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Bachelor Thesis

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Modelling antibody orientation at bacterial surfaces

Vibha Kumra

Supervised by Pontus Nordenfelt[1] and Tobias Ambjörnsson[2]

[2]Department of Astronomy and Theoretical Physics

[1]Department of Immunology at Biomedical Centre

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Abstract

Bacteria and humans have co-evolved since their common existence. They have thereby developed very specific defence and target mechanisms against one another. Understanding the complex biomolecular processes behind these interactions can be of great use for developing treatments against bacterial diseases. The aim of this thesis project was to verify, and introduce a model for understanding, the implications of previous findings regarding the binding tendencies of antibodies to bacterial surface proteins. Antibodies typically bind to substances foreign to the host via regions referred to as Fab, exposing the Fc region that can then be recognized by the immune system. However, certain bacterial proteins express a so called IgGFc-binding region enabling additional binding via Fc. This can prevent these bacteria from being recognized and eliminated from the host organism. The goal of this model was to calculate the binding orientation probability of antibodies at bacterial surfaces. The binding probability was calculated for S types of antibodies characterized by their concentration c and binding constant K to a specific site i on the bacterial protein of length N. Transfer matrices, describing the probability of site i being in a certain state m, provided site $i+1$ is in state m', were generated. Each antibody was ascribed a binding constant from a gaussian distribution of standard deviation σ and mean value $\overline{K_D}$. Additional experiments were performed in order to confirm whether or not the previous experimental findings were reproducible. The model was found to best fit the experimental values for the parameters $\sigma = 45$ $(\mu g/ml)^{-1}$ and mean $\overline{K_D} = 140$ $(\mu g/ml)^{-1}$. The dissociation constant for the Fc binding region was determined to be 1500 $(\mu g/ml)^{-1}$ Both our experimental and theoretical results imply that bacterial proteins with a IgGFc-binding region effectively reduce the amount of Fab-binding. The theoretically obtained values were consistent with the measured experimental values. This model incorporated many important aspects of the antibody-bacterial protein binding system and was an improvement from the previous model. However, in order to draw any general conclusions about the model it is necessary to perform additional experiments with a higher level of precision.

Populärvetenskaplig sammanfattning

Varför gör vissa bakterier oss sjuka men inte andra? Hur kommer det sig att vissa bakterier lyckas överleva och infektera särskilda delar av kroppen men väldigt sällan andra? Baketerier och människor har evolverat länge tillsammans och har därmed lyckats utveckla specifika angrepps och försvarsmekanismer mot varandra.

Det är känt sedan tidigare att några bakterier uttrycker proteiner med förmågan att binda humana proteiner på ett sätt som gör det möjligt för dessa bakterier att undgå vårt immunförsvar. Dessa bakterier överlever vanligtvis i hals och på hud men kan ge upphov till allvarliga invasiva infektioner.

Detta arbetet ämnar undersöka interaktionen mellan bakterier med den ovannämnda förmågan och människor. Inbinding till bakterie mättes i olika halter av humant protein, så kallade antikroppar. En teoretisk model för att räkna ut sannolikheten för typ av inbinding att ske har utvecklats. Interaktionen involverar många olika typer av antikroppar som har olika benägenhet att binda in till olika platser på det bakteriella proteinet. Dessutom kan alla antikroppar "vändas om" och binda starkt till en plats på det bakteriella proteinet som på så sätt inaktiverar antikropparnas försvar. Proteinerna vinner på att hamna i ett lägre energitillstånd. De frigör energi vid inbindning och benägenhet att binda in är beroende av hur mycket energi kan frigöras.

Resultaten visade att den normala antikroppsbindingen minskas signifikant i närvaro av bakteriernas proteindel med förmågan att undgå immunförsvaret. Modellen visar sig stämma väl överens med mätningarna. Mätningarna kunde dock variera lite för mycket och det behövs flera experimentella resultat för att kunna dra någon slutsats om hur väl modellen beskriver den verkliga interaktionen.

Contents

1 Introduction

The human immune system comprises many different biological processes involving multiple organs and a large variety of specialised cells. A functional immune system includes detection and elimination of foreign substances in the body such as bacteria, viruses and parasites. Microorganisms with the ability to cause damage to the host organism are known as pathogens. The immune system can be divided into the innate immune system and the adaptive immune system. The innate immune system consists of various in-born, non specific host defence mechanisms while the adaptive immune system has the ability to develop specialized cells and proteins to better target pathogens. [1]

One common protein involved in adaptive immunity is an antibody, also referred to as an immunoglobulin. Antibodies are approximately 150 kiloDalton globular Y-shaped proteins. Their main purpose is to recognize and bind to protein structures on foreign microorganisms known as antigens [2]. They therefore have variable regions that are different depending on which antigen they are designed to recognize. Immunoglobulins bind to antigen with a certain binding energy, i.e. affinity, via one of the two so called Fragment antigen binding (Fab) regions. The remaining part, referred to as the Fragment crystallizable (Fc) region, is then exposed on the bacterial surface. The Fc part can be identified by so called phagocytic immunocells via their Fc-receptors and the pathogen can thus be engulfed and destroyed by this cell. Humans have five different types of antibodies and immunoglobulin G (IgG) is the most abundant and the primary antibody involved in clearance of pathogens via phagocytosis. [1]

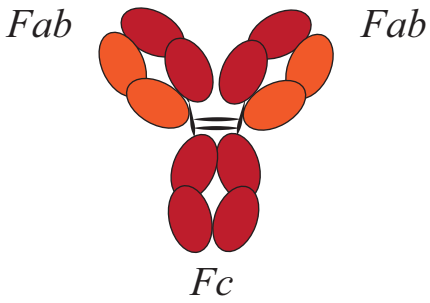

Figure 1: A schematic image of an antibody showing the two Fab regions and the Fc region marked out. The Fc region is seperated from the Fab regions by the hinge region (in black). The heavy chain is shown in red and the light chain in orange.

Several significant pathogenic bacteria exhibit surface proteins expressing anti-phagocytic IgGFc-binding regions [3] [4] [5] [6]. By evolving surface proteins with a high affinity to the Fc-region on IgG antibodies, these bacteria prevent the antibodies from being recognized by phagocytic cells via their Fc-receptors. They are thus protected from elimination and can survive in the host organism. Common pathogenic IgGFc- binding surface proteins include protein

M1 and protein H from *Streptococcus pyogenes* [7]. Protein M1 and H are long, fairly rigid proteins and express one IgGFc-binding region.

As a consequence of the IgGFc-binding regions, there is an ongoing competition between binding to bacteria via Fab and binding via Fc. A human sample consists of many different types of IgG antibodies. This type of antibody sample is said to be polyclonal. Since the Fab region varies between IgG antibodies, they have different affinities to different antigens. The location of an antigen on a bacterial protein is referred to as an epitope. The Fc region is constant and more or less the same between the subgroups of IgG relevant to a bacterial infection.[1] Because the system involves many unknown affinities and antigens, the outcome of this interaction becomes quite hard to predict.

The study in [8] shows that the orientation of polyclonal IgG at bacterial surfaces vary in samples from saliva and plasma, wherein IgG is more inclined to bind via Fc than Fab in saliva and vice versa in plasma (see figure 2). The IgG concentration in plasma is about four magnitudes higher than the IgG concentration in saliva. The results imply that the binding tendencies of antibodies to bacterial proteins depend on the concentration of antibodies present.

