, \\
m, n = 6 : & 0 \quad \text{for} \quad z_{AB} \geq \omega, \\
m, n = 7 : & -\varepsilon_{AC} \quad \text{for} \quad z_{AC} < \omega, \\
m, n = 7 : & 0 \quad \text{for} \quad z_{AC} \geq \omega, \\
m, n = 6 : & -\varepsilon_{BB} \quad \text{for} \quad z_{BB} < \omega, \\
m, n = 7 : & 0 \quad \text{for} \quad z_{BB} \geq \omega, \\
m, n = 7 : & -\varepsilon_{BC} \quad \text{for} \quad z_{BC} < \omega, \\
m, n = 7 : & 0 \quad \text{for} \quad z_{BC} \geq \omega, \\
m, n = 7 : & -\varepsilon_{CC} \quad \text{for} \quad z_{CC} < \omega, \\
m, n = 7 : & 0 \quad \text{for} \quad z_{CC} \geq \omega
\end{cases}
\end{align*}
\tag{S4}
\]

where \( \varepsilon_{AA}, \varepsilon_{AB} = \varepsilon_{BA}, \varepsilon_{AC} = \varepsilon_{CA}, \varepsilon_{BB}, \varepsilon_{BC} = \varepsilon_{CB}, \) and \( \varepsilon_{CC} \) (all defined as positive) are their square–well depths, and
ω their range. Attraction between the sites causes for model antibodies to form clusters. Note again that two Fab ends are named A and B, while C stands for Fc end. The clustering is possible through attractive Fab–Fab and Fab–Fc interactions.

Wertheim’s thermodynamic perturbation theory for computing the particle concentrations from the underlying model of the energetics

To calculate properties of the model fluid we use Wertheim’s thermodynamic perturbation theory (TPTI) (42, 43, 44). In this approach we decompose the Helmholtz free energy $F$ in the ideal $F_{id}$, hard–sphere $F_{hs}$, and association term $F_{ass}$. The terms are given by the following expressions:

$$
\frac{\beta F_{id}}{V} = \sum_{i=1}^{7} \rho \left[ \ln(\lambda^3 \rho) - 1 \right],
$$

[S5]

$$
\frac{\beta F_{hs}}{V} = 4\eta - 3\eta^2 \left( 1 - \eta \right)^2 \rho \rho,
$$

[S6]

$$
\frac{\beta F_{ass}}{V} = \rho \left\{ \left[ \ln(\rho^3 g_{\text{hs}}^{\text{(ov)}}) - 1 \right] - 6 \left[ \ln(\rho^3 g_{\text{hs}}^{\text{(ov)}}) - 1 \right] \right\},
$$

[S7]

where $\rho$ is the number of antibody molecules, $\lambda$ is the de Broglie thermal wavelength (58), $\rho_0 = \eta \pi \rho \sigma^3/6$, $g_{\text{hs}}^{\text{(ov)}} = (1 + \eta/2)/(1 - \eta)^2$ is the Percus–Yevick expression for the contact value of the hard–sphere radial distribution function (59), and $X_A$, $X_B$, and $X_C$ are the fractions of the particles which do not bond via sites A, B, and C, respectively. They follow from the statistical–mechanical analogue of the mass action law (44):

$X_A = \left\{ 1 + \Delta_{AA} X_A + \rho \Delta_{AB} X_B + \rho \Delta_{AC} X_C \right\}^{-1}$,

[S8]

$X_B = \left\{ 1 + \rho \Delta_{AB} X_A + \rho \Delta_{BB} X_B + \rho \Delta_{BC} X_C \right\}^{-1}$,

[S9]

$X_C = \left\{ 1 + \rho \Delta_{AC} X_A + \rho \Delta_{BC} X_B + \rho \Delta_{CC} X_C \right\}^{-1}$,

[S10]

where

$$
\Delta_{ij} = 4\pi g_{\text{hs}}^{\text{(ov)}} \sigma^4 \omega \int \tilde{f}_{ij}(r) r^2 dr,
$$

[S11]

$$
\tilde{f}_{ij}(r) = \exp\left( \beta \varepsilon_{ij} \right) - 1 \frac{(\omega + \sigma - r)}{(2\omega - \sigma + r)} \frac{1}{(6\sigma r)},
$$

[S12]

for $i$ and $j$ to be A, B, or C. Here $\tilde{f}_{ij}(r)$ is the orientational average of the Mayer function for the square–well site–site interaction (60). The orientational average is taken over all possible positions of sites A, B, C, D, and E on the beads. This introduces certain flexibility into the model, as indicated in Fig. 2.

