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Development of an immunoassay for detection of IgG aggregates in Gyrolab Bioaffy

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Title (English)

Development of an immunoassay for detection of IgG aggregates in Gyrolab Bioaffy

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Abstract

An immunoassay for detection of IgG aggregates in Gyrolab Bioaffy was developed using dimeric anti-IgG Affibody as capturing reagent and the tetramer form as detecting reagent. The ratio between this assay and a reference assay using the same capturing reagent and a Fab reactive Fab'_2 fragment as detecting reagent was used to indicate the presence of IgG aggregates. IgG samples were heat treated to produce aggregates and the samples were analyzed using size exclusion chromatography to determine the aggregate proportion in the samples. About 3 to 4% aggregates were detectable using this method.

Keywords

Immunoassay, IgG aggregates, therapeutic monoclonal antibodies, size exclusion chromatography, ÄKTApurifier, anti-IgG affibody, Bioaffy 20 HC CD microlaboratory

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Sammanfattning

Antikroppar är en del av vårt immunsystem och är ansvariga för att känna igen främmande molekyler som till exempel kan sitta på ytan på bakterier, och aktivera resten av immunsystemet mot dessa. Antikroppar binder mycket specifikt till olika molekyler, vilket har utnyttjats terapeutiskt. Som läkemedel kan antikroppar fungera genom att binda och aktivera immunsystemet mot skadliga substanser eller genom att vara kopplade till gifter som då kan levereras specifikt exempelvis till en cancercell och förgöra den.

Ett problem som förekommer vid utvecklingen av terapeutiska antikroppar är att om det finns ihopklumpade eller aggregerade antikroppar i läkemedlet kan de orsaka immunreaktioner så som aktivering av det så kallade komplement systemet som kan orsaka utslag, andnöd och hjärtklappning. Dessutom kan aggregat av antikroppar göra att läkemedlet försvinner snabbare ur kroppen vilket gör att högre eller fler doser måste ges med risk för biverkningar.

Olika antikroppar har olika benägenhet att aggregera, därför vore det bra att i ett tidigt stadie i läkemedelsutvecklingen kunna undersöka vilka antikroppar som är mest benägna att aggregera och förkasta dem. På så sätt skulle stora besparingar kunna göras eftersom det finns en större chans att läkemedlet man utvecklar fungerar. Det finns en del metoder att detektera aggregerade antikroppar, men bara i prover som är renade från andra makromolekyler. Vid screening för att undersöka vilken av många antikroppar som har bäst egenskaper för att bota sjukdomen av intresse är antikropparna däremot inte renade, och att rena dem tar tid och möda. Det finns därför ett behov av en metod som kan detektera förekomsten av aggregerade antikroppar direkt i odlingssupernatanter, gärna med små provmängder och på ett sätt som lätt kan utföras på många prover.

En sådan metod har utvecklats här, där analysen sker i ett CD mikrolaboratorium. Ytterligare utvärderingar av metoden på mer relevanta prover är önskvärt. Dock verkar metoden som utvecklats mycket lovande med goda egenskaper, till exempel verkar förekomsten av så låga nivåer aggregerad antikropp som 3-4% kunna detekteras. Den totala IgG koncentrationen vari aggregerat IgG kan detekteras med denna metod är 2 till 200 µg/ml, vilket är ett rimligt arbetsområde. Samtliga aggregat innehållande prov verkar inte detekteras med denna metod, ytterligare experiment behövs för att få lite mer statistik på hur stor andel av aggregat innehållande prov som detekteras.

Examensarbete 20p

Civilingenjörs programmet i Molekylär Bioteknik

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Abbreviations

2AffibodyBiotin	**-**	**Dimeric anti-IgG Affibody labeled with Biotin**
4AffibodyBiotin	**-**	**Tetrameric anti-IgG Affibody labeled with Biotin**
2AffibodyAlexa	**-**	**Dimeric anti-IgG Affibody labeled with Alexa**
4AffibodyAlexa	**-**	**Tetrameric anti-IgG Affibody labeled with Alexa**
Fab'2Alexa	**-**	**Fab reactive Fab'$_2$ fragment from IgG labeled with Alexa**
IgG	**-**	**Immunoglobulin G**
IgG$_1$ κ	**-**	**Immunoglobulin G$_1$ light chain κ**
Fab´$_2$	**-**	**Fab reactive Fab'$_2$ fragment of IgG**
kDa	**-**	**Kilo Dalton**
MW	**-**	**Molecular Weight**
BSA	**-**	**Bovine Serum Albumin**
2AffibodyBiotin: 4AffibodyAlexa	**-**	**The assay using these reagents**
2AffibodyBiotin: 2AffibodyAlexa	**-**	**The assay using these reagents**
4AffibodyBiotin: 2AffibodyAlexa	**-**	**The assay using these reagents**
4AffibodyBiotin: 4AffibodyAlexa	**-**	**The assay using these reagents**
2AffibodyBiotin: Fab'2Alexa	**-**	**The assay using these reagents**
PBS	**-**	**15 mM phosphate buffer, 150 mM NaCl**
PBS-T	**-**	**15 mM phosphate buffer, 150 mM NaCl 0.01% Tween 20**

Unless otherwise stated the IgG used in this project was monoclonal human myeloma IgG$_1$ κ purchased from Biodesign, catalog number: A50183H.

In this paper aggregates of IgG are defined as anything larger than monomers, i.e. dimers, trimers and polymers are all grouped together and called aggregates.

Unless otherwise stated the CD microlaboratory used here was Bioaffy 20 HC.

1. Introduction

1.1 Therapeutic monoclonal antibodies

Antibodies are thought to possess a huge potential as drugs for many diseases[1]. In fact the majority of recombinant proteins in the clinic are monoclonal antibodies, with more than 150 products in clinical trials in 2005[2]. Antibodies ability to bind target molecules with high specificity and trigger immune responses toward these molecules and the cells they are attached to holds promise of great success, with a global market value predicted to reach 16.7 billion USD in 2008[3]. The potency of antibodies as therapeutic agents is reinforced by coupling toxins or enzymes to the antibody, thus targeting specific cells. Therapeutic antibodies can also act by interfering with signaling pathways, binding to soluble molecules such as cytokines, or by binding to receptors, thus blocking target molecules or activating receptors[4].

However the use of antibodies in therapeutics was not an immediate success. The first antibody approved for therapeutical use, Muromab-CD3 approved 1986, was of murine origin[5]. Early on most therapeutical antibodies were of murine origin and not as effective as anticipated due to immunogenicity and a poor ability to recruit immune effector mechanisms. Since then chimeric and humanized antibodies have been developed, with murine variable or complementary determining regions genetically fused into a human IgG framework. These types have met greater success. Even more promising are completely human antibodies, which currently compose the largest group of antibodies entering clinical trials. These are commonly derived from phage-display technologies or transgenic mice expressing human immunoglobulin genes[1].

IgG is the immunoglobulin class most commonly used in therapeutics and IgG_1 is the most frequently used isotype. IgG_1 is very effective at activating antibody-dependent cellular cytotoxicity (ADCC) and the complement system. Antibody-dependent cellular cytotoxicity is a mechanism by which antibodies bind to antigens on the surface of a cell, for instance a tumor cell, and the Fc part of bound antibodies in turn bind to Fc receptors on natural killer cells (NK cells). Cross linking of these receptors lead to the release of perforin and granzymes which lyse the target cell. The extent to which this mechanism contributes to the positive effect of therapeutic antibodies is not shown in any generic way. However this mechanism has a positive effect on the efficiency of the monoclonal antibody rituximab in the treatment of lymphoma and it is probably beneficential for trastuzumab and cetuximab as well[6].