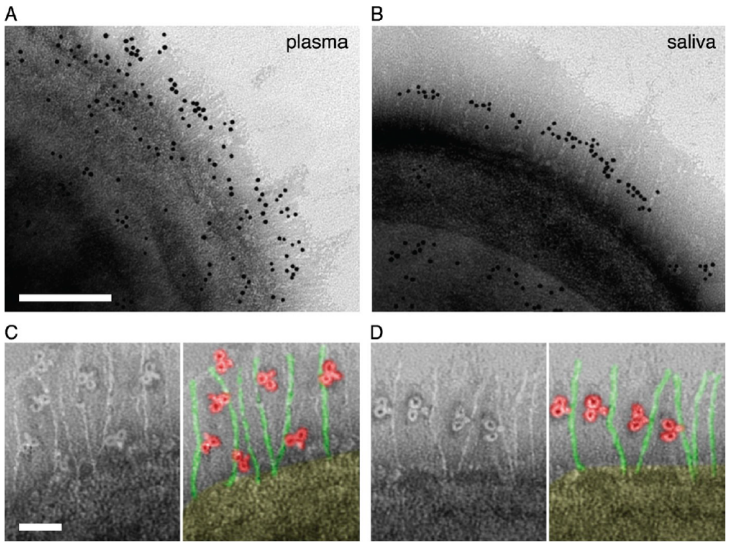

Figure 2: IgG molecules bound to *S. pyogenes* is plasma (A and C) and saliva (B and D) observed by negative staining electron microscopy. High magnification (C and D) reveals the orientation of bound IgG. The antibodies in saliva are well aligned at the IgGFc-binding regions, located in the middle on both protein M1 and H, whereas the antibodies in plasma bind to many different epitopes via Fab on the same surface proteins. Colouring in C and D is simply for illustrative purposes. All figures are taken from [8].

This project aims to evaluate whether or not it is possible to verify the implications of the previous experimental findings by creating a computational model as well as by trying to reproduce the experimental results.

IgGFc-binding bacteria such as *S. pyogenes* cause more than 700 million uncomplicated throat and skin infections annually and are at the root of uncommon but serious invasive infections. Understanding the mechanism behind the binding tendencies may thus help explain what causes this significant difference in infectious severity between saliva and plasma. This knowledge can in turn be valuable for treatment-oriented research. Knowing how pathogens interact with our cells and proteins will assist is understanding how this system can be manipulated with pharmaceuticals.

Furthermore, additional experimental results regarding the binding tendencies of polyclonal IgG to bacterial surface proteins are required for a more thorough analyses. The simulation will be evaluated by comparison to the experimental data. It is hence of importance to verify whether or not the previous experimental data is reproducible.

The specific aspect of this thesis, analysing the orientation of antibodies on bacterial surface proteins, may also assist in evaluating the pathogenic impact of the bacterial proteins. Do these bacterial proteins play an essential role in throat and skin infections?

2 Background

The project is, to begin with, approached by attempting to describe the physical aspects of protein binding and reviewing existing theoretical models for various types of molecular binding. Previous experimental results are thereafter presented and discussed.

2.1 Protein Binding

Protein-protein binding is in essence an interaction resulting in a stable complex with lower free energy than when the proteins are unbound. The proteins of interest in this project are large complex molecules. The binding is formed through cumulative attractive forces between the atoms in the proteins, mainly caused by various non-covalent interactions such as electrostatic attraction, hydrogen bonding and van der Waals. These protein interactions are primarily enthalpy-driven [9]. The complexity of large molecular energy systems requires these to be described statistically.

The binding force between a long, linear protein and a globular protein can be calculated using the binding statistical weight $Z(k, n)$, which is derived by calculating the Boltzmann weighted number of ways k to bind n number of antibodies and divide this by a so called reference statistical weight Z_{ref} [10]. Z_{ref} describes the possible states of the free antibodies in the absence of any bacterial proteins. Similar to in the study by Ambjörnsson et al. [10]

assume that the antibodies, in the absence of the linear protein, are confined to a compartment with the volume V and that each antibody occupies the volume v_0. Consequently there exist $N_{tot} = V/v_0$ number of voxels the antibodies can occupy. If there are N number of antibodies present the number of possible ways to arrange the antibodies, Z_{ref}, is

$$Z_{ref} = \binom{N_{tot}}{N} = \frac{N_{tot}!}{N!\,(N_{tot} - N)!} \tag{1}$$

Consider now the antibodies in the presence of the bacterial protein, neglecting the volume it takes up in the compartment to which the antibodies are confined. The bacterial protein is divided into k sites. Note that k is in our case a fixed number. When bound, an antibody manages to cover the integer value λ number of binding sites on the bacterial protein. ϵ indicates the total binding energy of an antibody bound to a bacterial protein. The maximum number of antibodies that can attach to the bacterial protein n_{max} is the largest integer value smaller than or equal to k/λ. The number of possibilities to arrange n antibodies on k binding sites is denoted $\Omega_{Bind}(k, n)$. The binding statistical weight $Z_{Bind}(k, n)$ is obtained by multiplication with the binding associated Boltzmann weight;

$$Z_{Bind}(n|k) = \binom{k}{n} e^{-B\epsilon n} = \Omega_{Bind}(k, n) e^{-B\epsilon n} \tag{2}$$

where $B = 1/K_B T$. K_B is the Boltzmann constant and T is the temperature. As antibodies bind to the bacterial protein, the number of free antibodies reduces to $N - n$. This is accounted for with the expression

$$Z_{cor} = \frac{N_{tot}!}{(N - n)!\,(N_{tot} - (N - n))!} \tag{3}$$

For large N a known identity [10] to solve equation 3 can be used, explicitly

$$\frac{(N + a)!}{(N + b)!} \approx N^{a-b}(1 + (a - b)(a + b + 1)/(2N) + ...) \tag{4}$$

As a result, for large number of antibodies, the volume fraction $\Phi^n \equiv N/(N_{tot} - N)$ is introduced such that

$$Z_{cor} = \Phi^n Z_{ref} \tag{5}$$

Ultimately, by combining equation 2 and equation 3 the statistical weight can be given as

$$Z(n|k) = Z_{cor} Z_{Bind}(k, n) = \Omega_{Bind}(k, n) \kappa^n Z_{ref} \tag{6}$$

where the effective binding strength κ has been introduced. Explicitly,

$$\kappa = \Phi e^{B|\epsilon|} \tag{7}$$

In the case of dilute solutions, κ can be expressed in the form

$$\kappa = c K_{eq} \tag{8}$$

where c is the available concentration of antibodies N/V and

$$K_{eq} = v_0 e^{B|\epsilon|} \tag{9}$$

is the equilibrium binding constant. Equation 6 together with 8 expresses the statistical weight for binding. The expectation value of n is

$$\langle n \rangle = \sum_n nP(n|k) \tag{10}$$

where $P(n|k)$ is the probability to find n bound ligands on the linear protein. $P(n,k)$ is given as

$$P(n|k) = \frac{Z(n|k)}{\sum_n Z(n|k)} \tag{11}$$

where $Z(n|k)$ is given in equation (5).

2.2 Binding curves

The expression for an ideal binding curve is derived and discussed in the following section. The expectation value (see equation 10) can be rewritten with equation 11 as

$$\langle n \rangle = \sum_n^k nP(n|k) = \frac{\sum_{n=0}^k nZ(n|k)}{\sum_{n=0}^k Z(n|k)} \tag{12}$$

Using equation 6 to express $Z(n|k)$ we have

$$\langle n \rangle = \frac{\sum_{n=0}^k n\binom{n}{k}\kappa^n}{\sum_{n=0}^k \binom{n}{k}\kappa^n} = \frac{\kappa\frac{\partial}{\partial\kappa}(1+\kappa)^k}{(1+\kappa)^k} = \frac{\kappa k(1+\kappa)^{k-1}}{(1+\kappa)^k} = \frac{k\kappa}{1+\kappa} \tag{13}$$

For a dilute sample (see equation 8) containing the ligand concentration c the binding probability for a particular site i is the expectation value divided by number of sites k. This gives

$$p(c) = \frac{cK_{eq}}{1+cK_{eq}} \tag{14}$$

This is the expression for an ideal binding curve. Plotted with a logarithmic x-axis, it yields a classical sigmoidal curve typical for an ideal binding [11]. However, if steric properties are taken into consideration, such as in the McGhee von Hippel model,[12] the binding curve has a slightly different appearance. The McGhee von Hippel model describes binding to a one dimensional lattice for one type of ligand, incorporating cases where $\lambda > 1$ and assuming equal binding constants for all sites. Additionally, the model assumes that the ligands are present in excess relative to the linear protein.

