SHEAR THINNING IN MONOCLONAL ANTIBODIES

by

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Abstract

Antibodies are large Y-shaped proteins which are used by immune system to identify and neutralize pathogens. Monoclonal antibody therapy is used to treat different patient conditions. There are problems associated with the manufacturability and deliverability of mAb solutions due to the viscous nature of the protein. The viscosity of antibody solutions increases with the increase in concentration and decreases with applied shear. We want to know why these behaviours are seen and to address this problem we have developed a theory describing the rapid viscosity increase with increasing concentration. We use the polymer theory to explain this behaviour. Here antibodies are treated as polymers. The length of the polymer depend on the aggregation. The reptation time increases approximately as the cubic power of size of aggregate (N^3). We see the shear thinning behaviour is dependent on the Ab-Ab binding energy and find the relationship between the size of the aggregate and the binding energy. We find aggregate size and morphology using several models for Ab-Ab interaction sites. We use the head to head binding (fAb-fAb binding) model to describe aggregation state in our viscosity theory. The size of the aggregate and hence the reptation time is captured by the binding energy. When the binding energy increases the zero shear viscosity increases and the reptation time decreases. Likewise when the binding energy decreases the zero shear viscosity decreases and the reptation time increases. We have yet to find the correct exponents for the shear thinning behaviour of different mAbs which would be our future work.

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Acknowledgments

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Chapter 1

Introduction

1.1 Structure of Antibody and role in immune system

The immune system protects the body against various germs which are present in the surrounding. It protects the body by blocking the entry of microorganisms to the body and if by any chance pathogens enter the body it attacks it by producing antibodies. Thus if antigens attacks the immune system, antibodies act against it and will block its function.

Antibodies are large Y-shaped proteins which are used by immune system to identify and neutralize pathogens like bacteria and virus[1]. Antibodies are also called Immunoglobin(IG)[1]. They are heavy globular proteins with weight 550kDa[1]. They are made of two structural units- two light chain and two heavy chain. There are five types of mammalian heavy chain- γ, δ, α, ν and ϵ with the classes IgG, IgD, IgA, IgM and IgE respectively[1]. Heavy chains have approximately 1000 amino acids. Light chains have an approximate length of 211 to 217 amino acids. Each light chain and heavy chain are made of two regions, the constant region and the variable region.[1]. The constant region is same in the antibodies of the same isotopes whereas the variable region is different for the antibodies formed from different B cells[1].

Each antibody has unique target known as antigen. The antigen is the foreign substance and it acts like a key for antibody in identifying the organism. The antibody contain three

domains arranged in Y shaped. The antibody binds to specific antigens. The strength of binding between antibody and antigen at the single binding site is known as the antibody's affinity for the antigen. The structure of an antibody is given below.

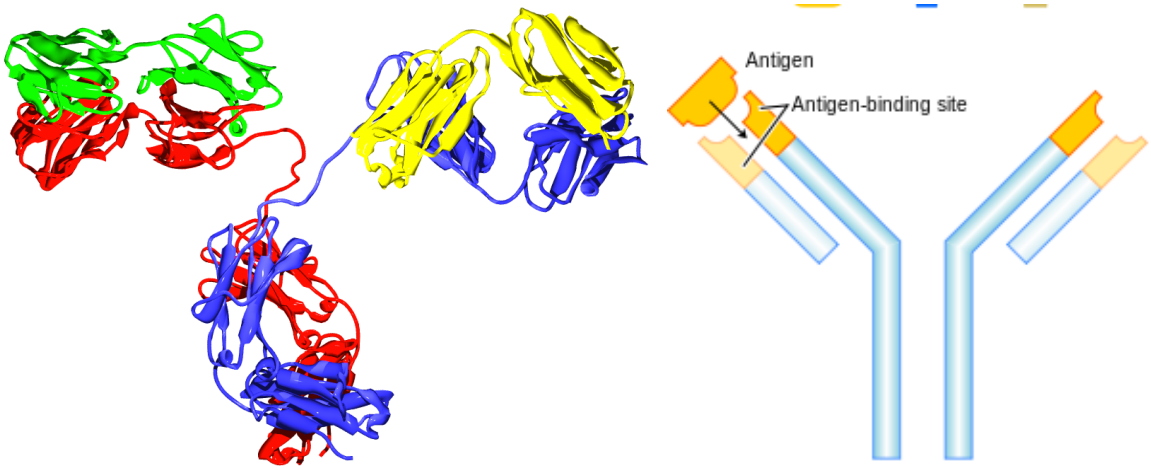

Figure 1.1: *structure of Antibody. The figure in the left shows the molecular structure.Red and blue are heavy chains whereas green and yellow are light chains of the antiboy. The figure in the right is the schematic diagram (picture source wikipedia) . The antigen binding domains are on the tip of the light chain and the heavy chain on the variable domain. The constant domain are present in both heavy chain and light chain*

1.2 Monoclonal Antibodies and its uses

Monoclonal antibodies (mAbs) are made by similar immune cells which are clones of a unique parent cell[1]. For any substance monoclonal antibodies can be produced that specifically bind to that substance which then is used to identify or purify that substance.

After the Nobel prize-winning breakthrough in physiology and medicine by Sir Bernard Katz in the 1970s, the use of antibodies in medicine formed the scientific basis for its mass production. By the mid 1980s, the FDA had approved the first therapeutic antibody, and by the late 1990s, scientists and doctors had developed the first therapeutic antibody for cancer (Genentech[26]). Today, biology has moved far beyond from a descriptive science to one that allows engineers and scientists to develop new types of therapeutic antibodies. One way certain therapeutic antibodies may work against cancer is by binding to proteins on the

surface of tumor cells. Another way is cancer medicines that combine the targeting ability of antibodies with chemotherapies designed to destroy cancer cells so the medicines are directly delivered to cancer cells which decreases the effects of chemotherapy on healthy cells.

Molecular engineering has contributed to the clinical success of antibodies in the recent years. With the help of antibody engineering, it has been possible to modify the molecular size, binding affinity, specificity and effector function of antibodies. Nowadays engineers are producing small antibody fragments. The large molecular size of IgG resulted in high circulation time and high background in medical imaging[22]. The motive to develop small antibody fragments was to make these molecules suitable for the radioimmunotherapy of cancer. Thus antibody engineering has helped for improving their clinical utility

Antibodies are also used to reduce the risk of organ transplantation. When any organ like lungs or kidneys are transplanted to the patient there is always risk of organ rejection. So when patients with lungs transplantation are given antibodies against T cells (cell that plays vital role for immunity) within two weeks of lung transplantation the probability of rejection decreases[23]. Antibodies also show promise as treatment of AIDS. The approach called passive immunization is infusing antibodies in the patient's blood. The studies are ongoing and experts believe it will not only decrease the virus in the body but would cure it[24]. There are many uses of mAbs. Some of them are summarized below.

1) They are used in diagnostic tests like pregnancy test kits(Biotech 1989). They are also used in diagonosis of AIDS by ELISA test (oris 1987)

2) They are used in detection of cancer for example radioimmunodetection and radioimmunotherapy of cancer. Nowadays many cancer drugs are based on technology of mAbs.[3]

3) They are used to alleviate the problems of organ rejection to those who have organ transplant[23]

4) They are used in treating viral diseases traditionally considered untreatable. There is evidence antibodies may lead to treatment of AIDS[24]

5) Researchers use mAbs to identify and to trace specific cells or molecules in an organism.