Aggregates of IgG in therapeutic antibodies can be responsible for side effects such as activation of the complement system leading to tachycardia, dyspnea and exanthema[7]. Protein aggregates may also enhance the immune response to monomers of the same protein, by increasing the risk of anti-drug antibody (ADA) development, thus increasing the risk for an enhanced clearance rate for the therapeutic antibody[8]. This is not desirable when administrating a drug, since it might lose effectiveness or a higher dosage might be needed, which in turn can cause more side effects. Different monoclonal antibodies have different tendencies to form aggregates. The purpose of this project is to find a way to measure the amount of aggregated IgG present in monoclonal immunoglobulin G in an early phase of antibody development, thus making screening possible so that monoclonal antibodies prone to aggregate formation can be abolished early on.

1.2 Purification of monoclonal antibodies

Purification of monoclonal antibodies is often performed in at least three steps. Beginning with a capture step on protein A affinity chromatography, and often followed by several polishing steps such as cationic exchange chromatography, flow through anion exchange chromatography and possibly size exclusion chromatography[9]**.** Purification can influence the chemical and physical properties of these proteins making them slightly denatured, exposing hydrophobic amino acids thus making them susceptible to partial aggregation. For instance the pH change that the immunoglobulins will go through during purification can induce aggregate formation[7]**.**

1.3 Biotin – Streptavidin

Biotin is a small biomolecule also referred to as a vitamin B. Biotin is often used in different biochemical assays because of high affinity to avidin and streptavidin[10,11]. When used in assays, biotin is conjugated to a molecule of interest and streptavidin, or avidin, is conjugated to a solid phase, facilitating a way for the molecule of interest to be attached to the solid phase. In Gyrolab Bioaffy CD microlaboratory there are gel particles in a chromatographic column to which streptavidin is attached. The capturing reagent is labeled with biotin which facilitates a means of attachment through affinity.

1.4 Protein A – Fragment Z – Anti-IgG Affibody

Staphylococcal protein A, often referred to simply as protein A, is a cell surface protein from a bacterium called *Staphylococcal aureus*[12]. It binds to the Fc portion of antibodies, more specifically to the interface between domain two and three of the heavy chain[13], and is involved in a number of immunological activities such as the activation of the complement system and cell mediated cytotoxicity[14]. Protein A was initially thought only to bind to the Fc portion of IgG. However later studies have showed reactivity towards the heavy chain variable domain in antibodies belonging to the V_H3 gene family. Since protein A binds IgG very effectively it is often used in affinity chromatography to purify IgG[13].

Protein A is a highly repetitive protein with two structurally and functionally distinct parts, one responsible for IgG binding and one responsible for association to the cell wall of *Staphylococcus aureus*. The part responsible for IgG binding consists of a number of highly homologous domains, E, D, A, B and C, each capable of binding to IgG on their own. In 1987 a synthetic protein, fragment Z, was designed based upon the best known of these domains, domain B. This domain lacked a methionine residue present in domain E, D and A making it resistant to cyanogen bromide treatment, unlike protein A. A glycine in an asparagine-glycine sequence was changed to an alanin in the designed protein, making it resistant to hydroxylamine treatment as well. This engineered protein based on domain B of protein A binds strongly to the Fc portion of IgG, much like protein A[14]. However it has been shown that the reactivity towards the heavy chain variable domain present in protein A is non-significant in fragment Z[15]. In this project a commercially available version of this molecule called anti-IgG Affibody was used, which is the genetically designed fragment Z with an additional modification, a cysteine has been added at the C-terminal, facilitating further modifications such as for example the conjugation to a biotin molecule[16]**.**

1.5 Immunoassays

An immunoassay is a procedure that utilizes the exquisite specificity of antibodies to detect and quantify antigens. The most commonly used principles are called sandwich and competitive immunoassays. The assays used in this project are all sandwich immunoassays.

In a sandwich immunoassay the antigen is allowed to react with an immobilized antibody and detection is performed by adding labeled antibody that will react to another epitope of the bound antigen[17]. Here anti-IgG Affibody molecule is used instead of antibodies immobilized for capture and labeled for detection. This molecule has been used in dimeric and tetrameric form and is a very small molecule compared to antibodies, 14 and 28 kDa for dimer and tetramer form respectively, compared to 150 kDa which is the size of antibodies.

1.6 Assays for aggregated proteins

For proteins that functionally are monomers, aggregation is reasonably simple to identify using a sandwich immunoassay. Capturing and detecting reagents that have identical epitope specificities for the protein should not be able to generate signal except from the fraction of molecules that are in aggregated form. This principle does however not apply for homodimeric proteins.

IgG is a homodimeric protein with regards to the heavy chains. As mentioned in section 1.4 Anti-IgG Affibody interacts with the interface between domain 2 and 3 of the heavy chain[13]. It has been shown that protein A, consisting of 5 IgG binding domains, as well as a dimer of fragment Z are unable to bind to both heavy chains of one IgG molecule[12]. However with a sufficiently high concentration of capturing reagent, the binding of separate anti-IgG Affibodies to each heavy chain of one IgG molecule might be enabled. This phenomenon and the fact that the capturing reaction is temporally favored in a sandwich immunoassay compared to the detection reaction, makes the interaction of the detecting reagent less favored, particularly for monomeric IgG molecules. In contrast, multimers of IgG that are present in sample may hypothetically have an advantage of generating signal in an immunoassay because of the increased number of binding sites for the anti-IgG Affibody molecule in a complex that is not saturated during the capture step of the assay.

The molecular format of the capturing and detecting reagents is a key question for the design of an assay that overestimates the IgG contents in samples containing aggregated IgG. In this project there has been an opportunity to label anti-IgG Affibody in dimer or tetramer format, with biotin and Alexa Fluor® 647 respectively. Another issue that needs to be addressed is the expectation that the concentration of IgG which partly may be in different physical forms will have an impact on the response level in the system. One approach to overcome this variability would be to study the ratio of IgG generated from assays exaggerating the response from the aggregated form of IgG and assays insensitive to the molecular form of IgG. This approach would not only accurately quantify the amount of IgG in the sample but also disclose the proportion of IgG that appear in aggregated form.

1.7 Methods to analyze aggregate content in monoclonal antibodies

There are a number of different methods available to analyze the aggregate content of IgG samples. High performance size exclusion chromatography (SEC) combined with UV detection is one. A limitation this method has is that very large aggregates will go undetected as they will not penetrate the matrix. Light scattering is another method used to analyze the aggregate proportion in immunoglobulin containing samples often coupled to SEC[18]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is still another commonly used method to analyze the aggregate content of IgG samples. A limitation that all these methods experience is that the sample has to be purified before any of these analysis can be performed. However SDS-PAGE can be coupled to Western Blott to facilitate an analysis of the amount of aggregates present in a sample containing unknown molecules. This approach is quite laborious and time consuming though.

Analytical Ultracentrifugation and Field Flow Fractionation are two other methods capable of detecting IgG aggregates. These methods cover a wide range of aggregate sizes from 0.001 to 50 µm, this should be compared to SEC which has a quite limited detection range. However Ultracentrifugation and Field Flow Fractionation are both very complicated methods requiring highly trained personnel[19]. Furthermore unpurified material is not suitable for detection using these methods either.