In order to describe binding between a polyclonal antibody solution and fairly linear and stiff bacterial surface proteins, it is necessary to consider site dependence, varying binding constants and competition among antibody types as well as between the regions of each antibody. Including such effects is the purpose of this thesis.

Binding of polyclonal IgG to *S. pyogenes* was measured for varying concentrations of antibodies in the study by Nordenfelt et al. [8]. In contrast to the sigmoidal shape typical for a binding curve a biphasic curve was obtained,

implying there were more than one sort of binding present in the sample (see figure 3). This was confirmed with additional experiments, as can be seen in figure 2. For mutant bacteria lacking protein M1 the binding curve did not differ much. Bacteria lacking protein H did however exhibit lower binding at low IgG concentration. For the mutant missing both surface proteins, the binding curve had a classical sigmoidal shape. The less the bacterial surface protein the more the binding at high concentrations. This is possibly because of exposure of epitopes the antibodies recognize. Due to the change in bacterial surface structure, it becomes hard to compare these binding curves to one another for any extensive quantitative analysis.

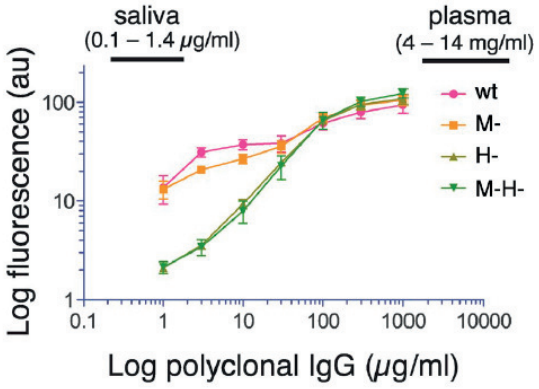

Figure 3: Fluorescence as a measure of bound polyclonal IgG given for varying IgG concentration. Binding of IgG was measured for *S. pyogenes* strain AP1 that contains IgGFc-binding protein M1 and protein H (wt), mutant lacking protein M1 (M-), mutant lacking protein H (H-) and mutant missing both proteins(M-H-). Image taken from [8].

A model has previously been created, but as it solely depends on classic ligand-receptor kinetics it does not incorporate other vital contributing factors such as the steric properties of the system. The main issue with this model is that it assumes all binding sites may can be filled simultaneously giving rise to results with a lot more binding than actually possible. The steric properties may be taken into consideration by deriving a method for describing the binding constants as location-dependent and introducing binding probabilities for each antibody.

3 Method and models

Experiments for measuring the concentration dependence of binding were performed. A model based on the transfer matrix method for competitive binding was implemented and simulated using Matlab.

3.1 Experiment

The experiments aim to measure the concentration dependence of binding between polyclonal IgG and the bacterial surface proteins of *S. pyogenes*. This is done by analysing bacteria samples with varying IgG concentration. The polyclonal IgG used in all experiments here is IVIgG; human IgG antibodies pooled from >3500 individuals. Moreover, all experiments are performed with the *S. pyogenes* strain AP1.

To begin with, a bacterial culture was prepared. An overnight culture of AP1 was grown. A sample from the overnight culture was extracted and grown to its exponential phase. The bacterial population is at this point highly reproductive and healthy. In order to preserve the surface structure of the AP1 bacteria in the exponential phase the bacteria is heat killed by incubation at 80 deg C for 5 mins. Since exclusively the binding tendencies of the surface proteins are of interest it is not required to keep the bacteria alive. See figure 2 in [8] for details. The heat killed AP1 sample is sonicated as means to disentangle them from one another and homogenise the sample. Two sets of experiments have been performed. The first one involves measuring the total binding of IgG to the bacterial protein whereas the other one aims to measure the orientation of the bound antibodies by cleavage at the hinge region (see figure 1)using enzyme protein IdeS [13].

3.1.1 Measuring total binding of IVIgG to *S. pyogenes*

Samples with varying IVIgG concentration and an equal amount of AP1 were prepared. Furthermore a control sample consisting of only AP1 was prepared. The samples were incubated at 37 degrees C under shaking conditions for 30 mins, allowing the mixtures to reach equilibrium. Prior to adding fluorescent protein (Dylight 647 anti-IgG) with high affinity and specificity to IgG antibodies, unbound antibodies were washed away from the samples, ensuring that the fluorescent protein was not saturated by binding to free antibodies. Washing away unbound IgG may disrupt the initial equilibrium state of the antibodies, primarily dissociating those bound with low to average affinity to the bacterial surface. In order to give an idea of the impact of the washing steps, the analysis was done on samples right after labelling, and then repeated after additional washing. The binding tendencies of the fluorescent protein to IVIgG is similar to that of IVIgG to AP1 and can thus be examined to evaluate the initial washing steps. Moreover the samples are stained with a fluorescent protein with high specificity and affinity to DNA, Syto9, marking the bacteria in the sample. Once the samples have been labelled with fluorescent protein, they are analysed in a flow cytometer.(see section 3.1.3) For additional steps and quantities, see appendix 1.

3.1.2 Measuring competitive and non-competitive binding of Fab and Fc regions to *S. pyogenes*

Samples with varying IVIgG concentration and an equal amount of AP1 were prepared. Additional samples with varying IVIgG concentration were set up. Prior to adding AP1 bacteria to the IVIgG samples, they were treated with the enzyme IdeS that cleaves human IgG at the hinge region (see figure 1),

separating the Fab regions from the Fc region. The binding measured from the latter sample set will be non-competitive between the Fab and the Fc regions. The first samples, containing antibody and bacteria, were treated with IdeS following the incubation allowing the mixtures to reach equilibrium; the binding measured from this sample set represents the competitive binding between the Fab and Fc regions. The samples were washed and divided into two aliquots wherein one was labelled with fluorescent protein with high specificity to the Fab region and the other labelled with a fluorescent protein with high specificity to the Fc region. In both cases the absorption peak wavelength is 647 nm. All samples were labelled with Syto9. In these experiments the Alexa 647-labelled anti-Fab and anti-Fc proteins were used. Once the samples had been labelled with fluorescent protein, they were analysed in a flow cytometer.(see section 3.1.3) See appendix 1 for additional details on the experiment.

3.1.3 Flow cytometry

Flow cytometry is a common method of analysis wherein the particles intended for analysis is injected in a fluid stream through a laser beam. By measuring how the incident beam is scattered and/or emitted in various angles, it is possible to resolve properties such as the particles relative size and the fluorescence intensity [14]. The flow cytometer used had four different laser channels irradiating light with wavelengths ranging from about 400 to 650 nm. The samples were gated by analysing the forward and side scattered light, ensuring only the fluorescence intensity from particles of more or less the same size were being evaluated. The fluorescence intensity was measured from channel 1 and channel 4, representing the emission from Syto9 and Dylight/Alexa 647 respectively.

3.2 Transfer matrix method

By treating different binding possibilities on the pathogenic protein as statistical weights, a formula for the probability of a type of binding to occur at a specific site could be derived. This is approached by attempting to implement the multi-ligand transfer matrix theory for competitive binding.

Protein M and protein H are long and fairly rigid proteins. We therefore assume that these can be modelled as one dimensional lattices. Protein M is about 500 aminoacids long and protein H about 400. An antigen is typically composed of at least 10-12 aminoacids. The affinity between any two proteins is commonly described with a dissociation constant, K_D, which is the ratio between the concentration of bound and unbound proteins in a system.