1.3 Problems Observed at High Concentration Biologic Formulation

While developing of biopharmaceuticals high protein concentration are commonly encountered and pursued for several reasons[6]. The reasons to develop high protein concentration are target requiring high dose and easy delivery approaches for convenience to patient[6]. Antibodies are large and large proteins require large mass for therapeutic dose. Developing moderate to high concentration formulations is challenging as there are many problems encountered like high viscosity, protein instability due to formation of soluble and insoluble aggregates during formulation process and storage. These hamper the manufacturability, deliverability, stability and self life of the product[6]. High viscosity also requires high injection force which is painful to the patient. Thus addressing this issue has been one of the main challenge pharmaceutical companies are struggling with[6]. Efficient dosing of therapeutic antibodies often requires concentrations in excess of 100 or even 200 mg/ml. However, many antibodies have a sharp rise in the viscosity that renders production and delivery prohibitive at these concentrations. Unfortunately, this problem is only apparent late in the development pipeline when it is not feasible to alter the sequence to mitigate the viscosity. A better approach would be to choose low viscosity target molecules early in the pipeline so that the problem can be avoided altogether. To achieve this goal it is necessary to understand how the minor sequence perturbations within the complementarity determining regions contribute to the many-body interactions responsible for the elevated viscosity. The sharp rise in antibody viscosity has several characteristics that are very different from solutions of rigid bodies. First, the onset of the nonlinear regime occurs at volume fractions on the order of 5–10% which is much less than the jamming transition for comparably shaped rigid bodies (e.g. 58% for spheres). Secondly, the viscosity of a given molecule correlates well with attractive intermolecular interactions[5]. While this is intuitively reasonable, the viscosity of a flocculated solution is primarily a function of the solute volume fraction. The volume fraction does not change upon aggregation, although entrained water cavities could

account for a factor of 2 or 3 increase. To explain these discrepancies, we recently proposed an alternative model in which transient interactions between antigen binding domains result in long, flexible antibody complexes[1]. These complexes entangle with each other giving the solution the viscosity characteristic of a semi-dilute polymer solution. Up to certain concentration the increase in viscosity is small but as soon as the concentration increases to certain value the mAbs show rapid increase in viscosity.

1.4 Introduction of Shear Thinning and Shear Thickening

There are two types of fluids newtonian and non-Newtonian. Those fluids in which the viscosity of fluids remains constant with the shear strain are called Newtonian fluids and those fluids in which the viscosity of fluids changing with the shear strain are called non-Newtonian fluids. In Newtonian fluids the relation between shear stress and shear rate is linear with constant of proportionality being the coefficient of viscosity. In non-Newtonian fluids the relation between shear stress and shear rate is different and we can't define the coefficient of viscosity. The relationship between shear stress and the viscosity is given by

$$\tau = \eta * \gamma \tag{1.1}$$

where τ is the shear stress, η is the viscosity and $\dot{\gamma}$ is the shear rate. The example of Newtonian fluids are water, alcohol, glycerine etc. In these fluids shear strain does not affect the viscosity. The example of Non-Newtonian fluids are ketchup, custard, starch, toothpaste etc. Non-Newtonian fluids can be categorized into two ways, those fluids whose viscosity increases with the shear rate are called shear thickening fluids and those fluids whose viscosity decreases with the shear rate are called shear thinning fluids. Example of shear thickening fluids are corn starch, custard etc. When you pour water in corn starch and shear it after sometime you will get a thick fluid and continuous shearing will eventually make it hard.

In shear thinning fluids the contiuous shearing will decrease the viscosity. Example of shear thinning fluids are blood, ketchup, paint etc. The ketchup is thick inside the bottle but when you shear it becomes thin and flows.

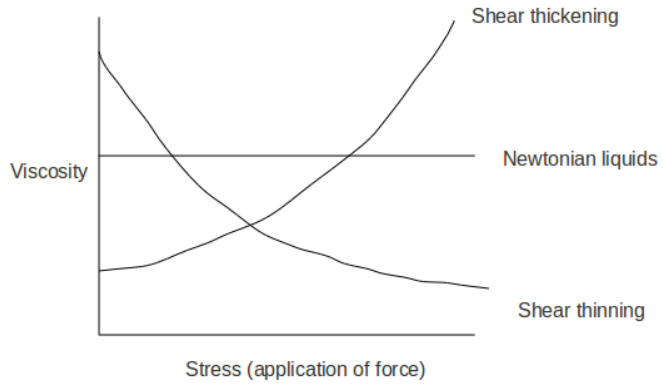

Figure 1.2: *Relationship between stress and viscosity. Shear thinning fluids show decrease in viscosity with stress while shear thickening fluid show increase in viscosity with stress*

1.5 Classification of Polymer and Polymer Model of Antibody Viscosity

Grassley[16] has classified polymer solutions into five different regimes(see figure 1.5) according to concentration and molecular weight. There are theories for three of these regimes-Zimm model for dilute solutions, Rouse model for concentrated but not entangled solutions and Doi Edwards model for concentrated entangled solutions. Other two regimes are mixed theories by applying Rouse model to semidilute unentangled solutions[17–19] and Doi-Edwards semidilute entangled solutions[9;17;18]. Broadly speaking the polymer solutions are classified into three different categories according to the extent of the interaction- dilute polymer solutions, semidilute polymer solutions and concentrated polymer solutions. Those solutions which have concentration less than the overlap concentration(c^*) are called dilute polymers, those with concentration near the overlap concentration are called semi dilute and those

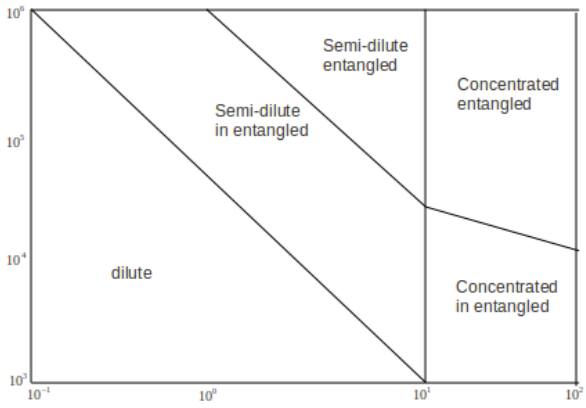

Figure 1.3: *classification of polymers in terms of concentration and molecular weight which shows different regimes and each regimes can be explained by different theories*[16]

with greater concentration than the overlap concentration are called concentrated polymer solutions[11].(see figure 1.6)

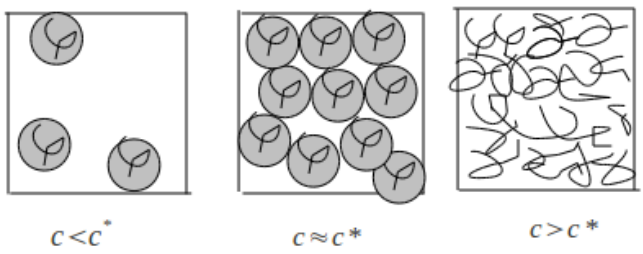

Figure 1.4: *classification of polymer solutions according to the extent of interaction of polymer chains (de Gennes*[11]*)*

The dynamics of the polymer solutions is limited by entanglements. In dilute solutions there is no entanglement. In concentrated solutions molecules are entangled hindering the flow. In semidilute solutions the entanglement is just started. There are different polymer theories which have been explained briefly below-

1.5.1 Rouse Model

The Rouse Model describes the dynamics of the ideal chain. It is applicable to short chains which consists of gaussian worm-like chain. It excludes the hydrodynamic interaction. A long chain is divided into short chains which obey the gaussian statistics with end to end distance a_R. Then these chains are attached with imaginary springs and the restoring force f as a that of a whole chain is given by

$$f = \frac{3k_BT}{< \delta r^2 >}\delta r \tag{1.2}$$

where δr is the mean square displacement.

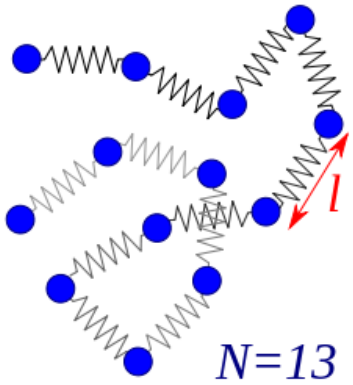

Figure 1.5: *Schematic view of Rouse model. The beads(N=13) are connected by springs(picture source wikipedia)*

The finite chain can be divided into different subsections with indistinguishable units to get the different Rouse modes. The motion of the chain is superpostion of independent intra-chain or Rouse Modes which can be found by the Fourier transformation (M.Doi[9]). I have not calculated the Rouse modes and the diffusion coefficient but included the results[9]. The diffusion coefficient is given by

$$D = \frac{k_BT}{N\zeta} \tag{1.3}$$

and the Rouse relaxation time for the zeroth mode is given by[9]

$$\tau_r = \frac{\zeta N^2 b^2}{3\Pi^2 k_BT} \tag{1.4}$$

where ζ is the friction coefficient on the chain due to the surrounding medium. From this relation we find $D \sim N^{-1}$ and $\tau_r \sim N^2$. Since N is proportional to the molecular mass, we have $D \sim M^{-1}$ and $\tau_r \sim M^2$. These are not consistent with the experimental results. Experiments in dilute conditions gives $D \sim M^{-1/2}$ and $\tau_r \sim M^{3/2}$. This failure is because we excluded hydrodynamic interaction. Thus Rouse model is regarded inappropriate in dilute solutions.