Note that the association free energy, Eq. (S7), contains the intermolecular association term and six intramolecular terms each equal to $[\ln(\rho^3 g_{\text{hs}}^{\text{(ov)}}) - 1]$. Osmotic pressure $\Pi$ is now readily available using standard thermodynamic relations (58). Note again that $\rho_0$ stands for the number density of individual spheres $i = 1 \cdots 7$, being equal to the number density of the Ab molecules $\rho$. It is necessary to emphasize that such theories are only applicable in the domain of concentrations and pH values where the proteins do not undergo major conformational changes (61, 62).

Fractions of non–bonded particles determine the viscosities of antibody solutions

Modeling monospecific antibodies: both A and B arms bind identically, and $C$ does not. The simplest possible case to examine is the one where the sites A and B are physically equal so $\varepsilon_{AA} = \varepsilon_{AB} = \varepsilon_{BB}$. Fab sites interact only in–between (there is no interaction with Fc site) so: $\varepsilon_{AC} = \varepsilon_{BC} = \varepsilon_{CC} = 0$. The parameter which completely determines the $n$–distribution, $H(n, \gamma)$, and weight fraction distribution, $P(n, \gamma)$, is the fraction of molecules, not bonded through site $A$, $X_A$ ($X_A = X_B$).

From polymer physics it follows (45)

$$
H(n, \gamma) = X_A(1 - X_A)^{n-1},
$$

[S13]

while expression (6) yields an exact result for $\langle n \rangle$,

$$
\langle n \rangle = \frac{1}{X_A},
$$

[S14]

which can, as shown in Section “Solution viscosity depends on the antibody cluster–size distribution”, easily be calculated.

Modeling bispecific antibodies: sites A and B bind differently, and $C$ does not. Distinction among sites A and B can be introduced in several ways. Here we select for $\varepsilon_{AA} \neq \varepsilon_{BB}$ and for the cross interaction to be zero: $\varepsilon_{AB} = 0$. As before, there is no interaction with Fc region. The $n$–distribution $H(n, \gamma)$ is now determined not only by $X_A$ but also by value of $X_B$. Clusters of size $n$ can be terminated by two A sites ($A \cdots A$), by one A and one B site ($A \cdots B$), or two B sites ($B \cdots B$), depending on the parity of the $n$–mer cluster. If $n$ is odd, only $A \cdots B$ terminated clusters are possible. For even $n$, the cluster starts and ends with same type of site. The $n$–distribution, $H(n, \gamma)$, depends on the cluster parity (45)

$$
H(n, \gamma) = \langle n \rangle \begin{cases} \frac{1}{2} \left( \frac{1}{X_A} \right)^{n-2}/X_A^2(1 - X_B) & \text{for even } n, \\ \frac{2(X_A + X_B - X_A X_B)}{X_A^2(1 - X_B) + X_B^2(1 - X_A) + 2X_A X_B} & \text{for odd } n, \end{cases}
$$

[S15]

[Distributions $P(n, \gamma)$ and $H(n, \gamma)$ are normalized as described in “Solution viscosity depends on the antibody cluster–size distribution”; Notice that in this case the two distributions and $\langle n \rangle$ depend on values of $\varepsilon_{AA}$ and $\varepsilon_{BB}$.