The methods mentioned here to analyze the aggregate portion of IgG all need purification of the antibody prior to analysis. When developing therapeutical antibodies the tendency for the different antibodies to form aggregates needs to be addressed at an early stage, thus avoiding spending huge resources on immunoglobulins doomed to poor performance in the clinic due to the presence of aggregates. This can be achieved by screening for antibodies with low tendencies to aggregate at an early stage. When screening for different properties the molecules have not been purified, therefore a method where the amounts of aggregates present can be determined directly in the cell supernatant is desirable. Another desirable property of such a method is an ease to perform the analysis on a large amount of different samples. The development of a method fulfilling these criteria has been attempted in this project. However, here an immunoassay has been developed, utilizing the specific interactions between different biomolecules as compared to the physical chemical properties of the molecules, as the other methods described here do. Immunoassays are easily performed in Gyrolab Bioaffy CD microlaboratories where 112 samples can be analyzed in one CD microlaboratory. In the Bioaffy 20 HC microlaboratory cell supernatants can easily be analyzed, thus an assay for detection of IgG aggregates in Gyrolab Bioaffy 20 HC microlaboratories would fulfill the above mentioned criteria.

2. Aim and analytical strategy

The aim of this project is to develop an assay or a combination of assays with which the amount of aggregated IgG in an IgG containing sample can be quantified using a Gyrolab Bioaffy CD microlaboratory in a fast and simple way.

Previous studies using the dimer form of anti-IgG Affibody as capturing reagent and the tetramer form as detecting reagent gave indications that aggregated IgG, that may form from monomeric IgG during cultivation and/or purification, generated higher IgG responses compared to monomeric IgG[20]. These observations warranted further investigation and optimization using various assay formats and reagent combinations. One such assay, i.e. the one where dimeric anti-IgG Affibody is used as capturing reagent and Fab´$_2$ anti-human IgG as detecting reagent, is believed to react proportionally to the amount of IgG regardless of the molecular form, therefore this assay is used as reference. Combinations of the above mentioned assays and other assays using the dimer and the tetramer of anti-IgG Affibody in all possible combinations were examined in this project with the aim to identify one reagent combination that is superior for quantification of IgG aggregates.

3. Materials and Methods

3.1 Gyrolab Bioaffy®

3.1.1 Gyrolab Bioaffy® CD microlaboratory

A schematic picture of Gyrolab Bioaffy® CD microlaboratory can be seen in Figure 1. The shape is that of an ordinary music CD, but with a complex microstructure network aimed at performing various forms of analytical processes. There are 14 different segments, each segment consists of 8 individual structures, shown in Figure 2. The individual structures have two functions for volume definition, both generating liquid volumes of 200 and 20 nL respectively for the Bioaffy 200 microlaboratory and the Bioaffy 20 HC microlaboratory. The common channel serves 8 individual microstructures with liquids such as capturing and detecting reagents and washing liquids. The volume defining chamber is connected to the individual inlet, where the volume of the sample is defined. Excess of sample is discarded through an overflow channel connected to each individual inlet. There is also an overflow channel connected to the common channel for each segment of 8 structures. As well as two hydrophobic barriers, preventing liquids from moving unless pushed through by the force generated by spinning the microlaboratory. There is a column filled with streptavidin-coated beads towards the periphery of each structure. This simplifies assay development since streptavidin binds strongly and specifically to biotin. Biotin can easily be conjugated to reagent molecules, such as antibodies, hence this microlaboratory is easily used in a multitude of assays.

***Figure 1**. A Gyrolab Bioaffy® CD microlaboratory. 14 segments are visible, of which the slice is one.* [21] *Illustration used with permission from Mats Inganäs at Gyros AB.*

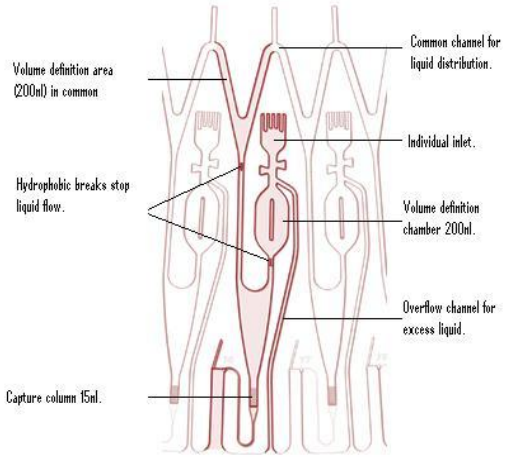

***Figure2**. An individual structure from a Bioaffy 200 microlaboratory where one sample can be analyzed. There are 112 structures like the one above grouped into 14 segments in one microlaboratory*[21]*. Illustration used with permission from Mats Inganäs at Gyros AB.*

3.1.2 Gyrolab® Workstation LIF

Gyrolab® Workstation LIF is the instrument where the Gyrolab Bioaffy® CD microlaboratory is analyzed. Reagents and samples are prepared before the run and placed in a microtiter plate in the instrument from which they are transferred to the micro laboratory by a robotic system in an automated fashion. For detection a Laser Induced Fluorescence (LIF) detector is used. A photomultiplier tube (PMT) is used in the system and the settings used in this study were 0.1% amplification.

3.1.3 Gyrolab® software system

Evaluation of data is facilitated by a program called the Gyrolab Evaluator which essentially convert response levels to analyte concentration. Moreover, a program called Gyrolab Viewer can be used to get a visual illustration of the fluorescence distribution in the column, assisting in data evaluation.

To perform assays using this instrument a certain workflow is used. Firstly, create a method in the software system, or identify an existing method suitable for the specific assay. Secondly, create a transfer list, that is a list describing how you want your reagents and standards placed in the microlaboratory. Thirdly, create a reagent list, that is a list of where you are going to put your reagents and standards in the reagents microtiter plate. Both lists are excel lists written in a specified manner, see the Gyrolab Bioaffy® User Guide[22]. Fourthly, create a batch in the software system. This can be done in two ways called the Bioaffy 1 SIA application or the Development application. In this project the Development application has been used. In the Development application a method is selected as well as a transfer list and a reagent list. Fifth, run the batch, putting the microlaboratories and the prepared reagent microtiter plate into the instrument. One CD microlaboratory takes approximately one hour to run. If unknown samples shall be tested a separate sample list must be written in a specific manner. This list will then be imported when running the batch. In this particular project a standard method called Bioaffy 20 HC v1 with a change in detecting PMT have been used. For a more extensive description of how to use the Gyrolab Workstation with its microlaboratories see the Gyrolab Bioaffy® User Guide.

3.2 Absorbance measurements 280 nm

Absorbance measurements are often used to quantify proteins in solutions. Light with a wavelength of 280 nm is absorbed by aromatic amino acids i.e. tryptophan, tyrosine and phenylalanine, the absorbed energy cause a delocalization of electrons. The aromatic amino acids each absorb different amounts of light with tryptophan absorbing most, followed by tyrosine, and phenylalanine. Therefore different proteins absorb different amounts of light wavelength 280 nm depending of their amino acid content. Anti-IgG Affibody contains no tryptophans and only one tyrosine and three phenylalanines[14], making absorbance measurements at 280 nm unreliable at reasonable concentrations. Fab´$_2$ fragments of IgG at the other hand have more aromatic amino acids making quantification by this method applicable.

3.3 Reagents

Human Myeloma IgG$_1$ κ was purchased from Biodesign (A50183H, through BioSite, Täby, Sweden). Fab´$_2$ fragments of goat-anti human IgG (Fab´$_2$ specific) was purchased from Jackson ImmunoResearch (109-006-097, West Grove, Pennsylvania, USA). Anti-IgG Affibody biotinylated and unconjugated was purchased from Affibody (10.0623.02.0005 and 10.0623.01.0010, Bromma, Sweden).

3.3.1 Preparation of reagents – Labeling and separation

Tetrameric and dimeric anti-IgG Affibody were both labeled with Alexa 647 (Invitrogen A20186, A20347, Lidingö, Sweden) as detecting flourophore and Biotin (Pierce 21335, 21902, Illinois, USA) as capturing reagent using NHS[23] and maleimide[24] chemistry, respectively. Fab´$_2$ was labeled with Alexa 647 using NHS chemistry (Invitrogen A20186 Lidingö, Sweden). An overview can be seen in Table 1.