1.5.2 Zimm Model

Zimm model is applicable to polymers in dilute solutions. Zimm model takes account into hydrodynamic drag force on the polymer due to solvent. Force acting on the polymer creates flow field with net components in the same direction as the primary force. We can use Stokes'

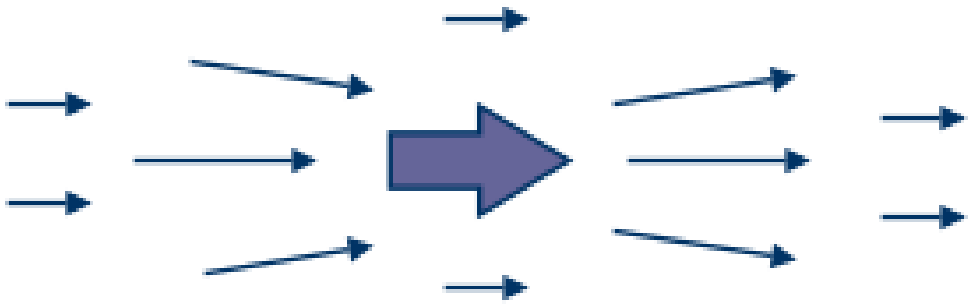

Figure 1.6: *Hydrodyanamic force in zimm model. Force acting on polymer coil creates flow field with net component in the same direction as the primary force. Here the bigger arrow is the primary force and other arrows are the flow in the direction of the primary force. The figure is only to show the hydronamic drag*

law which gives

$$\zeta = 6\Pi R\eta \tag{1.5}$$

where ζ is the friction coefficient. Consider polymer coil as group of particles or beads. Group of particles move more easily than the isolated particles. In Zimm model group of the whole coil moves as one including the solvent molecules. The effective hydrodyanamic

radius of the coil is given by

$$R_h = \zeta_p / 6\Pi\eta \tag{1.6}$$

where ζ_p is the friction of coefficient of the polymer which is given by

$$\zeta_p = 4\Pi\eta Rg \tag{1.7}$$

Einstein's theory gives diffusion coefficient to be

$$D = k_B T / \zeta_p \Rightarrow D \approx N^{-0.6} \tag{1.8}$$

These results are consistent with the experimental results in dilute polymer solutions.

1.5.3 Reptation Model

Reptation is the thermal motion of very long linear entanglement macromolecules in polymer melts or concentrated polymer solutions.[7]. Reptation is used as a mechanism to explain viscous flow in an amorphous solution.[8]. Reptation suggests the movement of the entanglement polymer chain as that of snakes slithering through one another.[8;14]. Long chain polymers contain entanglements which constrain the movement and thus their response to mechanical deformation. The chain only can move in the longitudinal direction and its transverse motion is restricted. The entanglements can be thought of long bars which restrict the chain in two dimensions and the diffusion is only along its length. This wriggling motion called reptation governs the time taken by the polymer chains to respond to any applied stress which leads non-linear response. This non-linear behaviour can be explained by the scaling arguments(see calculations below) based on time taken for the chain to escape the tube confining entanglements.

The mobility μ_{tube} of the tube is given by

$$\mu_{tube} = \mu_1 * /N \tag{1.9}$$

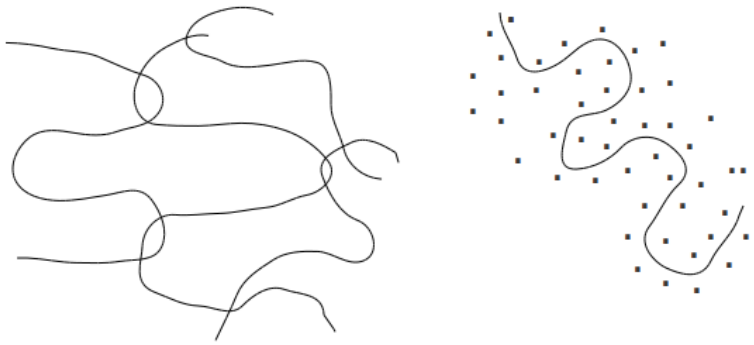

Figure 1.7: *The figure in the left shows entanglement. The figure in the right shows transverse motion is restricted and motion is only along its length*

Figure 1.8: *motion of chain along the tube showing the initial conformation of the primitive chain and the tube which we call the original tube. Here due to entanglement we can assume the polymer chain moves along the virtual tube.*

where μ_1 is the mobility of the chain segment.

The tube diffusion coefficient can be calculated as

$$D_{tube} = \mu_1 T/N = D_1/N \tag{1.10}$$

The time taken by for a chain to diffuse over a length comparable to that of tube is

$$T_t = L^2/D_{tube} = NL^2/D_1 \sim N^3 \tag{1.11}$$

The prediction that terminal time and hence the shear viscosity scales like N^3 is in reasonable agreement with the experiment $N^{3.3}$. Reptation theory gives the relationship

between the molecular mass and the relaxation time.[9]. The relaxation time τ is given by $\tau \sim M^3$ where M is the molecular mass[9].

Here we treat the antibodies as highly flexible molecule. The goal is to determine if the elongated shape and flexibility has effect in the viscosity. The antibody viscosites are found consistent with the reptation dynamics as would be expected in the semidilute solution of polymers[10].

Chapter 2

Different binding Models of antibody

2.1 Introduction

Numerous experimental studies have established that the steep rise in the viscosity of antibody solutions is caused by the formation of supramolecular complexes of antibodies[5]. These complexes increase the viscosity either through polymer-like entanglements or by the formation of an extended network[10]. Regardless of the viscosity mechanism, it seems clear that the first step toward predicting antibody viscosity is to understand the size and shape of the complexes. Antibodies are Y-shaped molecules so there are different ways in which the molecules can interact. In $f_{ab} - f_{ab}$ binding the head of one molecule binds with the head of another molecule which we call here as $f_{ab} - f_{ab}$ or head-head binding. Similarly head of one molecule can bind with the tail of another molecule which we call $f_{ab} - F_c$ interaction or head-tail binding. Similarly tail of one molecule can bind with tail of another molecule which we call $F_c - F_c$ binding or tail-tail binding. The different binding models are described below.

2.2 Binding models

Here we derive the equilibrium distribution of antibody complexes. The input parameters for our calculation are two-body binding constants that can be directly measured by techniques like SPR. We consider two binding constants in the model corresponding to F_{ab}-F_{ab} interactions and F_{ab}-F_c interactions. In the following we consider each of these interactions separately before presenting a theory that accounts for both interactions simultaneously.

2.2.1 Head to head binding

First we consider the association between two F_{ab} domains, which we refer to as "head to head" (HH) binding. As discussed previously, HH binding results in the formation of linear chains of antibodies [Schmit et al. 2014]. The two simplest HH complexes are shown schematically in Fig. 2.1. In these cartoons model the head of one polymer antibody attaches with the head of another and so on.