As the antibody-antigen affinities are unknown for a polyclonal IgG sample from human plasma, these are to begin with modelled as a normal distribution of binding strengths. We have S number of antibody types with varying binding strengths according to a normal distribution ρ such that

$$\rho(K_D) = \frac{1}{\sqrt{2\pi\sigma^2}} e^{-\frac{(K_D - \overline{K_D})^2}{2\sigma^2}} \tag{15}$$

where $\overline{K_D}$ is the mean dissociation constant and σ the standard deviation. [15] The concentration c_s of each of these antibodies is distributed in accordance

with equation 15. Each antibody is ascribed an epitope on the bacterial protein. Even though the antibody only binds to about 10 aminoacids it is a large globular protein and manages to cover a considerable part of the bacterial protein, potentially preventing other antibodies to bind to their respective epitopes (see figure 4). The length of the part the antibody manages to cover on the bacterial protein is referred to as λ. We here describe a simple model in which

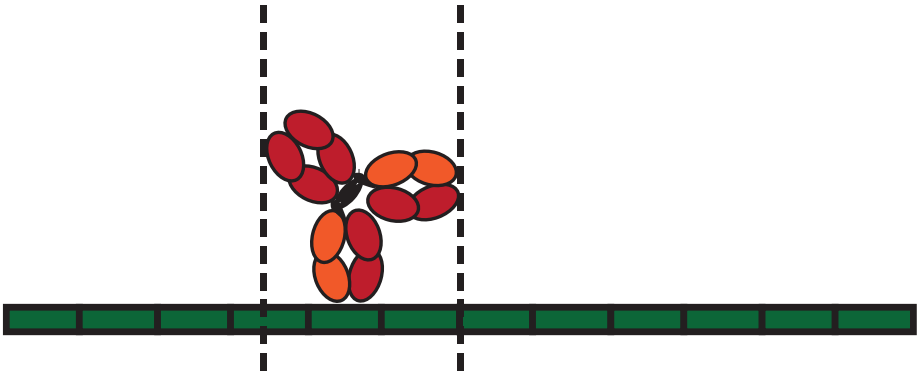

Figure 4: A schematic image illustrating an antibody bound to one site on the linear bacterial protein while covering three binding sites. The dashed lines show the area the antibody mangaes to cover when bound. This is the case for $\lambda = 3$.

a straight protein is divided into equally large sites i consisting of 10 amino acids. $\lambda = 3$ for all types of antibodies. c_s is the bulk concentration of each antibody and K_s is the binding constant for each antibody to their respective epitope. The goal here is to resolve the probability $p(i)$ of i being bound to any antibody via Fab or Fc. The probability $p(i)$ is calculated as

$$p(i) = \frac{Z(i)}{Z} \tag{16}$$

where Z is the partition function and $Z(i)$ is the sum over all allowed Boltzmann weighted states for site i. This equation is derived in section 2.1. Z and $Z(i)$ are calculated using transfer matrices describing the binding possibilities at each state. Each antibody can bind via three sites; the Fc-region and the two Fab-regions. Every site i on the protein can exist in $M = 1 + 3 \cdot \sum_{k=1}^{S} \lambda_k$ possible states. Each state has a statistical weight for site i on the bacterial protein. The statistical weight for site i being empty has been defined as 1 while the weight for ligand s to be bound to site i depends on the binding constant K_s of s to i and the present bulk concentration c_s of ligand s (see figure 5). In the above described case $\lambda = 3$ and $S = 1$; thus $M = 10$. For each site i the $M \times M$ transfer matrix $T(i, m, m')$ is defined. $T(i, m, m')$ describes the statistical weight of i being in state m provided $i + 1$ is in state m'.

Similar to in the study [16] by Nilsson et al., the choice of enumeration of possible states and their respective statistical weights is illustrated in figure 6. Explicitly, the elements of the transfer matrices can be described as follows:

- Site i and $i + 1$ are both empty
  T(i,1,1) = 1

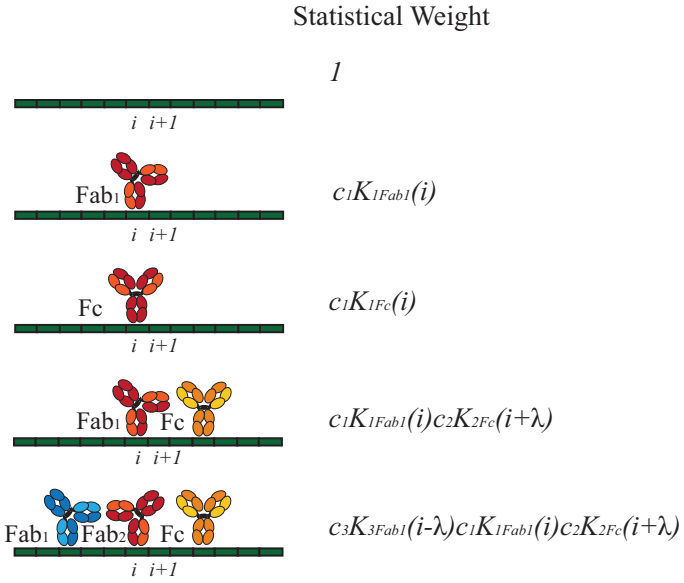

Figure 5: Examples of statistical weights. Different antibody types are shown in different colours. The state in which no antibodies are bound to the bacterial protein has the statistical weight 1, whereas the statistical weigth for a bound protein depends on the concentration of that protein c_s and the binding constant to the site for that region $KFab/Fc(i)$. Note that the statistical weight for binding with the two Fab regions is identical.

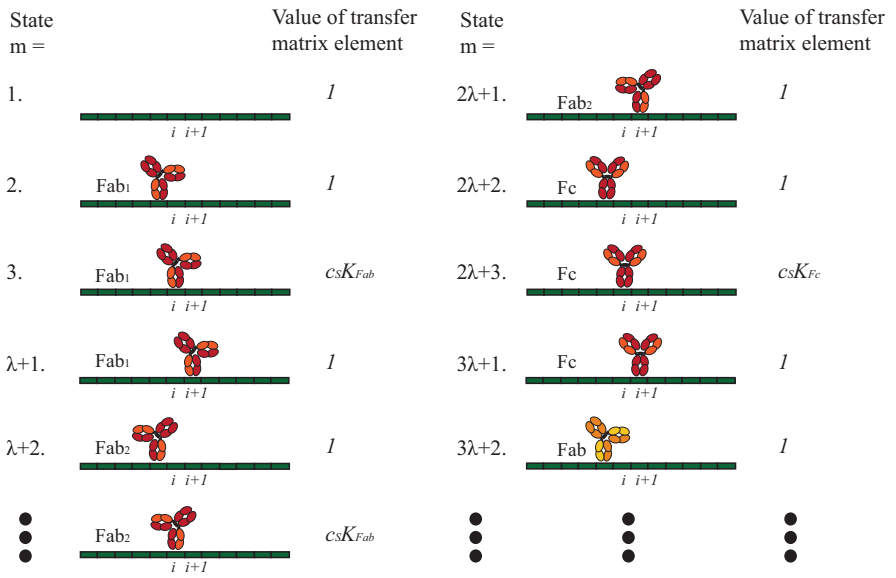

Figure 6: The choice of enumeration for state m together with the transfer matrix elements for site i. The schematic images illustrates binding via the region Fab_1, Fab_2 and Fc as stated in the figure. All states for the first antibdoy types (in red) are shown. The first one for the other antibody (in yellow) type is shown. The states for all antibodies follow the same pattern.