Table 1. *This table shows a summary of the capturing and detecting reagents used and the conjugation procedure used to label them.*

Capturing Reagent	**Conjugation Procedure**	**Detecting Reagent**	**Conjugation Procedure**
Dimeric anti-IgG Affibody - Biotin	DTT reduction, Maleimide-PEO$_2$-Biotin	Dimeric anti-IgG Affibody -Alexa 647	DTT reduction Alexa Fluor® 647 C$_2$-maleimide
Tetrameric anti-IgG Affibody - Biotin	Sulfo-NHS-LC-Biotin	Tetrameric anti-IgG Affibody - Alexa 647	Alexa Fluor® 647 Monoclonal Antibody Labeling Kit
		Fab´$_2$ Alexa 647	Alexa Fluor® 647 Monoclonal Antibody Labeling Kit

3.3.2 Labeling with Alexa Fluor® 647 Monoclonal Antibody Labeling Kit

Alexa 647 is a bright and photostable flourophore which has an excitation maximum of approximately 650 nm and an emission maximum of approximately 668 nm. In this kit the reactive dye has a succinimidyl ester moiety which reacts efficiently with primary amines of proteins to form a stable Alexa/protein conjugate[25]**.**

Fab´$_2$ was labeled with Alexa 647 according to the Alexa Fluor® 647 Monoclonal Antibody Labeling Kit (A-20186, Invitrogen, Lidingö, Sweden) experimental protocol. The tetramer of anti-IgG-affibody was labeled according to the same protocol, except for the separation from unreacted Alexa where a Nanosep Centrifugal Device with a molecular weight cutoff of 10 kDa was used, spinning a total of four times adding 100 µl PBS between each spin (OD003C33 Pall Corporation, Michigan, USA).

3.3.3 Labeling the tetramer of anti-IgG Affibody

Sulfo-NHS esters, such as Sulfo-NHS-LC-Biotin, react with primary amines forming amine crosslinks[22]. Here the tetramer of anti-IgG Affibody was labeled using EZ-Link Sulfo-NHS-LC-Biotin (21335, Pierce, Illinois, USA) according to the Gyrolab Bioaffy® User Guide Version 1.0, with the exception that the labeled protein was separated from unlabeled reagents using a Nanosep Centrifugal Device with a molecular weight cutoff of 10 kDa (OD010C33, Pall Corporation, Michigan, USA), repeating the spin adding 100 µl PBS each time until the sample had been spun a total of four times.

3.3.4 Labeling the dimer of anti-IgG Affibody

Anti-IgG Affibody is purchased as a tetramer which consists of two dimers connected via a disulphide bridge. Reducing the disulphide will lead to the formation of two dimers containing sulfhydryl groups that can be addressed for conjugation. Reduction was achieved using DTT. The resulting dimers were labeled with either Alexa using a reagent called Alexa Fluor® 647 C$_2$-maleimide (A20347, Invitrogen, Lidingö, Sweden), or with Biotin using another maleimide called Maleimide-PEO$_2$-Biotin (21902, Pierce, Michigan, USA). In both cases the attached protocol was followed, except for the removal of excess labeling reagent.

Here a Nanosep Centrifugal device with a molecular weight cutoff of 3 kDa was used (OD003C33, Pall Corporation, Michigan, USA), repeating the spin adding 100 µl PBS until the sample had been spun a total of four times. The maleimides on the reactive dye/biotin react with the cysteins on the anti-IgG Affibody dimer that have been made available by reduction with DTT.

3.4 Aggregation of IgG

The aggregation properties of a protein, such as the onset and the rate of aggregation, are dependent on the proteins solution environment, such as the temperature, pH, salt types, salt concentrations and so on[26]. In this study exposure to elevated temperatures during limited time was used to induce the formation of aggregates.

3.4.1 Heat treatment of IgG

To produce aggregates of IgG, IgG at 2 mg/ml was heated in a 63°C water-bath. To determine a reasonable time to heat the samples 160 µl IgG at 2 mg/ml was heated for 30 minutes aspirating 40 µl out of the sample after 5, 10 and 20 minutes. These samples were analyzed using size exclusion chromatography in an ÄKTApurifier system connected to a HR 10/30 column containing Superdex 200 gel. A flow rate of 0.5 ml/min of PBS, 0.002% sodium azid, was applied for elution and fractions of 100 µl were collected for further analysis in Gyrolab Bioaffy.

3.5 Preparation and characterization of monomeric/aggregated IgG using ÄKTApurifier

The aggregate content of the IgG samples was separated from monomeric IgG using size exclusion chromatography on a Superdex^{TM} 200 HR 10/30 column in an ÄKTApurifier system. This column has an exclusion limit of 1300 kDa for globular molecules[27], that means that aggregates consisting of approximately 8 monomers will be unable to penetrate the matrix. Fractions from separations were collected in microtiter plates, 50 or 100 µl in each well. The methods used were created using UNICORN software.

3.6 Assay setups

3.6.1 Assay for quantification of IgG

To quantify IgG an assay using biotinylated dimeric anti-IgG Affibody as capturing reagent and Alexa 647 labeled Fab specific Fab´$_2$ fragment of IgG as detecting reagent was used. The capturing reagent was diluted to a working concentration of 0.1 mg/ml and the detecting reagent was diluted to a working concentration of 75 nM. A setup that is found to work very well and that increased the working range of the assay upwards is adding 3000 nM unlabeled Fab´$_2$.

3.6.2 Assay for quantification of aggregated IgG

Various reagent combinations based on biotinylated and Alexa 647 labeled anti-IgG Affibodies (di- and tetramer) were tested to generate an assay that tend to respond strongly to aggregated IgG while generating modest response to monomeric IgG. Assays with all combinations of labeled anti-IgG Affibodies were performed.

The concentration of the biotinylated dimeric anti-IgG Affibody should be 0.1 mg/ml diluted in PBS-T. The concentration of the tetrameric Alexa 647 labeled anti-IgG Affibody was not determined due to difficulties in determining the anti-IgG Affibody concentration. An estimated value was however determined to 130 nM. This estimation is achieved by

calculating the loss of proteins when biotinylating dimeric anti-IgG Affibody, assuming the working concentration achieved by titration is 0.1 mg/ml. The loss of proteins in the labeling process is assumed to be equal when Alexa 647 is labeled to tetrameric anti-IgG Affibody. The concentrations used for biotinylated tetrameric anti-IgG Affibody and Alexa 647 labeled dimeric anti-IgG Affibody were not determined either, but an estimation can be performed as above. Titrations were used to determine suitable dilutions and titration is advisable for every new batch of labeled reagent.

4. Results

4.1 Reagent preparation – Labeling and separation

Unlabelled anti-IgG Affibody is provided as a functional tetramer consisting of two dimers held together by a disulphide bridge. In order to label the dimer through the sulfhydryl group the preparation has to be reduced with DTT. Initial attempts to effectively reduce the protein during a short period of time proved unsuccessful. Hence the time for reduction was extended to 1 hour. Another note of importance is that anti-IgG Affibody is a quite small molecule, 14 kDa for the dimer and 28 kDa for the tetramer, and the separation methods proposed in the protocols accompanying the different labeling molecules are suitable for antibodies which are much larger. Therefore a few trials were done to find the best method to separate labeled protein from free label, using mini dialysis units and spin columns, before the method employing Nanosep centrifugal devices was chosen.