Figure 2.1: *The head to head binding model describes antibodies that aggregate by forming reversible contacts between F_{ab} fragments. The two simplest structures are the dimer (left) and trimer (right). Further aggregation results in the formation of long chained antibodies.*

The equilibrium constant for HH association is defined by

$$k = \frac{c_2^{HH}}{c_1^2} \tag{2.1}$$

where c_2^{HH} is the concentration of dimers formed by HH association. Therefore, the con-

centration of dimers is kc_1^2, the concentration of trimers is $k^2c_1^3$, etc. Summing over all HH complexes, the grand partition function is

$$q_{HH} = c_1 + c_1^2 k + c_1^3 k^2 + \ldots \tag{2.2}$$

where the monomer concentration plays the role of the fugacity $c_1 = e^{\mu/k_B T}$. The partition function can be written as the recursion relation

$$q_{HH} = c_1(1 + k q_{HH}) \tag{2.3}$$

The two terms on the right hand side of Eq. 2.3 correspond to the two possible states for an antibody 'head'. First, the head can be unbound, which terminates the complex and results in the factor of 1. Secondly, the head can be bound to another molecule which, in turn, can be bound to another, and so on. In the grand canonical formalism, the number of molecules in this chain can range from one to infinity. Since this is the same range of sizes considered in the partition function, the sum of possible outcomes can be replaced by the factor q_{HH} on the right hand side of Eq. 2.3. Rearranging Eq. 2.3, we arrive at a simple expression for the partition function

$$q_{HH} = \frac{c_1}{1 - kc_1} \tag{2.4}$$

2.2.2 Head to tail binding

The second model of binding we consider is the association of a F_{ab} domain with a F_c domain. We refer to this as "head to tail" (HT) binding. There are two possible dimers resulting from HT binding which are shown in Fig. 2.3 and 2.4.

We define s as the equilibrium constant for HT binding. The partition function for complexes formed entirely by HT binding can be written as the following recursion relation

$$q_{HT} = c_1(1 + 2s q_{HT} + s^2 q_{HT}^2) \tag{2.5}$$

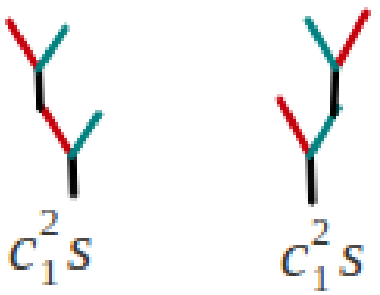

Figure 2.2: *head tail binding model describes antibodies that aggregate by forming contacts between $F_{ab} - F_c$ fragments. In the above figure head of of antibody attaches with tail of the other to form dimer*

The three terms correspond to the possible head binding states of a single molecule. The first term is the state where both heads are unbound, the second term is when only one of the heads binds to another molecule, and the third term corresponds to the state where both heads binding to other molecules. Since each bound molecules can initiate a cluster of any number of molecules, this introduces factors of q_{HT}. The recursion relation can be solved for q_{HT}

$$q_{HT} = \frac{1 - 2sc_1 - \sqrt{1 - 4sc_1}}{2s^2 c_1} \tag{2.6}$$

which has the Taylor expansion

$$q_{HT} = c_1 + 2c_1^2 s + 5c_1^3 s^2 + \dots \tag{2.7}$$

This predicts 5 different trimer states which can be diagrammed as follows

2.2.3 Tail to tail binding

A third mode of binding, association between F_c domains, is also possible. While "tail to tail" (TT) binding may be responsible for the formation of larger structures that are important for the viscosity, we omit it from the current model. TT binding will be included in future versions of the theory.

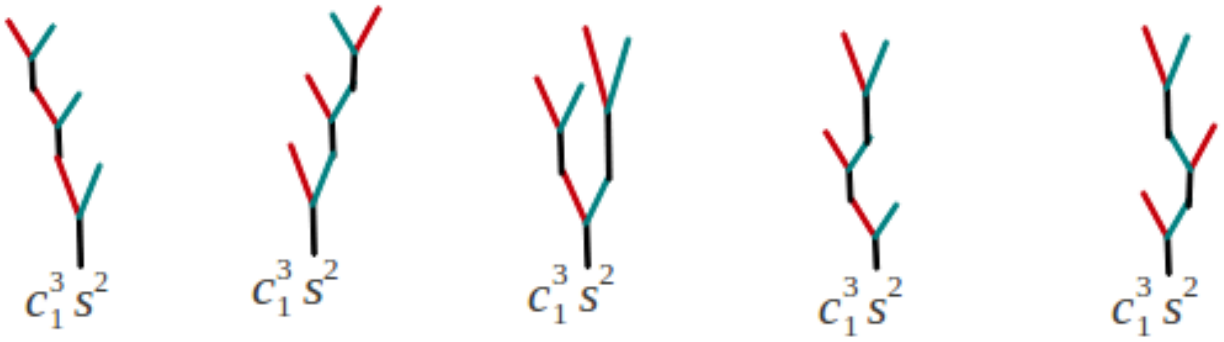

Figure 2.3: *Head tail binding for trimer is shown in the figure in which head of one antibody attach with tail of another and so on. The structure shown is trimer. Further aggregation in the similar way forms long chain of antibodies*

2.2.4 Mixed binding

We now compute the distribution of complexes formed by molecules that can bind by both HH and HT interactions. We expect that this model will capture most of the variability between different antibody sequences and will be quantitatively accurate under conditions where TT binding is weak.

The partition function for the mixed HH/HT model is given by the recursion relation

$$q = c_1(1 + sq + kq)(1 + sq) \tag{2.8}$$

To derive this formula, we assume the existence of a molecule in each cluster that has an unbound head. The partition function is a summation over the possible states of molecules bound to this starting molecule. Specifically, we need to account for the binding states of the remaining head and tail. The first term in Eq. 2.9 represents the states for the remaining head which can be unbound, bound to the tail of another molecule, or bound to another head. The second term represents the states of the tail, which can be unbound or bound to a head. Since each binding event ties up one head on the following molecule, the summation over molecules bound to each site is equivalent to a factor of q. Eq. 2.9 is a quadratic

equation for q which can be solved to give

$$q = \frac{1 - c_1 k - 2c_1 s - \sqrt{1 - 2c_1 k + c_1^2 k^2 - 4c_1 s}}{2(c_1 k s + c_1 s^2)} \tag{2.9}$$

This has the Taylor expansion

$$q = c_1 + 2c_1^2 s + c_1^2 k + c_1^3 k^2 + 5c_1^3 s^2 + 5c_1^3 sk + \ldots \tag{2.10}$$

where the enumerated states are schematically represented in figure **??**

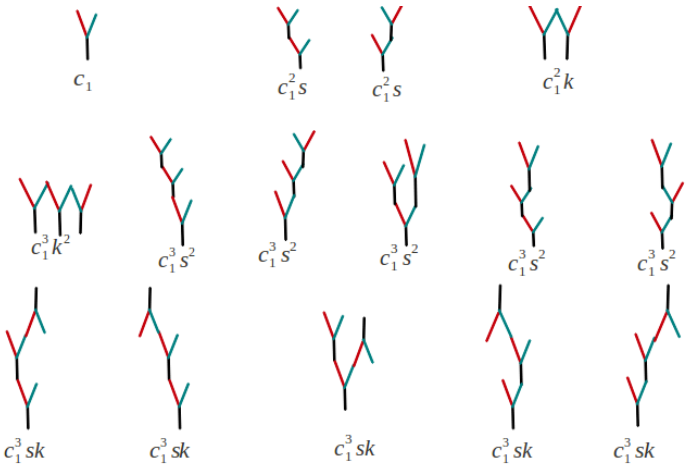

Figure 2.4: *HT and HH model. They either bind head head, they can bind head tail and they can bind either head or tail in the same molecule as shown in figure.*

In these structures we have shown all types of binding. For dimers there can be either head head binding or head tail binding. While we go to higher structure like trimers and above we see either head of one antibody binds with tail of other or head of one antibody with head of other and so on (see Fig 2.4).

The average size of the structures is given by

$$< n >= c_1 \frac{dln(q)}{dc_1} \tag{2.11}$$

where c_1 can be found from the total concentration $c_{tot} = c_1 dq/dc_1$. Once we find the average size we can find the viscosity which is described in chapter 3. To calculate the distribution

of complexes, we first express the equilibrium constants in terms of the antibody mass m and the free energy of the binding interactions

$$k = \frac{1}{m} e^{v_1} \tag{2.12}$$

$$s = \frac{1}{m} e^{v_2} \tag{2.13}$$

where v_1 and v_2 are the free energies for HH and HT binding, respectively, in units of $k_B T$.