- Site i is empty and $i+1$ is covered by the first part of the three antibody parts s
  T(i,1,1 + $\sum_{k=1}^{s} \lambda_k$) = 1, If $i+1$ is covered by a first part of any antibody, i can either be empty or covered by the last part of any other antibody.
- Site i is covered by the last part of antibody s and site $i+1$ is empty
  T(i,2 + $\sum_{k=1}^{s-1} \lambda_k$,1) = 1
- Both site i and $i+1$ are covered by antibody s; excluding all cases in which the centermost region of s is bound to- and not merely covered by- site i.
  T(i,m+1,m) = 1 for all m>0.
- Site i is covered by the last part of antibody s and site $i+1$ is covered by the first part of another antibody s'.
  T(i,$\sum_{k=1}^{s-1} \lambda_k$,$\sum_{k=1}^{s'} \lambda_k$) = 1
- Site i is bound to the centermost region of antibody s, $i+1$ is hence covered by the following part of antibody s.
  T(i,1+ $\sum_{k=1}^{s} \lambda_k$,$\sum_{k=1}^{s} \lambda_k$) = $c_s K_s(i)$

The forms described above are sufficient for automated computation of all transfer matrix elements. For the case with one type of antibody $S = 1$ and $\lambda = 3$ each transfer matrix takes the form:

$$T = \begin{pmatrix} 1 & 0 & 0\,1 & 0 & 0\,1 & 0 & 0\,1 \\ 1 & 0 & 0\,1 & 0 & 0\,1 & 0 & 0\,1 \\ 0\,c_1K_1(i) & 0\,0 & 0 & 0\,0 & 0 & 0\,0 \\ 0 & 0 & 1\,0 & 0 & 0\,0 & 0 & 0\,0 \\ 1 & 0 & 0\,1 & 0 & 0\,1 & 0 & 0\,1 \\ 0 & 0 & 0\,0 & c_2K_2(i) & 0\,0 & 0 & 0\,0 \\ 0 & 0 & 0\,0 & 0 & 1\,0 & 0 & 1\,0 \\ 1 & 0 & 0\,1 & 0 & 0\,1 & 0 & 0\,1 \\ 0 & 0 & 0\,0 & 0 & 0\,0 & c_3K_3(i) & 0\,0 \\ 0 & 0 & 0\,0 & 0 & 0\,0 & 0 & 0\,1 \end{pmatrix}$$

Typically, antibodies have been designed to recognize a particular sequence of amino acids, and thus exhibit high affinity to a specific epitope. There may nevertheless occur binding to other epitopes on the bacterial protein with lower affinities. All these aspects are incorporated in the formalism above. However, considering results from experiments, we have made the simplification that each antibody only bind to a specific site i. As a consequence of the site dependence of the antibodies, transfer matrix T_i will only include the states in M in which antibody s has been assigned to cover site i. For illustrative purposes, only a few antibody types have been shown in figure 7. In more extensive calculations, S is set to be about $100 - 1000$. Regardless of the resolution chosen for i, the length of the bacterial proteins of interest is about 500 aminoacids and the size of an epitope about 10. It is evident that with these figures, each site i will be covered by more than one sort of antibody s, giving rise to not only competitive binding between the Fab regions and the Fc region, but also amongst the Fab regions of different antibodies s sharing sites. Once these matrices have been produced, Z,

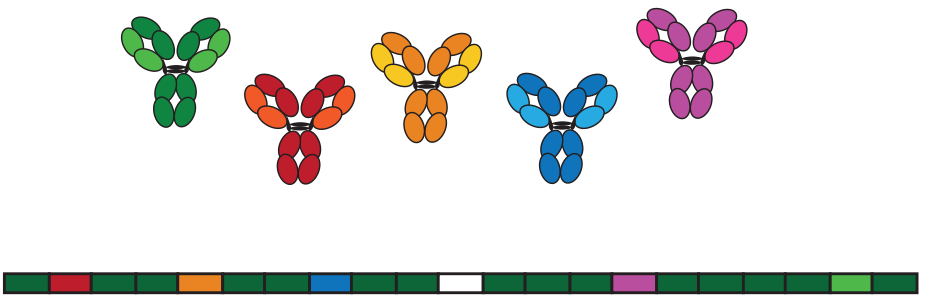

Figure 7: Each antibody binds via Fab to its specific epitope as illustrated with the colour coding, whereas all antibodies bind via Fc to the same site (shown in white). Each antibody s has a specific binding constant $K_s(i)$ For simplicity, binding with lower affinities to other sites than the specific epitopes has been neglected.

$Z_s(i)$ and thereof $p_s(i)$ can be calculated for each site. The partition function Z is

$$Z = v(1)^T \cdot T(1) \cdot T(2) \cdot T(3) ... \cdot T(k) \cdot v(k+1) \tag{17}$$

where $v(1)$ and $v(k+1)$ are column vectors with M elements, ensuring that the first and last site only exists in its possible states. As the antibodies bind via their middle part, the allowed sites for the first site include all states corresponding to no binding or coverage via the parts larger than or equal to the

middle part on the antibody. For $\lambda = 3$ $v(1)$ is

$$v(1) = \begin{pmatrix} 1\,0\,1\,1\,0\,1\,1\,0\,1\,1 \end{pmatrix}^T$$

The last site is fixed to the bacterial surface protein. There is not space for antibodies lying outside of the linear protein on this site. The allowed states on this site are those corresponding to no binding or coverage via the first part of the antibody. For $\lambda = 3$ $v(k+1)$ is

$$v(k+1) = \begin{pmatrix} 1\,1\,0\,0\,1\,0\,0\,1\,0\,0 \end{pmatrix}^T$$

$Z_s(i)$ can be described as

$$Z_s(i) = v(1)^T \cdot T(1).. \cdot T(i-1) \cdot \mathcal{O}_s \cdot T(i)... \cdot T(k) \cdot v(k+1) \tag{18}$$

where $\mathcal{O}_s$ is the projection operator guaranteeing that states in which antibodies bound via either Fab or Fc are retained. The projection operator $\mathcal{O}_{Fab}$ for all states associated with Fab-binding would, in accordance with the previously defined choice of enumeration (see figure 6), have the form

$$\mathcal{O}_{Fab} = \begin{pmatrix} 0\,0\,0\,0\,0\,0\,0\,0\,0\,0 \\ 0\,1\,0\,0\,0\,0\,0\,0\,0\,0 \\ 0\,0\,1\,0\,0\,0\,0\,0\,0\,0 \\ 0\,0\,0\,1\,0\,0\,0\,0\,0\,0 \\ 0\,0\,0\,0\,1\,0\,0\,0\,0\,0 \\ 0\,0\,0\,0\,0\,1\,0\,0\,0\,0 \\ 0\,0\,0\,0\,0\,0\,1\,0\,0\,0 \\ 0\,0\,0\,0\,0\,0\,0\,0\,0\,0 \\ 0\,0\,0\,0\,0\,0\,0\,0\,0\,0 \\ 0\,0\,0\,0\,0\,0\,0\,0\,0\,0 \end{pmatrix}$$

Similarly, $\mathcal{O}_{Fc}$ is zero for all elements except for those corresponding to states associated with binding via Fc, which are one. The probability of Fab and Fc binding is calculated for each site i. The mean probability for the entire bacterial protein length is calculated as

$$\frac{1}{k} \sum_{i=1}^{k} p(i) \tag{19}$$

where k is the number of sites i on the bacterial protein. This is done for several values of total antibody concentrations $C = \sum_S^{l=1} c_s$ ranging from 1 to $10000 \mu g/ml$. Typically the concentration of IgG in saliva lies around $10 \mu g/ml$ while the concentration in plasma is about $10000 \mu g/ml$. This corresponds to about $4 \cdot 10^{-9}$ and $4 \cdot 10^{-6}$ antibodies per nm^3 in saliva and plasma respectively.

4 Results

Experimental results are presented below. The code is tested for different scenarios. Various assumptions and aspects of the model are evaluated. Results from the model are compared to the experimental results and unknown parameters such as the affinity distribution are analysed.

4.1 Experimental results

The effect of washing was evaluated by analysing nine samples with varying IVIgG concentrations before and after washing of the fluorescent protein. Figure 8 shows the measured fluorescence ratio between Dylight 647 and Syto9 for anti-IgG for the samples before and after washing. As expected, a decrease is seen after washing off the fluorescent protein. Interestingly the decrease seems more or less constant for all concentrations apart from a slight increasing difference between the curves for higher concentrations. Considering the standard deviation obtained from the total binding curve (see figure 9) this decrease in binding due to wash is relatively small.