To make sure the labeling went well and to decide how much the reagents should be diluted, titrations of the products were performed in a Bioaffy 20 HC microlaboratory. Absorbance measurements were conducted for labeled Fab'$_2$, but as mentioned before anti-IgG Affibody can not be analyzed in 280 nm absorbance measurements because of its low extinction coefficient. The results from labeling anti-IgG Affibody are summarized in Table 2. The results from the labeling of Fab´$_2$ are shown in Table 3 and Figure 3.

Table 2. *Here the results from the labeling efforts of anti-IgG Affibody are summarized. These results are as expected.*

Labeled reagent	**Label**	**Reaction size [µg]**	**Final volume [µl]**	**Dilution**
Dimeric anti-IgG Affibody	Alexa 647	100 µg	35 µl	1 : 640
Dimeric anti-IgG Affibody	Biotin	100 µg	20 µl	1 : 40
Tetrameric anti-IgG Affibody	Alexa 647	100 µg	50 µl	1 : 640
Tetrameric anti-IgG Affibody	Biotin	100 µg	30 µl	1 : 80

Labeling of Fab'$_2$ with Alexa 647 worked well. This can be seen by the results of the absorbance measurement in Table 3, where the labeled reagent was diluted 10 times in PBS. At 280 nm the protein content was measured to 0.495 mg/ml. After labeling there are 0.773 Alexa molecules on each Fab´$_2$ molecule. The successful labeling was confirmed by performing an assay in Gyrolab Bioaffy where the labeled reagent was used, see Figure 3. A nice standard curve was achieved, as expected.

Table 3*. Absorbance measurement of Alexa labeled Fab´$_2$ diluted 10 times on PBS.*

A_{280}	**A_{650}**	**ε Fab'$_2$ 280nm**	**ε Alexa 650 nm**	**Protein concentration**	**Alexa / Fab´$_2$**
0.074 A	0.231 A	1.48	223 000 cm^{-1} M^{-1}	0.495 mg/ml	0.773 mol/mol

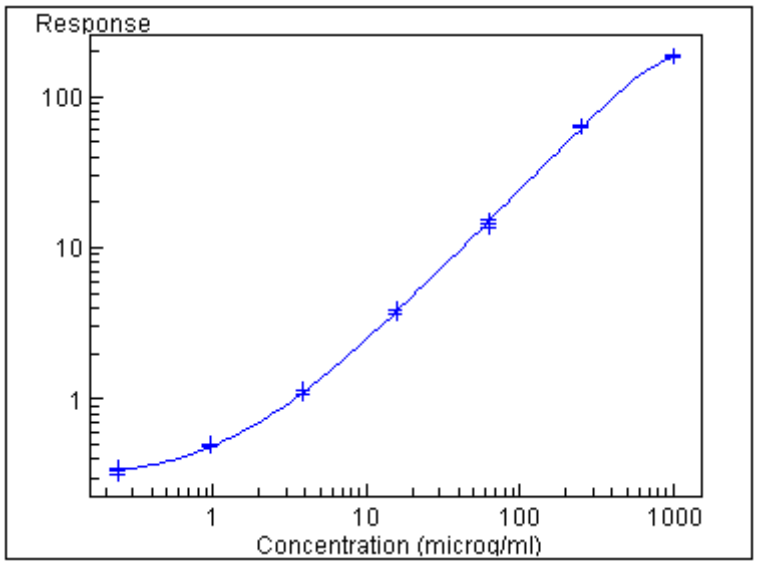

Figure 3*. Assay using the above labeled Fab´$_2$ Alexa, and biotinylated dimeric anti-IgG Affibody as capturing reagent. Human myeloma IgG was used as a standard with concentrations from 1000 µg/ml diluted in steps of four to 0.244 µg/ml. Gives a nice curve, which indicates that the reagents are labeled successfully.*

4.2 Analysis of untreated IgG using ÄKTApurifier

Chromatograms of untreated monoclonal (A50183H, through BioSite, Täby, Sweden) and polyclonal (30-A106, Fitzgerald, Massachusetts, USA) IgG obtained by an analytical size exclusion separation in an ÄKTApurifier system is seen in Figure 4. As can be seen by the small peak in the high molecular weight fractions in both chromatograms there were aggregated species in both of these IgG samples. The proportion of aggregated material was significantly higher in the polyclonal sample 12 % compared to 3 % for the monoclonal.

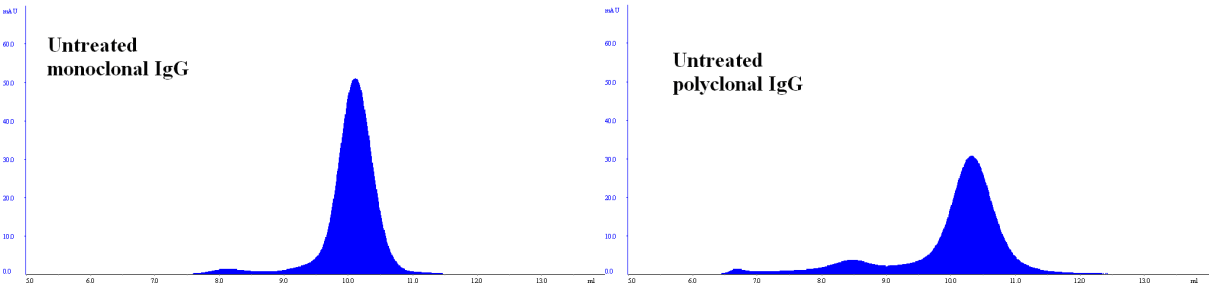

Figure 4*.Left: Chromatogram showing untreated monoclonal IgG. Right: Chromatogram showing untreated polyclonal IgG. The small peaks in the high molecular weight fractions in both chromatograms consist of aggregated IgG and the large peaks consist of monomeric IgG. The area under the peaks allow calculation of the amount of aggregated IgG in the untreated monoclonal and polyclonal IgG samples to 3% and 12% respectively.*

4.3 Aggregation

4.3.1 Heat-treatment of IgG

160 µl at IgG 2 mg/ml was heat treated at 63°C and 40 µl was withdrawn after 5, 10, 20 and 30 minutes. The different samples were analyzed using size exclusion chromatography in a Superdex 200 HR 10/30 column connected to an ÄKTApurifier system to see the amount of aggregates present in the different samples. The procedure has been repeated for monoclonal (A50183H, through BioSite, Täby, Sweden) and polyclonal (30-A106, Fitzgerald, Massachusetts, USA) IgG samples. From the chromatograms in Figure 5 and 6 it is obvious that increased time for heat treatment gives more aggregated material for both monoclonal

and polyclonal IgG. Monoclonal IgG treated this way seem to have an almost linear correlation between the amount of aggregates formed and the time at elevated temperature. The polyclonal sample did not show such a clear correlation. Unfortunately this method can not display aggregates that are so large they get stuck at the top of the column. The exclusion limit for this column is 1.3 MDa for globular proteins[27]. Thus the largest aggregates able to penetrate the matrix consist of approximately eight IgG monomers. The polyclonal sample seems to have formed some very large aggregates when heated for 30 min, the total area in the corresponding chromatogram is considerably smaller than the area in the chromatograms from the samples heated for a shorter time, see Figure 6. Monoclonal IgG was chosen for continued experiments and 200 µl at 2mg/ml was heat treated at 63 °C continuously for 30 minutes, this lead to the formation of approximately 40% detectable aggregates, most of it being eluted in the exclusion volume, see chromatogram in Figure 7. This procedure has been repeated a few times and the aggregate content has ranged from 37% to 44%.