Fig. 2.6 plots the average size of the antibody complexes as a function of the binding interactions at a constant antibody concentration of 150 mg/ml. Very little aggregation occurs for free energies of 0 or 3 $k_B T$, but significant association occurs when the energy reaches 6 $k_B T$. The effect is particularly dramatic when HT binding is strong because this allows the antibodies to form highly branched structures with high entropy (see graphs 2.5 and 2.6).

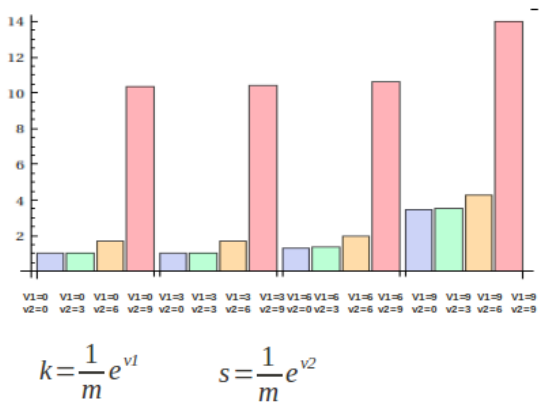

Figure 2.5: *average size as function of binding energy. The size is large particularly when HT binding is strong.*

The following plots show the concentrations of monomers, dimers, and trimers as a function of binding energies. At high binding energies all three species show a drop in concentration indicating that most of the protein in the system is tied up in larger aggregates.

The different binding models of the antibody is explained above. In the following chapter we consider only the head-head binding of the antibody and leave other types of binding for

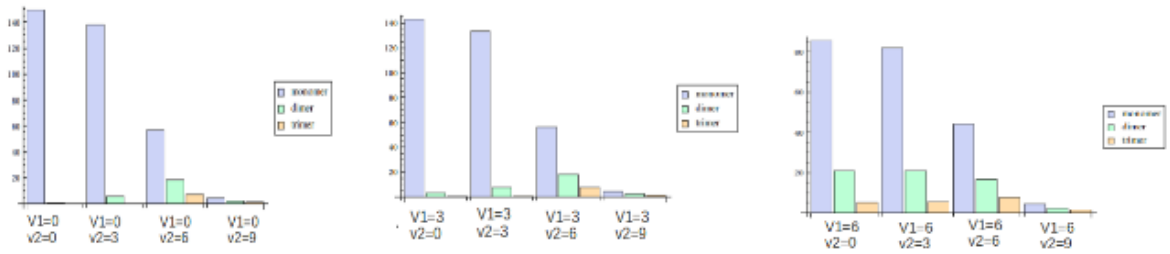

Figure 2.6: *concentration of monomers, dimers and trimers as function of binding energies. At high binding energy all the species show a drop in concentration indicating most of the protein in the system is tied up to larger aggregate*

future work.

Chapter 3

Shear thinning in Monoclonal Antibodies

3.1 Introduction

Due to the growth of monoclonal antibody therapy the viscosity behaviour of mAbs are one of the great interest and topic of research[12]. While injecting the protein drugs to the patients, force is needed to push the antibody drug through the needle. The antibodies act as shear thinning fluid which means upon shearing the viscosity of antibody decreases.

3.2 Model

We begin by summarizing previous theory for the zero shear viscosity[10]. According to the reptation theory polymer is confined to an effective tube due to constraints imposed by neighbouring molecules. As time passes, the polymer diffuses along the tube ("reptates"), so after some time the ends of the polymer are disengaged from the original tube while some part in the middle is still confined in the tube. We take antibody as polymer in which fAb region of one antibody binds with the fAb region of another.(see figure below).

After a strain is applied to the solution, the confining tube will be deformed. The

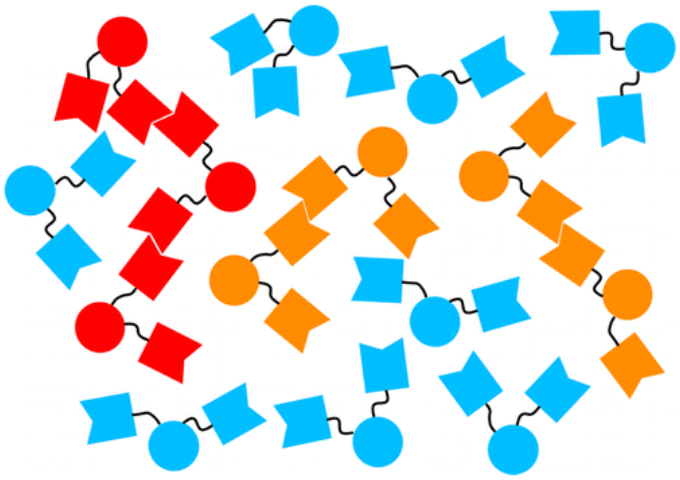

Figure 3.1: *Antibody forms long polymer structure in head head binding. Here fab region of antibody binds with fab region of another antibody to form long elongated structure. Reprinted with permission from Schmit, J.D.: He,Feng; Entanglement model of antibody viscosity. Copyright 2014. The Journal of Physical Chemistry*

subsequent reptation motion will establish a new equilibrium tube and only the polymer segments confined to the original deformed tube will contribute to the stress. So the stress after strain $d\gamma$ in time dt is given by

$$\begin{aligned} \sigma &\sim G(t)d\gamma && (3.1)\\ &= G(t)\frac{d\gamma}{dt}dt && (3.2)\\ &= G(t)\dot{\gamma}dt && (3.3) \end{aligned}$$

where G is the elastic modulus. Thus we can write the instantaneous viscosity as $\eta_{\text{instantaneous}} = \frac{\sigma}{\dot{\gamma}} = G(t)dt$. Only the segments of this deformed tube contribute to the stress so the relaxation modulus is proportional to the fraction $\psi(t)$ of the polymers still confined in the tube.

$$G(t) = G_N^0 * \psi(t) \tag{3.4}$$

Here G_N^0 is some constant and $\psi(t)$ is the fraction of the polymer still confined in the tube. As the system evolves after the application of strain, the molecules will release their entanglements by diffusion. Therefore, the stress relaxation will occur with a characteristic

timescale of the molecular diffusion. In an entangled solution this diffusion is described by the reptation time, $\tau_{\rm rep}$, so

$$\psi(t) = e^{-\frac{t}{\tau_{rep}}} \tag{3.5}$$

The zero shear viscosity of the antibody is given by the $\omega = 0$ mode of the fourier transform

$$\begin{aligned} \eta_0 &= \int_0^\infty \eta_{\rm instantaneous} dt && (3.6)\\ &= \int_0^\infty G(t) dt && (3.7)\\ &= G_N^0 \int_0^\infty dt \psi(t) && (3.8)\\ &= G_N^0 * \tau_{rep} && (3.9) \end{aligned}$$

The elastic modulus gives the energy stored in the network. This can be estimated by counting the number of intermolecular entanglements, each of which is expected to store an energy on the order of $k_B T$. A scaling calculation to find G_N^0 is described below.(M. Doi[9]) Consider a original chain consisting of bond length b. The statistical property of the Gaussian chain doesn't depend on the local structure of the chain so instead of the original Gaussian chain with bond length b and consisting of N segments, we consider new chain consisting of $N^{`} = N/\lambda$ segments with bond length $\sqrt{\lambda} b$(see figure below). The transformation to the new chain from the old one is the change is parameter $N \longrightarrow N/\lambda$, $b \longrightarrow b\lambda^\nu$, $c \longrightarrow c/\lambda$

The overlap concentration c^*[9] is given by

$$c^* \simeq N/(R_g^{(0)})^{3*} \sim \frac{N^{1-3\nu}}{b^3} \tag{3.10}$$

where $R_g^{(0)} \simeq N^\nu\ b$ is the radius of gyration. We use the dimensional analysis to find G_N^0. The relevant parameters in the problem are b,c,N,$k_B T$, so we write

$$G_N^0 = \frac{c}{N} k_B T F(cb^3, N) \tag{3.11}$$

where $F(cb^3, N)$ is the parameter to match the dimension. After using the above transfor-

mation we get

$$G_N^0 = \frac{c}{N} k_B T F(cb^3 \lambda^{3\nu-1}), N/\lambda) \tag{3.12}$$

Since G_N^0 is invarient under the above transformation we have

$$\frac{c}{N} k_B T F(cb^3, N) = \frac{c}{N} k_B T F(cb^3 \lambda^{3\nu-1}), N/\lambda) \tag{3.13}$$