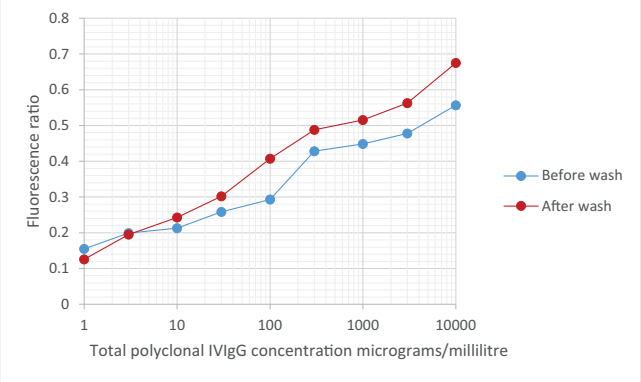

Figure 8: The fluorescence ratio between Dylight 647 and Syto9 for anti-IgG before (in red) and after (in blue) washing off the fluorescent protein. A decrease is seen for the values obtained from after washing of the fluorescent protein. Each point on the curve represents a median value from 10000 scattering events from one sample measured with a flow cytometer

The antibody binding and the binding orientation was measured for varying concentrations. Figure 9 shows the measured fluorescence ratio between Dylight 647 and Syto9 for anti-IgG, anti-Fab and anti-Fc illustrating the total binding, the Fab binding and the Fc binding respectively to AP1. Each point on the curve corresponds to the ratio between fluorescence from the labelled bacteria and the labelled antibodies taken as a median value of 10000 scattering events measured with the flow cytometer. The total binding curve is given as a mean with standard deviation from four samples measured at different occasions. The sum of the Fab and Fc binding corresponds to the total binding curve within a reasonable deviation considering the level of accuracy of the experiment (see figure 9).

The difference in competitive and non-competitive Fab and Fc binding was analysed. The fluorescence measured from bound Fab-fragments and Fc-fragments for IVIgG treated with IdeS before or after exposure to bacteria is seen in figure 10 for three different IVIgG concentrations. Each given value is the median value of 10000 scattering events measured with the flow cytometer. Notice that at the intermediate concentration regime, there is more binding via Fc than via Fab in the competitive binding and vice versa in the non-competitive.

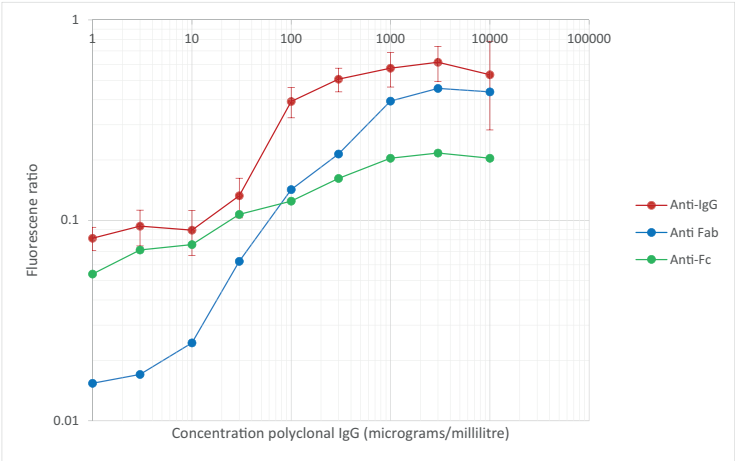

Figure 9: The fluorescence ratio between Dylight 647 and Syto9 for anti-IgG (in red), anti-Fab (in blue) and anti-Fc (in green) depicting the total binding, Fab-binding and Fc-binding respectively to AP1. The total binding is given a mean of four measurements with the standard deviation. The Fab and Fc binding is given for one measurement. Each point on the curve represents a median value from 10000 scattering events from one sample measured with a flow cytometer.

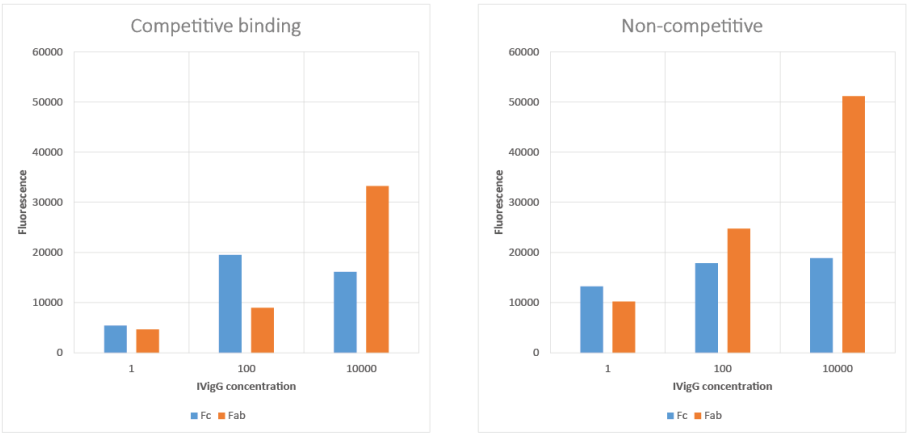

Figure 10: The figure depicts fluorescence in arbitratry units measured for three different concentrations of IVIgG as given in the figure. The lefthand diagram shows results for IVIgG treated with IdeS after exposure to AP1 while the righthand diagram shows results from IVIgG treated with IdeS before exposure to AP1. The lefthand image represents the competitive binding whereas the righthand image represents the non-competitive binding between Fab and Fc region. Notice that at a concentration of 100 $\mu g/ml$, there is more binding via Fc than via Fab in the competitive binding and vice versa in the non-competitive.

4.2 Code validation

In order to ensure that the code is in fact functional, cases for which the outcome is known are first calculated. When $\lambda = 1$ site i is independent of site $i+1$. For monoclonal IgG the result from the code should be the same as expressed in equation 14. The calculations from our model for monoclonal IgG and $lambda = 1$ yields results consistent with equation 14. The binding curve approaches 1 as the concentration goes to infinity. The binding curves behave as expected; lower affinity gives lower binding at same concentration level, binding increases with concentration and asymptotically approaches 1. When $\lim_{c\to 0}$ the expression in equation 14 approaches cK. Calculating binding for very small values of c gives a more or less linear binding curve in that range.

4.3 Binding model

To start with, variation in the results from the model was analysed for different number of antibodies. Since we do not have any information on which epitopes the different antibodies in the human IgG sample are designed to recognize, the epitopes are being randomly generated in the model. To test the variance in the results due to the difference in binding curve this causes and how this depends on the number of antibody types, 10 binding curves are calculated for 50 antibody types and 500 antibody types. According to these results the variance decreases with increasing number of epitopes. This seems reasonable because an increasing number of antibodies yields an increasing number of epitopes, thus covering most of the sites in an increasingly uniform manner. The deviation due to randomly generated sites will thus decrease. Notice how the overall shape of the curves differ, especially at lower concentrations where there is little standard deviation. At lower concentrations, their exists more Fc-binding. This implies that the number of antibody types give rise to more Fc-binding.

Considering the size of the antibody and the length of the bacterial proteins of interest, the antibody should cover about a fifth of the protein length. This seems to coincide with the results obtained from the experiments (see figure 9). There is only one Fc-binding region per antibody and the Fc-binding seems to saturate at a proportion of about 0.2 of the total binding curve.Therefore, for a bacterial protein with 50 sites, the antibody length λ is set to about 9-11 for the model.

It was determined for which σ and mean affinity value $\overline{K_D}$ the best theoretical fit to the experimental Fab-binding curve (see figure 9) is obtained. As mentioned in section 3.2, the antibody affinities in human IgG are unknown and have therefore been assumed to be a normal distribution of affinities. Figure 12 represents a heatmap of the goodness of fit for σ and $\overline{K_D}$. The goodness of fit is measured as the sum of the squared deviation between the experimental and theoretical Fab binding curves. According to the heat map, a change in $\overline{K_D}$ has a larger impact on the goodness of fit than σ. A minimum deviation is seen for values $\sigma = 45$ $(\mu g/ml)^{-1}$and $\overline{K_D} = 140$ $(\mu g/ml)^{-1}$. Figure 13 depicts the experimental results together with a theoretical fit for the values determined with the heat map.