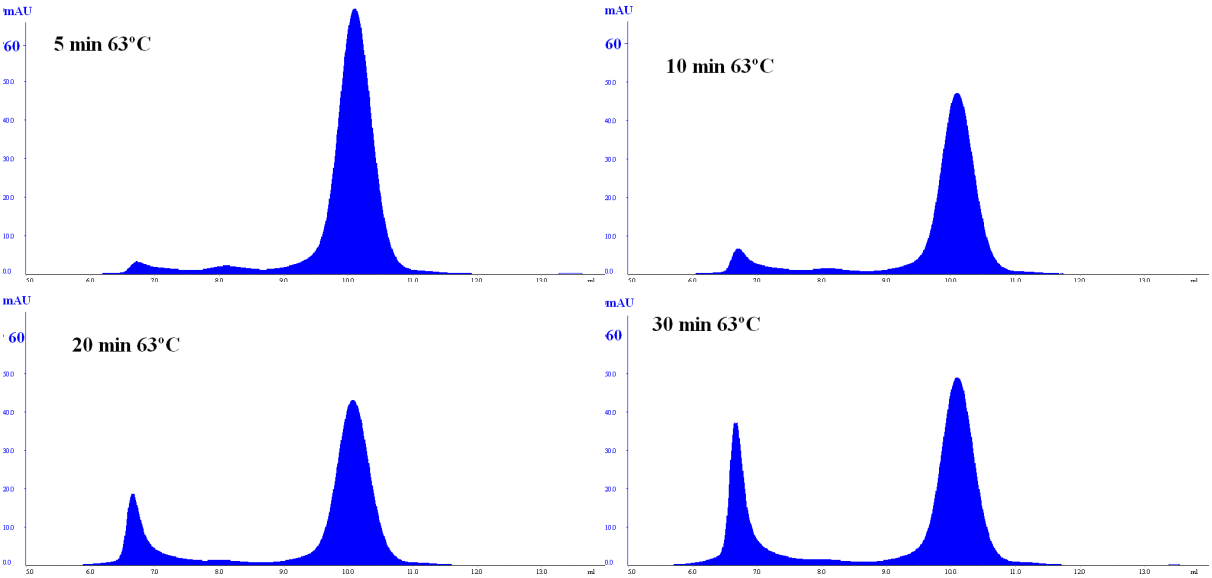

Figure 5. *Chromatograms showing a series of heat treated monoclonal IgG's, treated in a water bath at 63°C for different times. The area under the peaks let the aggregate contents be calculated to 5%, 10%, 21% and 31% respectively.*

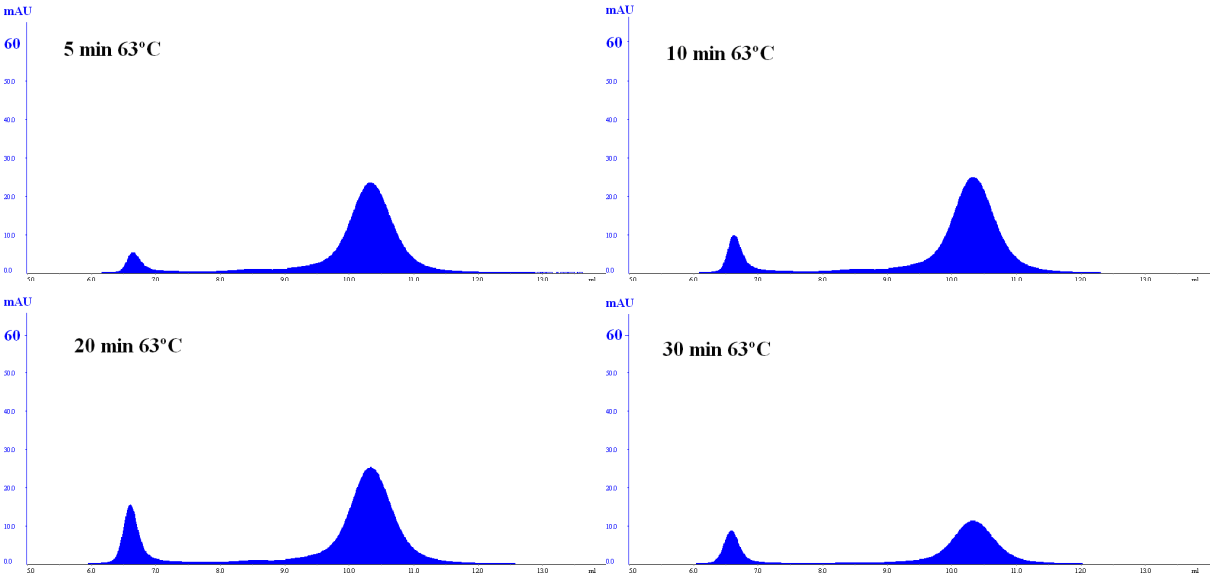

Figure 6. . *Chromatograms showing a series of heat treated polyclonal IgG's, treated in a water bath at 63°C for different times. The area under the peaks let the aggregate contents be calculated to 10%, 14%, 20% and 23% respectively.*

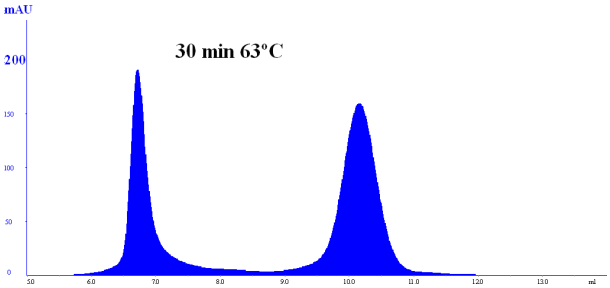

Figure 7. *Chromatogram showing 200 µl monoclonal IgG at 2mg/ml heat treated continuously for 30 minutes at 63 °C separated using size exclusion chromatography. From the areas underneath the peaks the aggregate content is calculated to 40 %.*

4.4 Assay evaluation

4.4.1 Diluents

Diluents are necessary for most assays performed in CD microlaboratories and this is true here as well. Aggregated IgG seems to be more sensitive to variations to the type of diluents used, probably because of denatured protein structures leading to more hydrophobic properties. An illustration of this is seen in Figure 8 where no diluents are used. Here the high molecular peak from the chromatogram is undetectable when fractions are analyzed using our reference assay in CD microlaboratories. This can be compared to Figure 9 where a few different diluents are used. Here a much greater portion of the high molecular peak is detectable using our reference assay in CD microlaboratories. The diluents used in this project are Gyros Detection Diluent (P0003779, Gyros, Uppsala) used to dilute the detecting reagent, Gyros Standard Diluent (P0003819, Gyros, Uppsala) used to dilute IgG samples for standard curves, and Gyros Bioprocess Diluent (P0004649, Gyros, Uppsala) used to dilute IgG samples analyzed as samples.