This condition is satisfied only when

$$G_N^0 = \frac{c}{N} k_B T F(cb^3 N^{3\nu-1}) \Longrightarrow G_N^0 = \frac{c}{N} k_B T F(\frac{c}{c^*}) \tag{3.14}$$

Here by assuming power law, $F(\frac{c}{c^*}) = (\frac{c}{c^*})^x$ In semidilute regime it is independent of N. so we write

$$(3\nu - 1)x - 1 = 0 \Rightarrow x = \frac{1}{3\nu - 1} \tag{3.15}$$

This gives

$$G_N^0 \sim \frac{c}{N} k_B T \left(\frac{c}{c^*}\right)^{\frac{1}{3\nu-1}} \tag{3.16}$$

It is well known that the reptation time is proportional to N^3 which I have described in chapter 1. Here $\nu \simeq 3/5$ is the Flory exponent, b is the bond length, c is the total concentration. Plugging equation 3.15 and equation 3.16 into equation. 3.9 we get

$$\eta_0 \sim c^{\frac{3}{3\nu-1}} N^3 \tag{3.17}$$

Here N is proportional to the aggregate size which can be calculated assuming that the antibodies form linear aggregates by the association of the antigen binding domains[10]. The calculation of N is based on head-head or fAb-fAb binding of the antibody polymer. I have calculated the partition function of the head-head binding of the antibody polymer in chapter 2 which I will recall here.

$$q = \frac{c_1}{1 - kc_1} \tag{3.18}$$

Here c_1 is the monomer concentration. The chemical potential of the monomer state with zero free energy is given by[10]

$$\mu = k_B T ln c_1 \tag{3.19}$$

The total protein concentration is given by

$$c = c_1 \frac{dq}{dc_1} = \frac{c_1}{(1 - c_1 k)^2} \tag{3.20}$$

we can solve this equation to find the monomer concentration c_1.

$$c_1 = \frac{1}{2k^2 c}(1 + 2kc - \sqrt{1 + 4kc}) \tag{3.21}$$

The average assembly size is given by

$$< N >= \frac{c}{q} \tag{3.22}$$

Thus the final expression for assembly size is

$$N = \frac{2 * k * c}{\sqrt{1 + 4 * k * c} - 1} \tag{3.23}$$

where k is the equilibrium constant for antibody dimerization. Thus finally we have the zero shear viscosity as

$$\eta_0 = P * c^{15/4} * (\frac{2 * k * c}{\sqrt{1 + 4 * k * c} - 1})^3 \tag{3.24}$$

where $P = 5.8 * 10^{-8} cP(mg/ml)^{-3.75}$[10] is a fitting constant that can not be obtained from scaling theories. Eq. 3.24 predicts that the viscosity varies with $c^{15/4}$ at low concentration and $c^{21/4}$ at high concentration. In practice, the antibody complexes are too short at low concentration to obey scaling relations and the observed exponent is closer to 2.7. As expected, the agreement with the scaling theory improves at high concentrations as the effective size of the antibodies increases.[10]

3.3 Introduction Of Shear

The viscous response of a polymer solution in the presence of shear is the product of two relaxation mechanisms. First, the shear will tend to stretch out the polymers by deforming the "tube" of entanglements that constrain them (T. Milner[15]). This will cause the polymers to contract back to their equilibrium size along the tube.

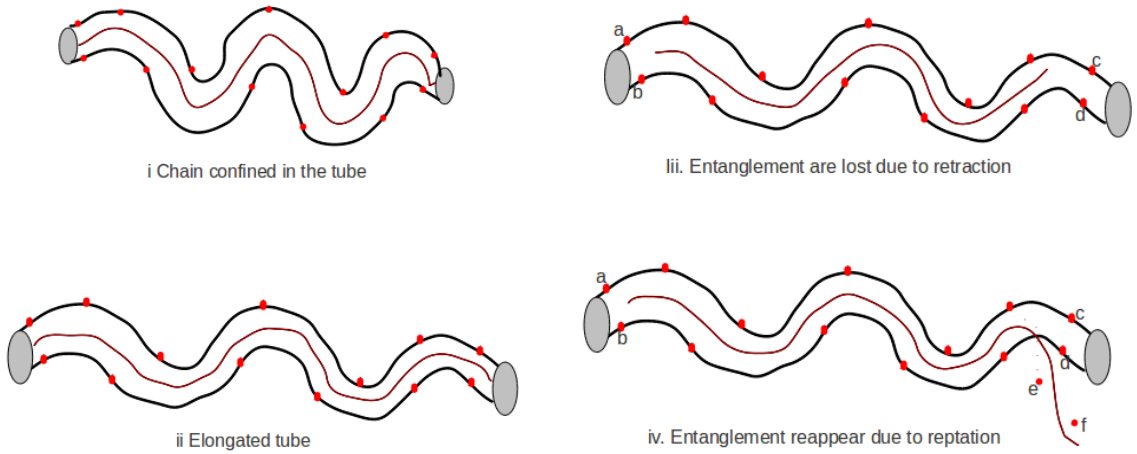

Figure 3.2: *chain retraction to equilibrium after strain. Here figure i shows the chain entangled inside the tube. Red circle denotes the entanglement points. After shear is applied the chain is elongated (figure ii) and as chain retracts the entanglements a,b,c,d are lost (figure iii). The entanglements e and f again come back as chain reptates (figure iv).*

This contraction releases entanglements and occurs on a timescale on the order of the Rouse time. Second, the polymers will undergo reptational diffusion to establish a new tube with the original number of entanglements. Therefore, these two processes oppose each other with the chain retraction releasing entanglements and reptation restoring them. In the scaling limit the Rouse time is proportional to R^2 so it can be treated as instantaneous compared to the reptation time which is proportional to R^3. Since our antibody complexes are too short for the scaling behavior to dominate, it is worth checking that this hierarchy is still appropriate. The Rouse time is given by

$$\tau_r = \frac{6\pi\eta a N^2 b^2}{3\pi^2 K_B T} \tag{3.25}$$

here, $L = Nb$ (b is the length of chain) and $L = 3Na$ where $a = 1.8$ nm is length of the arm of chain. After calculation we get $\tau_r = 2.6 * 10^{-7}$s for monomers, $\tau_r = 1.04 * 10^{-6}$s for dimers, and $\tau_r = 2.08 * 10^{-6}$s for trimers.

For the reptation time we estimate

$$\tau_{rep} \simeq \frac{L^2}{D} \tag{3.26}$$

where L is length of the polymer and D is the diffusion constant with units m^2/s. The diffusion constant is proportional to the number of segments in the polymer and the diffusion constant of a single segment. In our case, we identify a polymer segment with a single domain in the antibody. We can estimate the domain diffusion with the Stokes-Einstein equation

$$D_{domain} = \frac{k_B T}{6\pi\eta R} \tag{3.27}$$

here $\eta = 10^{-3} kg/(m.s)$, $2R = 5.5$nm is the diameter of an IgG domain. With these values we get $D_{domain} = 3.99 * 10^{-11} m^2/s$. Thus the longitudinal diffusion of polymer antibody is described by $D = D_{domain}/(3N)$. length L of polymer is $L = 3N \times 5.5$nm. With these substitutions we can rewrite the reptation time above as

$$\tau_{rep} = \frac{L^2}{D} = \frac{(3NR)^2}{D_{domain}/(3N)} \tag{3.28}$$

This takes the value $\tau_{rep} = 1.66 * 10^{-4}$ s for a dimer ($N = 2$),$\tau_{rep} = 5.61 * 10^{-4}$ s for a trimer and $\tau_{rep} = 1.33 * 10^{-3}$ s for a tetramer.