The cumulative affinity for Fc binding was calculated with the best fit of the

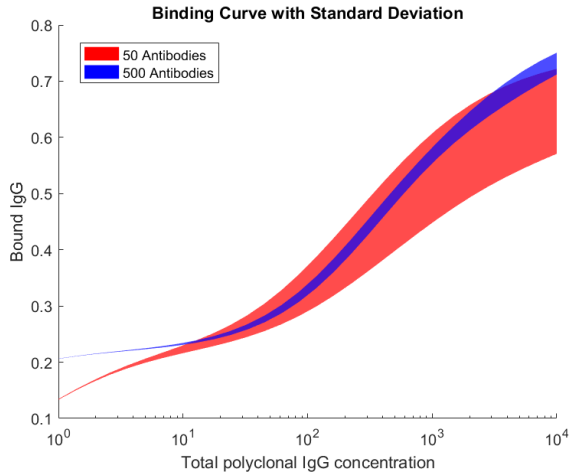

Figure 11: The standard deviation calculated from 10 computed binding curves, each with a different set of randomly generated epitopes, for 50 different antibody types (in red) and 500 different antibody types (in blue). Notice how the standard deviation decreases with a larger number of antibodies.

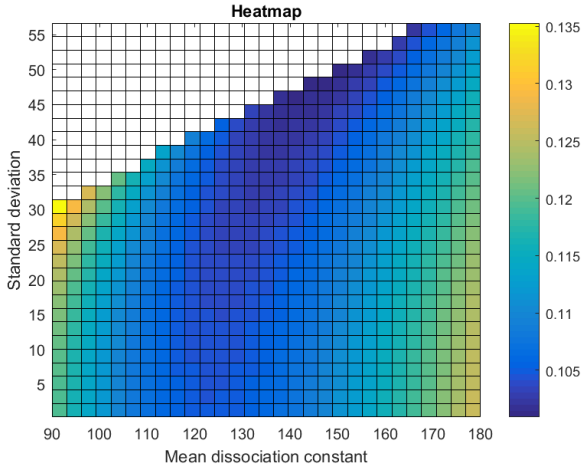

Figure 12: A heatmap over the goodness of fit for σ and $\overline{K_D}$ calcultated as the sum of the squared deviation between the experimental and theoretical Fab binding curves (see figure 9).The heatmap consists of 337 calculated values. The colourscale is given to the right on the image. A change in $\overline{K_D}$ seems to have a larger impact on the goodness of fit than σ. A minimum deviation is seen for values $\sigma = 45$ $(\mu g/ml)^{-1}$ and mean $\overline{K_D} = 140$ $(\mu g/ml)^{-1}$.

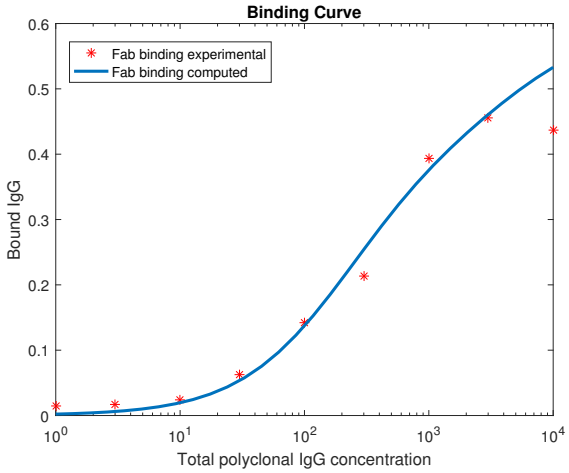

Figure 13: The experimentally measured values as seen in figure 9 together with the best theoretical fit for Fab binding, acquired for $\overline{K_D} = 140\ (\mu g/ml)^{-1}$, $\lambda = 11$, $K_{DFc} = 1500\ (\mu g/ml)^{-1}$ and $\sigma = 45\ (\mu g/ml)^{-1}$. $\overline{K_D}$ and σ have been determined by a goodness of fit (see figure 12). Notice that the last value does not fit very well with the theoretical value.

model for the experimental results. The affinity values for the Fc binding on protein M1 and Protein H have in previous studies been measured and quantified [17] [18]. The proportion of protein M1 and H is however unknown. We therefore calculate a goodness of fit as the summed squares of the deviation from the experimental values for the Fc-binding. Figure 14 illustrates the experimental binding values as seen in figure 9 together with the best theoretical fit for Fab and Fc binding. The calculated dissociation constant for the Fc-binding was $1500\ (\mu g/ml)^{-1}$

The non-competitive and competitive binding was evaluated with our model. Non-competitive binding curves are calculated for both Fab and Fc and can be seen in figure 15 together with the competitive binding curves. According to the binding curves in figure 15, Fab binding is clearly suppressed in the presence of Fc-binding whereas Fab-binding hardly seems to affect the Fc-binding. One can barely distinguish the two Fc-binding curves as they lie on top of one another.

5 Discussion

The results from the first measurements performed have not been included in the results as these were mainly for the purpose of learning the procedure and developing a functional method. Various quantities were changed and some substances replaced. Results from most of the first experiments are inconclusive. As a beginner, I found it difficult to distribute small volumes in to several samples using a pipette. Furthermore, it required some experience to be able to keep track and be consistent with handling of all the samples. It was in general quite hard to measure the binding at high concentrations, possibly due to the difficulty in washing away all free antibodies prior to the labelling. This

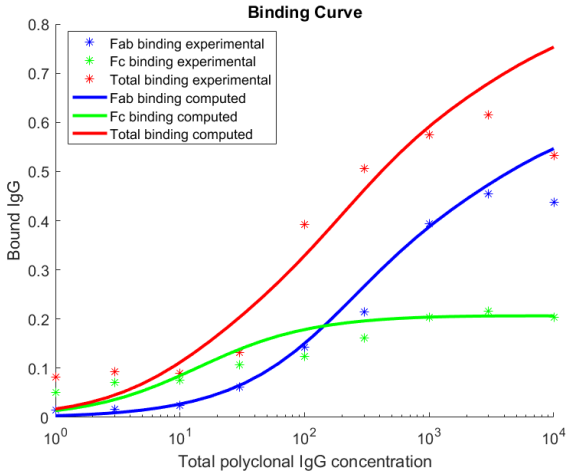

Figure 14: Experimental binding values for Fab, Fc and total binding together with their corresponding theoretical binding. For Fab-binding the values obtained from the heat map (see figure 12) were used and the Fc affinity was determined by minimising the summed squares of the deviation from the experimental results.

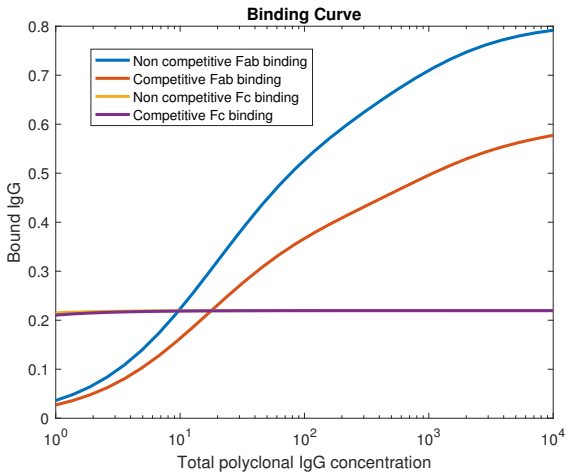

Figure 15: Binding curves calculated for competitive and non-competitive Fab and Fc binding. The blue binding curve represents Fab-binding in the absence of an Fc-binding region while the red binding curve depicts Fab-binding in the presence of an Fc-binding region. The two Fc-binding curves (in yellow and purple)are barely distinguishable from one another. This implies that the Fab-binding is suppressed by the Fc-binding, but hardly affects the Fc-binding.

typically resulted in a lower than expected value (see figure 9).