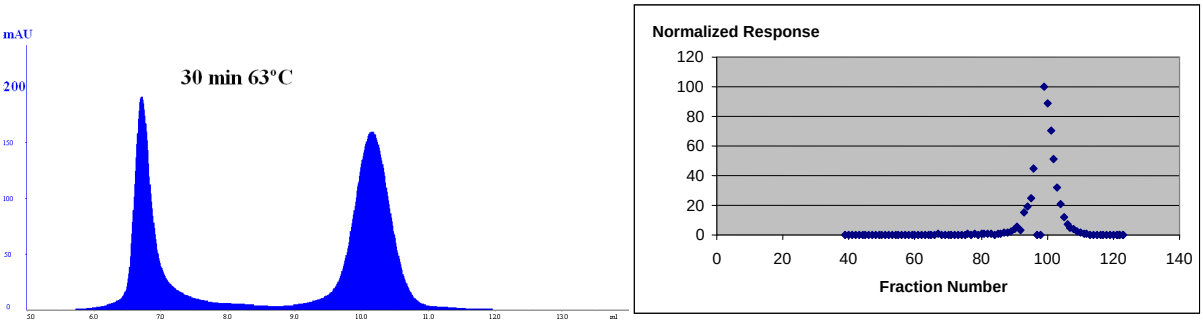

Figure 8. *Left: Chromatogram of IgG heat treated for 30 minutes in 63°C and fractionated on a Superdex 200 HR10/30 column. Right: graph where the fractions are analyzed in a CD microlaboratory. The assay used is the reference assay with dimeric anti-IgG Affibody as capturing reagent and Fab'$_2$ as detecting reagent. No diluent has been used in the fractions here. As can be seen the high molecular peak is not detectable.*

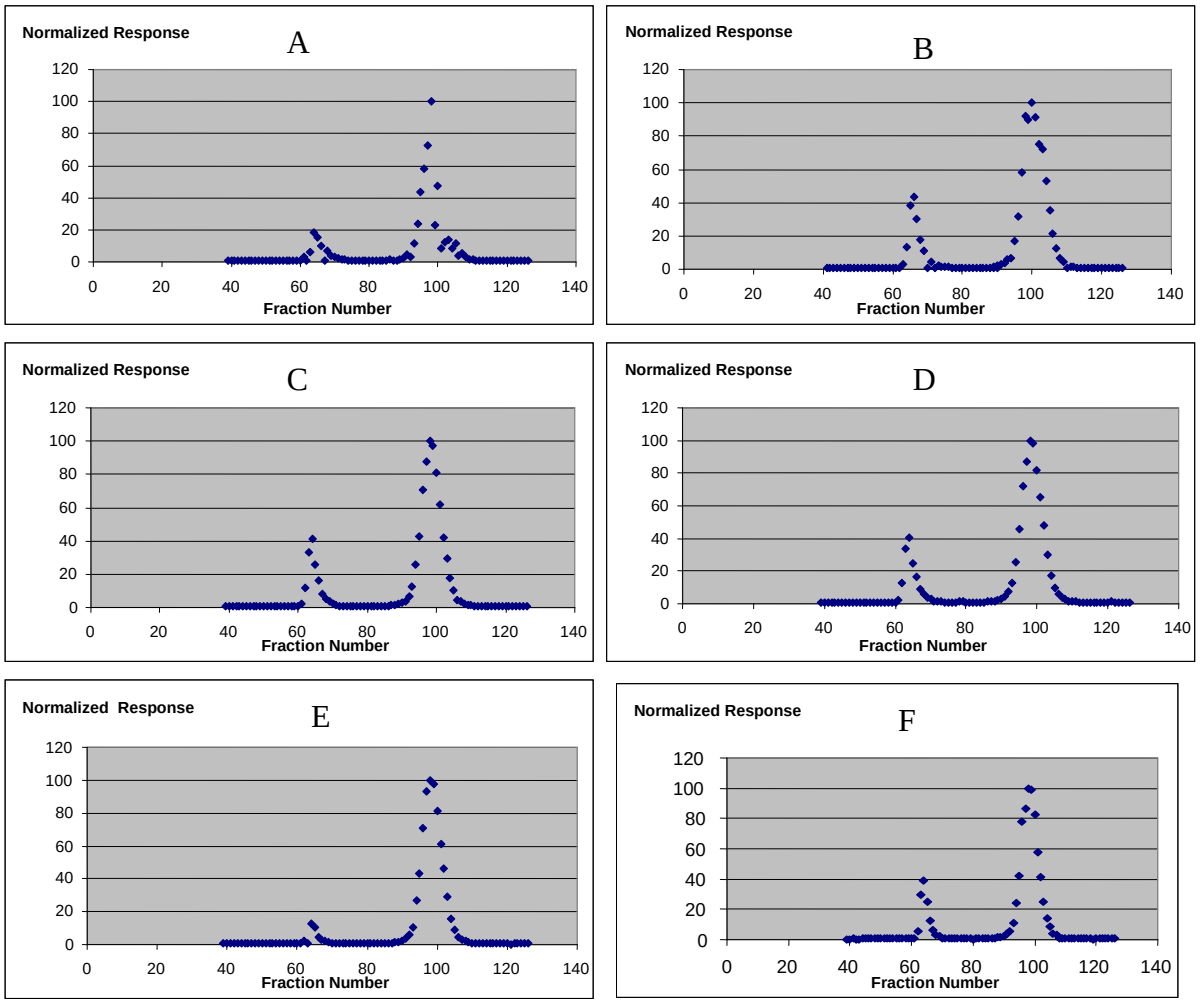

Figure 9. *Here the fractions seen in Figure 8 are analyzed again using the reference assay, diluted one to one in different diluents. In the different graphs the following diluents were used: A) Sample Diluent B)Bioprocess Diluent C) Human Serum and Plasma Diluent D) Standard Diluent E) PBS + 0.2% BSA F) Bioprocess Diluent + 10 mM EDTA.*

4.4.2 Assay design

All combinations of dimeric and tetrameric anti-IgG Affibodies as capturing and detecting reagents have been evaluated. Serial dilutions of untreated and heat treated, 30 min at 63 °C, monoclonal IgG were evaluated in all assays including the reference assay, i.e. dimeric anti-IgG Affibody as capturing and Fab'$_2$ as detecting reagent. The results are shown in Figure 10,

where heat treated and untreated IgG are grouped together in each assay, the heat treated sample shows a higher response in all assays except our reference assay where the aggregate containing sample shows a marginally lower response. All graphs also include the curve where untreated IgG was analyzed using the reference assay, blue stars. Among the assays the assay where dimeric anti-IgG Affibody was used as both capturing and detecting reagent, the assay where the dimer was used as capturing and the tetramer as detecting reagent and the assay where the tetramer form was used both as capturing and detecting reagent shows the greatest difference in response between untreated and heat treated IgG. The assay where the dimeric form was used both as capturing and detecting reagent gives very low response levels, thus this assay was rejected and the other assays amplifying the aggregated samples were tested further.