We conclude that it is still a good approximation to treat the Rouse time as much faster than the reptation time. The question, then, is how many entanglements does the chain retraction remove before the reptation relaxation restores them? To calculate this we need to know the amount the confining tube has elongated over the course of the reptation time. This is given by $d\gamma = \dot{\gamma}\tau_{rep}$, where $\dot{\gamma}$ is the shear rate. We write the elastic modulus as

$G(d\gamma) = G_0 h(d\gamma)$ where h is the damping function which describes the fraction of remaining entanglements. The damping function can be estimated as follows[15]. If n is tangent vector of the isotropic tube and is deformed by the deformation gradient E then the previous tangent vector is no longer a unit vector but it is $n' = E \cdot n$. Its length is $n'^2 = n \cdot E^T \cdot E \cdot n$. In shear the material is deformed which is characterized by the single parameter γ. All the stress components vanish except the diagonal components. So the deformation tensor under shear is

$$E = \begin{pmatrix} 1 & \gamma & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{pmatrix} \tag{3.29}$$

Thus the root mean squared tube length increases by factor of $(1+\gamma^2/3)^{\frac{1}{2}}$ immediately after strain. After retraction the length of the tube is reduced by a fraction $(1+\gamma^2/3)^{\frac{-1}{2}}$. Thus we can estimate $h(d\gamma)$ as $h(d\gamma) = (1+\gamma^2/3)^{-\frac{1}{2}}$.

The stress level after a deformation $d\gamma$ is given by

$$\sigma = G(d\gamma)d\gamma \tag{3.30}$$

$$= G_0 h(d\gamma)d\gamma \tag{3.31}$$

and the viscosity is

$$\eta = \frac{\sigma}{\dot{\gamma}} = G\tau_{rep} h(\dot{\gamma}\tau_{rep}) \tag{3.32}$$

which gives

$$\eta = \frac{\eta_0}{\sqrt{1 + \frac{\dot{\gamma}^2 \tau_{rep}^2}{3}}} \tag{3.33}$$

As with our original model[10], the risk with this analysis comes from the size of the antibody complexes which are too short for us to blindly trust polymer scaling calculations. An alternative model would be to treat the antibodies as rigid rods, which can still entangle and diffuse in a reptation-like manner, but cannot contract or expand. This model (multibead rod model) would give a damping fraction that scales like $\gamma^{-1/2}$[21] instead of the γ^{-1}

3.4 Model Behaviour

In our theory the differences between antibody molecules are contained in the equilibrium constant k. Increasing k corresponds to more attractive interactions which will result in larger antibody complexes. This has two effects on the shear dependent viscosity. First, larger complexes are able to form more entanglements, which will increase the zero shear viscosity predicted by Eq. 3.24. This can be seen by the height of the plateaus at low shear in Fig. 3.2. Secondly, the increased length will increase the reptation time of the polymers, which shifts the onset of shear thinning behavior to lower shear rates. This is because the slower diffusion prevents these complexes from re-establishing an equilibrium number of entanglements on the timescale of tube deformation. From Eq. 3.33 we can see that shear thinning will happen only when $\dot{\gamma} * \tau \geq 1$

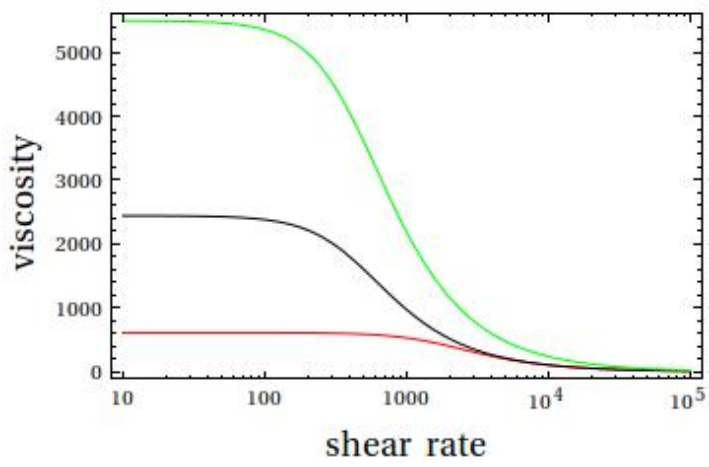

Figure 3.3: *Effect of antibody-antibody affinity on shear dependent viscosity. More attractive antibodies form larger complexes which have higher viscosity at low shear. However, these complexes also have a larger reptation time which shifts the shear thinning onset to lower shear rates. This is just the prediction curve with the k value's* $6.23 * 10^{-2}$,$4.7 * 10^{-2}$ *and* $3.02 * 10^{-2}$ *in units of ml/mg respectively*

3.5 Fitted with experiment

Now I try to fit equation 3.24 with the Genentech data[5]. The fitted plot for Mab1 at 150mg/ml for different NaCl concentration are shown in figure 3.3.

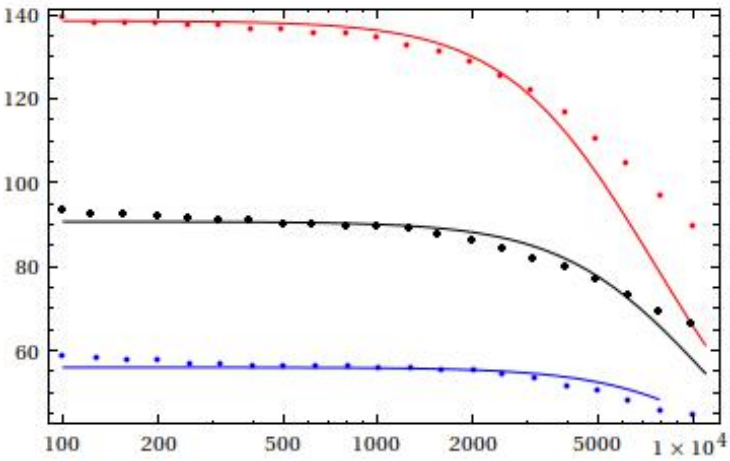

Figure 3.4: *Effect of omM, 25mM and 50mM NaCl from top to bottom on the viscosity of mAbs. The respective k values are* $2.79 * 10^{-2}$, $1.90 * 10^{-2}$ *and* $1.19 * 10^{-2}$

Fits capture correlation between plateau height and shear thinning onset. Mab1 is seen affected by the introduction of NaCl. I fitted extracted data with equation 3.24. We know Mabs have same IgG1 framework and they only differ in CDR sequence. So we can say the sequence changes vary the surface charge distribution which change the overall interaction behaviour. When $\gamma = 0$ equation 3.24 gives equation 3.15 i.e. the zero shear viscosity. We have experimental data for viscosity and concentration from Yadav's data[6]. When I fit these we get the curves which are fitted ok at high concentration. (see figure 3.4)

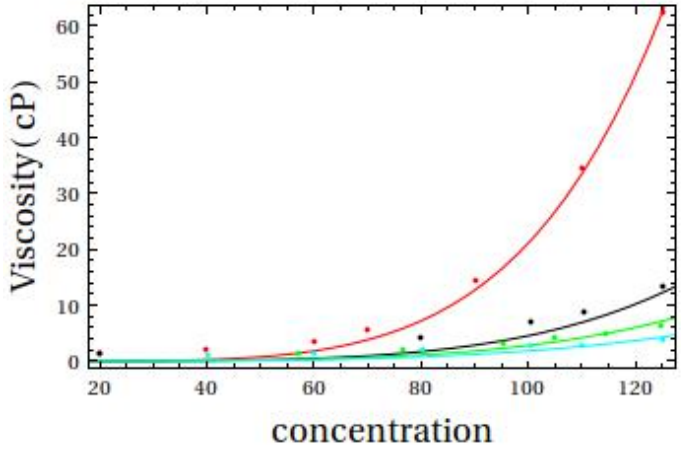

Figure 3.5: *Zero shear viscosity fitted with head to head model using yadav's data[5] for different mAbs. Fits are not good at low concentration but are ok at high concentration*

This curve in figure 3.4 is compared with data which I extracted from Yadav et al[5]. Fits are good at low concentration but not at high concentration.

3.6 Effect of temperature

We can also describe the effect of temperature on the viscosity of Mabs. The increase of temperature is going to decrease the binding energy which in turn decreases the reptation time. So as we increase the temperature the shear thinning occurs at higher shear rate (see fig. 3.5). At lower temperatures the binding energy is high that is why reptation time is high and shear thinning is seen at relatively low shear rates.