Modeling the Fab–Fc association. Here we assume that the sites A and B are bonded equally strongly to the C site ($\varepsilon_{AC} = \varepsilon_{BC}$) while all the other site–site attractions are set to 0. From the mass–action law, Eqs. (S8), (S9), and (S10), we obtain the relation $X_C = 2X_A - 1$. Three attractive interactions per Ab molecule allow formation of clusters with branched topology. Similarly as in the main text we can obtain the $n$–distribution $H(n, \gamma)$ and examine the average $\langle n \rangle$. Following Rubinstein and Colby (45), we write

$$
H(n, \gamma) = \frac{(2n)!}{n!(n+1)!} (1 - X_A)^{n-1} X_A^{n+1},
$$

[S17]
and, in the next step, the analytical result for \( \langle n \rangle \),

\[
\langle n \rangle = \frac{1}{2X_A - 1} = \frac{1}{X_C}.
\]

[S18]

In this example both distributions as also the average \( \langle n \rangle \) depend solely on \( \varepsilon_{pc} \).

**Monospecific antibodies: Extraction of \( \varepsilon_{AA} \) from experimental data.** In this Section we present the procedure used to extract the \( \varepsilon_{AA} \) values as a function of pH and salt content. We also present the whole set of \( \eta - \gamma \) curves as well as the \( P(n, \gamma) \) distributions for the Fab–Fab association for the Subsection “Viscosity increases as power law of the antibody concentration” of the main text.

Measured viscosities are for different pH and salt content and as a function of the mass concentration (\( \gamma \) axis) shown in Fig. S1 by symbols. Color of the symbol indicates the temperature. There are 6 different isotherms and 4 points per isotherm, yielding 24 measurements per panel. As explained in the main text, we assume fixed interaction range \( \omega = 0.18 \) nm for all conditions studied, while \( \varepsilon_{AA} \) varies with pH and salt content but not with the temperature and mass concentration (\( \varepsilon_{AA} \) is kept constant within each panel). Altogether we have to determine 18 parameters: (i) 16 values for \( \varepsilon_{AA} \), within each panel separately, and (ii) 2 parameters \( c \) and \( d \) of the function \( f(n) \), see Eq. (3) of the main text. For this purpose we define the objective function \( \chi^2 \) to be minimized during the fitting procedure as,

\[
\chi^2 = \frac{1}{16} \sum_{p=1}^{16} \sum_{t=1}^{4} \sum_{i=1}^{4} \left( \eta^{(T)}_{p,t,i}(\varepsilon_{AA}(p), c, d) - \eta^{(E)}_{p,t,i} \right) \times 2 W(p, t, i), \tag{S19}
\]

where indices \( p, t \) and \( i \) run over panels, temperatures and mass concentration points, respectively. \( \eta^{(E)} \) are viscosity data and \( \eta^{(T)} \) the corresponding theoretical values, while function \( W \) weights individual contributions according to uncertainties of measurements \( \Delta \eta^{(E)} \). For \( W \) we propose the form,

\[
W(p, t, i) = \exp \left( -\tau \frac{\Delta \eta^{(E)}_{p,t,i}}{\eta^{(E)}_{p,t,i}} \right), \tag{S20}
\]

while \( \tau \) is arbitrary chosen to be 4. Note that viscosity data for pure solvent, \( \eta^{(E)}_{p,t,1} = \eta(\gamma = 0) = \eta_0 \), are included in fit, therefore index \( i = 1 \) is omitted from \( \chi^2 \). On the other hand, indices \( i \) equal to 2, 3, and 4 correspond to viscosity data with mass concentrations 120, 145, and 175 mg/mL. Initially we chose \( \varepsilon_{AA}(p), c \) and \( d \) by hand, to qualitatively describe experimental data \( \eta^{(E)} \). Then we start the iteration procedure: at each step we minimize objective function \( \chi^2 \) by varying one randomly chosen parameter (from 18 possible). The minimization step is realized by the golden–section search. The procedure is repeated until \( \chi^2 \) does not reduce anymore. We check the robustness of the procedure by prescribing different initial values for \( \varepsilon_{AA}(p), c \) and \( d \). In all the cases we find the same solution \( c = 0.01205 \) mL/mg and \( d = 0.3762 \). The \( \varepsilon_{AA} \) varies from panel to panel as written in Fig. S1 and is visualized in Fig. S2. The whole set of \( \eta - \gamma \) curves, belonging to parameters \( \varepsilon_{AA} \), \( c \) and \( d \) are also a part of Fig. S1.