In figure 10, it seems as though more antibodies are bound when IgG fragments bind non-competitively than when they do compete. This may be due to the fact that once treated with IdeS, the resulting antibody fragments are smaller and no longer cover the same amount on the bacterial protein when bound, making room for more fragments to bind. Another contributing factor may be the increase in diffusivity due to the smaller particle size. According to the computed values in figure 15, Fab binding is clearly suppressed in the presence of Fc-binding whereas Fab-binding hardly seems to affect the Fc-binding. Both our experimental and theoretical results imply that bacterial proteins with a IgGFc-binding region effectively reduce the amount of Fab-binding. This may be of benefit in antibody concentrations around $100 - 1000\ \mu g/ml$, potentially making it easier for bacteria to handle transient increases in IgG concentrations.

From the results in figure 11 it is evident that our model holds best for larger number of antibody types. This corresponds well with the clinical scenario as the immune system has an astonishing capability to generate antibody diversity.

The heat map suggested that the best fit for the experimental Fab-binding curve would be for about $\sigma = 45$ $\overline{K_D}$ and $\overline{K_D} = 140\ (\mu g/ml)^{-1}$. The dissociation constant for Fc binding was found by minimising the summed squares of the deviation from the experimentally obtained values and was determined to be $1500\ (\mu g/ml)^{-1}$. Figure 14 shows how well the binding curves fit the experimental data for these values.

The theoretical curves seem consistent with the measured experimental values. However, considering the difficulties in executing the experiments, it is not yet possible to draw any broader conclusion about how well the model describes the antibody-bacterial protein interaction. Additional experiments need to be performed with higher level of precision. This may be attained by for example using a flow cytometer of higher accuracy.

6 Outlook

As discussed in section 2.2 a model based on classic receptor-ligand kinetics has previously been created. The new model based on the transfer matrix method incorporates more aspects of the antibody-linear protein binding system and is an improvement from the first attempt to model the system.

The model may be improved by adding certain aspects to better describe reality. As an example, λ has been set as a strict length in this model. In reality, it is probably very hard for an antibody to bind to a site more or less covered by another antibody, but it may still be possible for binding from another angle or direction. It is possible that the model may better describe the experiment if binding with overlapping site-coverage was possible with a low but non-zero statistical weight. Moreover, the model could possibly be expanded to describe binding to bacterial proteins with several Fc-binding regions, assuming they can be modelled as one dimensional lattices. If certain simplifying assumptions can be made about the binding to globular surface proteins such as protein G and

protein A, it may be possible to use this method to model antibody binding to these.

The model can be used for analysing binding tendencies of different types of antibody samples. The polyclonal IgG sample used, IVIgG, is human IgG antibodies pooled from >3500 individuals. This provides a good statistical representation. It can however be interesting to compare IgG samples from different individuals with varying pathogenic history.

A potential future application may be a diagnostic software for analysing a patients prognosis in relation to a certain pathogen. A blood and/or saliva sample could be taken from the patient and the interaction with the bacteria may be simulated for their specific set of IgG antibodies. Whether or not a patient is sensitive to certain infections can be of importance, especially for immunosuppressed individuals. Elders, patients with HIV/aids and patients with other chronic infections can have a suppressed immune system. This would require a method for accurately determining the distribution of affinities in any given antibody sample.

7 Acknowledgements

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A Protocol

The following section describes the procedure for the experiments with all necessary quantities, solutions and steps.

A.1 Solutions

The solutions used for the experiments are listed below.

- PBS
- BSA (10 mg/ml)
- IVigG (50 mg/ml) octagam
- IVigG (20000 $\mu g/ml$) 500 μl (200 μl IVIgG (50 mg/ml)+300 μl PBS)

- IVigG (2000 $\mu g/ml$) 500 μl (50 μl IVigG (20000 $\mu g/ml$) +450 μl BSA)
- IVigG (200 $\mu g/ml$) 500 μl (50 μl IVigG (2000 $\mu g/ml$) +450 μl BSA)
- IVigG (20 $\mu g/ml$) 500 μl (50 μl IVigG (200 $\mu g/ml$) +450 μl BSA)
- IVigG (2 $\mu g/ml$) 500 μl (50 μl IVigG (20 $\mu g/ml$) +450 μl BSA)
- DNA marker: Syto9 (Invitrogen) 1:1000
- Goat anti-human IgG 649, 1:100
- Goat anti-human IgGFab 649, 1:100
- Goat anti-human IgGFc 649, 1:100
- Bacteria

A.2 Heat kill bacteria

The procedure for preparing and heat-killing bacteria is described below.
Take 25 μl from stock to 225 μl PBS
S. pyogenes strain AP1 (50 μl)

- Set overnight culture: AP1 is placed in 10 ml Todd hewitt broth and incubated at 37C.
- Let grow to exponential phase (about 3 h)
- Heat-kill: 80C for 5 mins in heat block (agitation at 800 rpm)

A.3 Measuring total binding of IVIgG to *S. pyogenes*.

The procedure for measuring total binding of IVIgG to *S. pyogenes* strain AP1 by labelling IVIgG with fluorescent protein is listed below.

1. Add 100 μl bacteria to 400 μl PBS
2. Sonicate using 100% 0.5 cycle, (4 min for AP1).
3. Repeat sonication.
4. Take 100 μl bacteria and add IgG solution and PBS to a total volume of 200 μl. (see table 1) Use low binding tubes.
5. Incubate 30 min at 37C on shake (500 rpm).
6. Wash twice with PBS (5000g, 5 min, swing out rotor, soft deceleration).
7. Add 200 μl staining solutions 1:750 Syto9 and 1:50 goat anti-human IgG 649.
8. Incubate 15 min at room temperature.
9. Add 800 μl PBS.

10. Analyze in FACS.

Sample list
1. -10. AP1 + Syto9 + [0 1 3 10 30 100 300 1000 3000 10000] IVigG + 649 anti-igG

A.4 Measuring competitive and non-competitive binding of Fab and Fc regions to *S. pyogenes*

The procedure for measuring binding orientation of IVIgG to *S. pyogenes* strain AP1 by cleavage with IdeS and labelling IVIgG with fluorescent protein is listed below.

1. Samples 21-40: Add IgG solution and PBS to a volume of 200 μl. (see table) Use low binding tubes.
2. Samples 21 -40: Add IdeS to each tube. Incubate at 37C on shake (500rpm) for 2h. Continue with 6.
3. Add 100 μl bacteria to 400 μl PBS
4. Sonicate using 100% 0.5 cycle, (4 min for AP1).
5. Repeat sonication.
6. Take 100 μl bacteria and add IgG solution and PBS to a total volume of 200 μl. (see table 1) Use low binding tubes.
7. Incubate 30 min at 37C on shake (500 rpm).
8. Wash twice with PBS (5000g, 5 min, swing out rotor, soft deceleration).
9. Divide all samples into two tubes.
10. Samples 1-20: Add IdeS to each tube. Incubate at 37C on shake (500rpm) for 2h.
11. Add 200 μl staining solution to each tube in accordance with the samples list.
12. Incubate 15 min at room temperature.
13. Add 400 μl PBS.
14. Analyze in FACS.

Sample list
1. -10. AP1 + Syto9 + [0 1 3 10 30 100 300 1000 3000 10000] IVigG + 649 anti-Fab
11. -20. AP1 + Syto9 + [0 1 3 10 30 100 300 1000 3000 10000] IVigG + 649 anti-Fc
21. -30. AP1 + Syto9 + [0 1 3 10 30 100 300 1000 3000 10000] IVigG + 649 anti-Fab
31. -40. AP1 + Syto9 + [0 1 3 10 30 100 300 1000 3000 10000] IVigG + 649 anti-Fc

IgG-solution	20000	2000	200	20	2	BSA	AP1	V˙tot
IgG-conc							100	200
10 000	100						100	200
3000	30					70	100	200
1000		100					100	200
300		30				70	100	200
100			100				100	200
30			30			70	100	200
10				100			100	200
3				30		70	100	200
1					100		100	200
0						100	100	200

Table 1: Table of volumes of IgG and BSA required for the different concetrations.