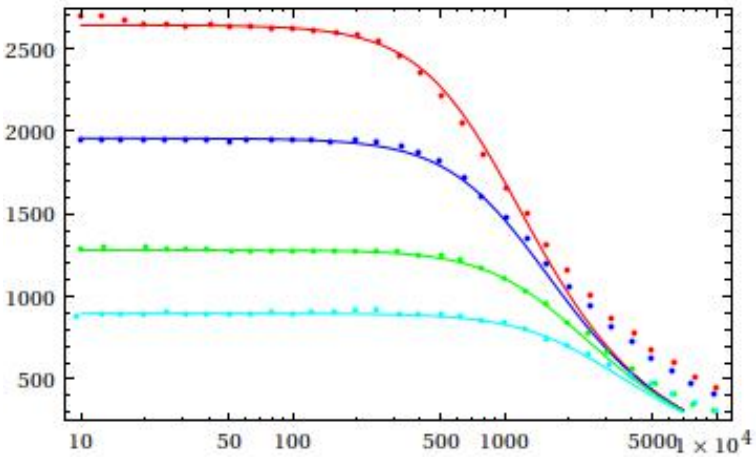

Figure 3.6: *Effect of temperature at concentration 200mg/ml (Top to bottom 5, 10, 15 and 20 degrees respectively) for mab1*[5] *and the values of k's are* $9.32 * 10^{-2}, 7.43 * 10^{-2}, 5.35 * 10^{-2}, 4.03 * 10^{-2}$ *in units of ml/mg respectively*

3.6.1 Explanation of fit in figure 3.5 and figure 3.6

At first I fit the equation

$$\eta = Pc^{15/4}(1 + \frac{2kc}{\sqrt{1+4kc}-1})^3(1 + \frac{\gamma^2 X^2(\frac{2kc}{\sqrt{1+4kc}-1})^6}{3})^{-1/2} \tag{3.34}$$

where $\tau_{rep} = Xn^3$ and $n = \frac{2kc}{\sqrt{1+4kc}-1}$ [1]. Here the reptation time I found was from sum of the square errors. I fitted four different viscosity vs shear data and the sum of square errors will give me constant X. The value of X found is $1.8 * 10^{-5}$.

Again I fit the equation 25 using the value of X and I find k's. These k's are used in curve 3.5 (see caption in curve 3.5 for values of k).

In the same way explained above I find k for figure 3.6. Now to account the temperature dependence of the binding affinity I fit the equation

$$k = m_w^{-1} e^{-(h-Ts)/k_b T} \tag{3.35}$$

where m_w is the molecular weight factor($m_w = 142KDa$). Then I find the fitted values of h and s. Here the motive is to reduce the parameters. Now I replot the curves with these fitted values of h and s.

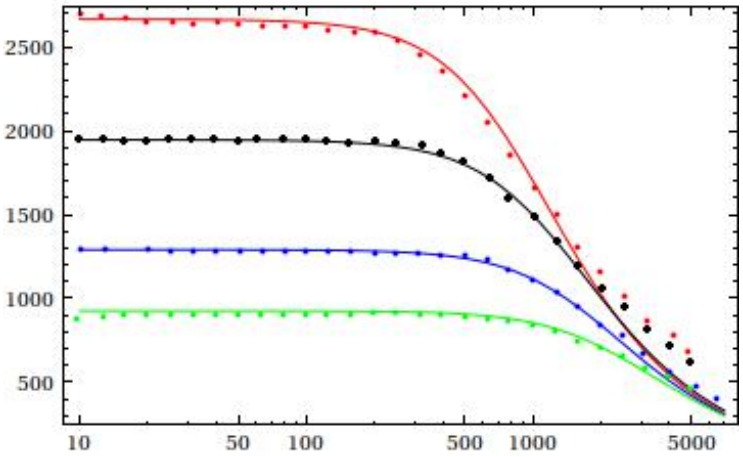

Figure 3.7: *Effect of temperature at concentration top to bottom 5, 10, 15 and 20 degree celsius respectively. The fitted values of h and s found from fitting were -37.03 KJ/Mole and -54 J/K/Mole respectively. The difference with figure 3.5 is in terms of parameters only. In figure 3.5 there are four k parameters while in figure 3.6 I reduced the parameters and fitted in terms of h and s (four k parameters are reduced to two parameters h and s)*

Now let's check the power law for rod model from the experimental data[21]. This gives the power law coefficient to be -0.5.

When I check the power law exponents for the mAb data for temperature dependence

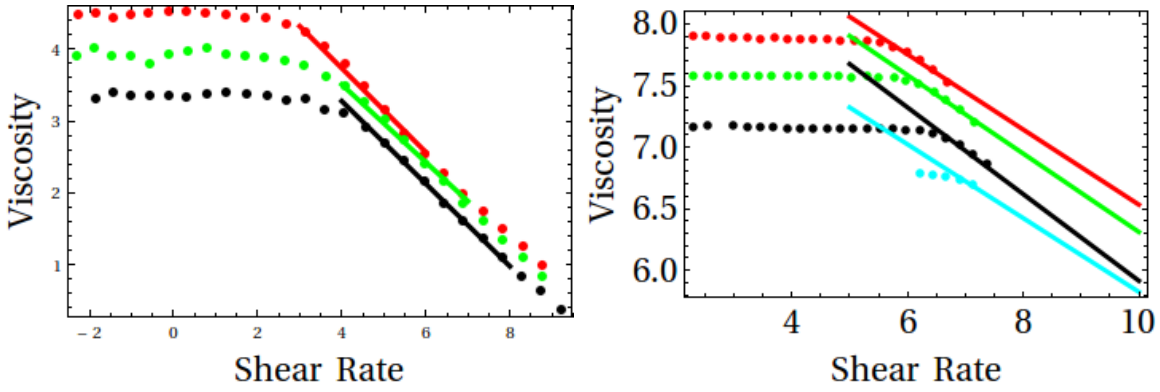

Figure 3.8: *fit for linear datas of rod model*[21] *and temperature dependent data. The exponents for rod model is -0.54 and that for temperature dependent data is -0.30*

and NaCl dependence I get the power law coefficient to be -0.30 and -0.20 respectively (see figures).

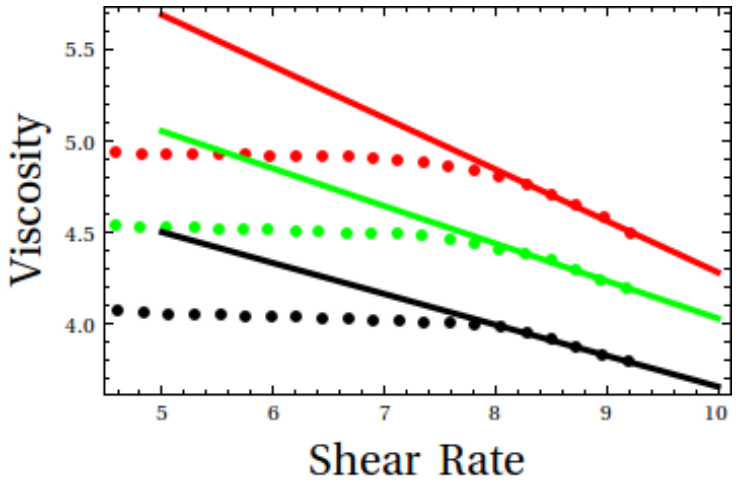

Figure 3.9: *Fit to get power law for NaCl dependence viscosity mAb. The exponent is -0.20*

3.7 Conclusion

The reptation time and the shear thinning is captured by binding energy k. When k increases the zero shear viscosity increases and the shear thinning occurs earlier. With the decrease in k, the value of the zero shear viscosity decreases and the shear thinning occurs at later time. We still need correct exponents to describe the theory. We have used the linear polymer model for the antibody which might have not given us the correct results. The failure of the prediction is because we considered antibody as flexible linear polymer. They might be short and branching might happen. In future we plan to work on the branching model of polymer and expect the correct exponents.

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