Fig. S2 shows an monotonic increase of \( \varepsilon_{AA} \) upon addition of NaCl at certain pH. The increase seems to be more pronounced at low pH values: the reason might be an enhanced role of counterions at low pH regime. Note that antistreptavidin IgG1 bears positive net charge in the region where measurements were performed (isoelectic point is around 8.0). The deviation from monotonic decrease is observed for the case where no NaCl is added.

Results for \( P(n, \gamma) \) are shown in Fig. S3 for two solution conditions from Fig. S1: (i) without added salt at pH = 5.0 and (ii) in mixture with 150 mM NaCl at pH = 6.5.

**Bispecific antibodies: Degree of asymmetry \( r \) modifies the distribution of clusters**

In Fig. S4 we show how the degree of asymmetry \( r \) affects the distribution \( P(n, \gamma) \) for the solution conditions described in Section “Bispecific antibodies have two different binding arms. Each arm contributes differently to viscosity of the main text.

Value \( r = 0 \) corresponds to the symmetric antibody molecule, so it is expected for results to be in qualitative agreement with those in Fig. 4 in the main text. When \( r > 0 \), we see the decrease in \( P(n, \gamma) \) for chains of odd parity on the expense of the chains of even parity. For \( r > 0 \) the A–A interaction becomes energetically more favorable, see Eq. (26) of the main text, and accordingly the B···B termination more probable. At higher antibody concentrations, the 4–mer chains start to form.

The distribution of cluster sizes when all 3 sites (2 Fab’s and 1 Fc) are sticky

In this Section we present the \( P(n, \gamma) \) distributions for the Arms–to–Fc binding in Section "When Fc is also sticky, the viscosity increases dramatically" of the main text. We concentrate on the case high viscosity, having the energy depth \( \varepsilon_{AC} \) 37.8 kJ/mol (see Fig. 3). For the particular energy value the \( P(n, \gamma) \) distributions are presented in Fig. S5 (solid lines), where they are compared with the results for the symmetric Fab–Fab association (dashed lines, see also Fig. S3).

We observe a decrease of probabilities to find the antibody molecule as a monomer and dimer, \( P(1, \gamma) \) and \( P(2, \gamma) \). On this account, the fraction of higher \( n \)–mers such as 5–mers increases. The propensity of antibodies to form clusters through Fab–Fc terminals is enhanced in comparison with the Fab–Fab type of association. Notice that in contrast to the Fab–Fab association, where only chain–like aggregates can be formed, the Fab–Fc association model allows the formation of branched clusters.
Fig. S1. Comparison of experimental data (35) (symbols) with the results of our model analysis (lines). NaCl concentrations are 0, 50, 100, and 150 mM, pH values studied here 5.0, 5.5, 6.0, 6.5, while the temperatures vary from 10 °C (top–blue) to 35 °C (bottom–red) in increments of 5 °C. The site–site interaction range $\omega$ is fixed to 0.18 nm for all calculations, while $\epsilon_{AA}$ varies from panel to panel. For the sake of completeness, we show the panels in Fig. 3 again.
Fig. S2. Values of potential depth $\varepsilon_{AA}$ for solution conditions given on panels of Fig. S1. Intermediate values on the surface are obtained upon interpolation and are colored to guide the eye.

Fig. S3. Weight fraction distribution $P(n,\gamma)$ for different cluster size $n$ (1 denotes the monomer, 2 dimer, ... etc; up to decamer denoted by 10) as a function of the protein mass concentration $\gamma$ at 10 $^\circ$C. (i) Upper panel shows the low viscosity trends – no salt present, pH = 5.0, and (ii) the bottom panel presents the high viscosity trends – concentration of added NaCl is 150 mM, pH = 6.5; see also Fig. 3.

Fig. S4. $P(n,\gamma)$ graphs for three values of the parameter $r$: (i) $r = 0$ (top left), (ii) $r = 0.1$ (top right), and (iii) $r = 0.2$ (bottom). When sites start to differentiate (that is for $r > 0$), we observe a decrease in probability of odd parity $n$ chains.

Fig. S5. Distributions $P(n,\gamma)$ for the model with three sites (solid lines) and the corresponding distributions of symmetric Fab–Fab association model from Fig. S3 (dashed lines). Figure shows the case of high viscosity. Probability for monomers and dimers are lower on the expense of higher clusters.