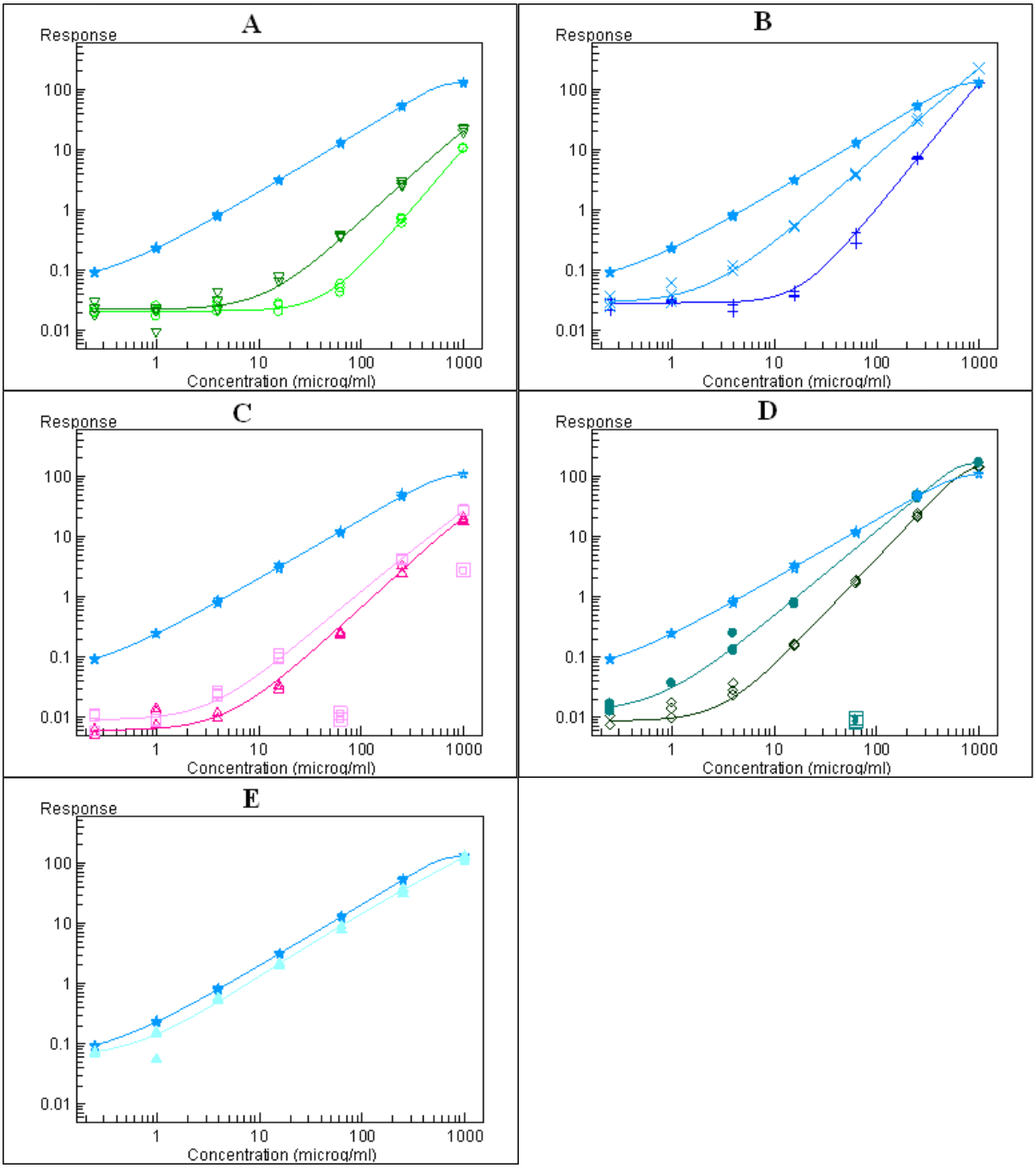

Figure 10. *Assays performed in CD microlaboratories using both untreated and heat treated. Untreated IgG analyzed using the reference assay is seen in all graphs, blue stars. Heat treated material is seen as the middle curve and untreated material is seen as the lowest curve in graph A to D. A) Dimeric anti-IgG Affibody as both capturing and detecting reagent. B) Dimeric anti-IgG Affibody as capturing reagent and the tetramer as detecting reagent. C) Tetrameric anti-IgG Affibody as capturing reagent and the dimer as detecting reagent. D) The tetramer form of anti-IgG Affibody was used both as capturing and detecting reagent. E) Dimeric anti-IgG Affibody used as capturing reagent and a Fab specific Fab'$_2$ fragment as detecting reagent, the reference assay. The light blue triangles depict heat treated IgG.*

Another experiment was performed to determine which of the assays using tetrameric anti-IgG Affibody as detecting reagent and the dimer form or the tetramer form as capturing reagent is best at detecting IgG aggregates. Here the heat treated IgG sample was separated using size exclusion chromatography into fractions that were collected into microtiter plates, 100 µl in each well. The fractions were analyzed using the two assays in CD microlaboratories and the results are shown in Figure 11. Concentrations were calculated

using a standard curve essentially containing monomeric IgG. The calculated concentrations and responses were plotted for all fractions analyzed. It is clear that the assay using the dimer form as capturing reagent gives a higher response for the high molecular peak. Thus this assay was chosen for further evaluations.

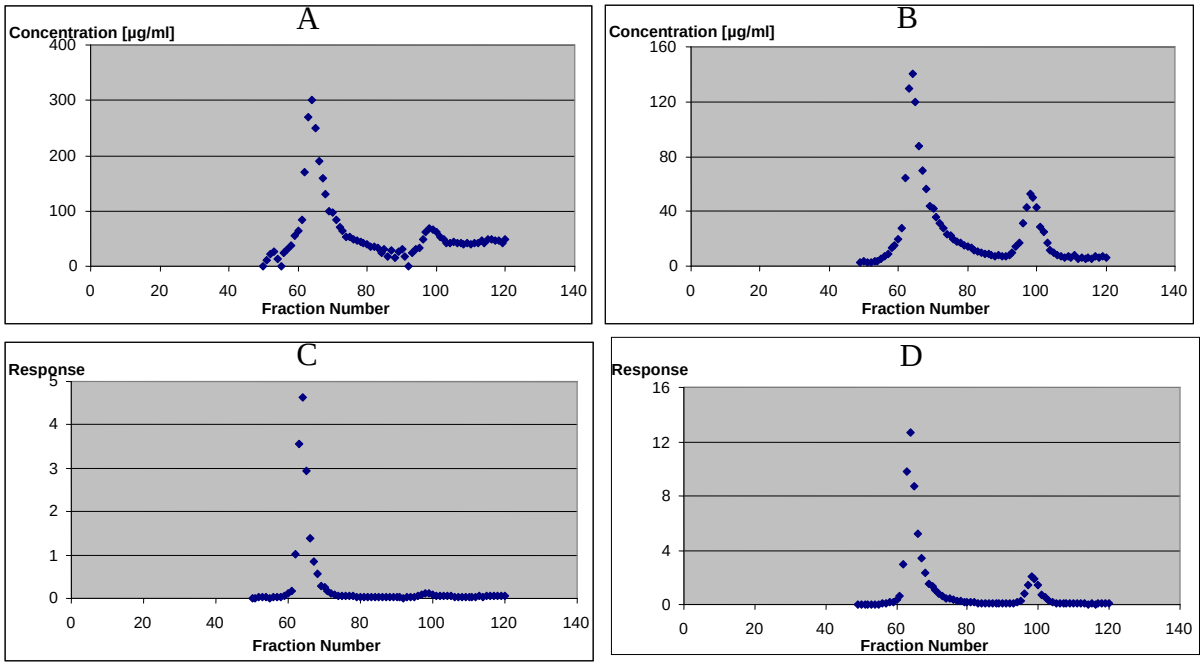

Figure 11. *Heat treated IgG samples, 30 min 63 °C containing 40% aggregates, was separated using size exclusion chromatography and fractionated into microtiter plates, 100 µl in each well. The fractions were analyzed using two assays. In graph A and C dimeric anti-IgG Affibody as capturing reagent and the tetramer form as detecting reagent. And in graph B and D Tetrameric anti-IgG Affibody both as detecting and capturing reagent.*

4.4.3 Detection of IgG aggregates

Heat treated IgG samples, 30 min 63°C, was separated using size exclusion chromatography with the fractions collected in microtiter plates. The fractions were analyzed in Bioaffy 20 HC using the assay where dimeric anti-IgG Affibody was used as capturing reagent and the tetramer form as detecting reagent, and the reference assay i.e. the assay using the same capturing reagent and Fab reactive Fab'$_2$ fragment of IgG as detecting reagent. Responses and concentrations vs. the fraction numbers are shown for both assays in Figure 12. It is clear that the first assay overrates the high molecular weight peak. The ratios between the responses from the first assay and the reference assay vs. the fraction numbers are also shown in Figure 12, as well as the corresponding ratios between the concentrations. Using the latter ratio, small aggregates such as dimers and trimers are also detectable, compare the concentrations calculated through the reference assay around fraction 70 to 80 and the ratio of concentrations for these fractions.

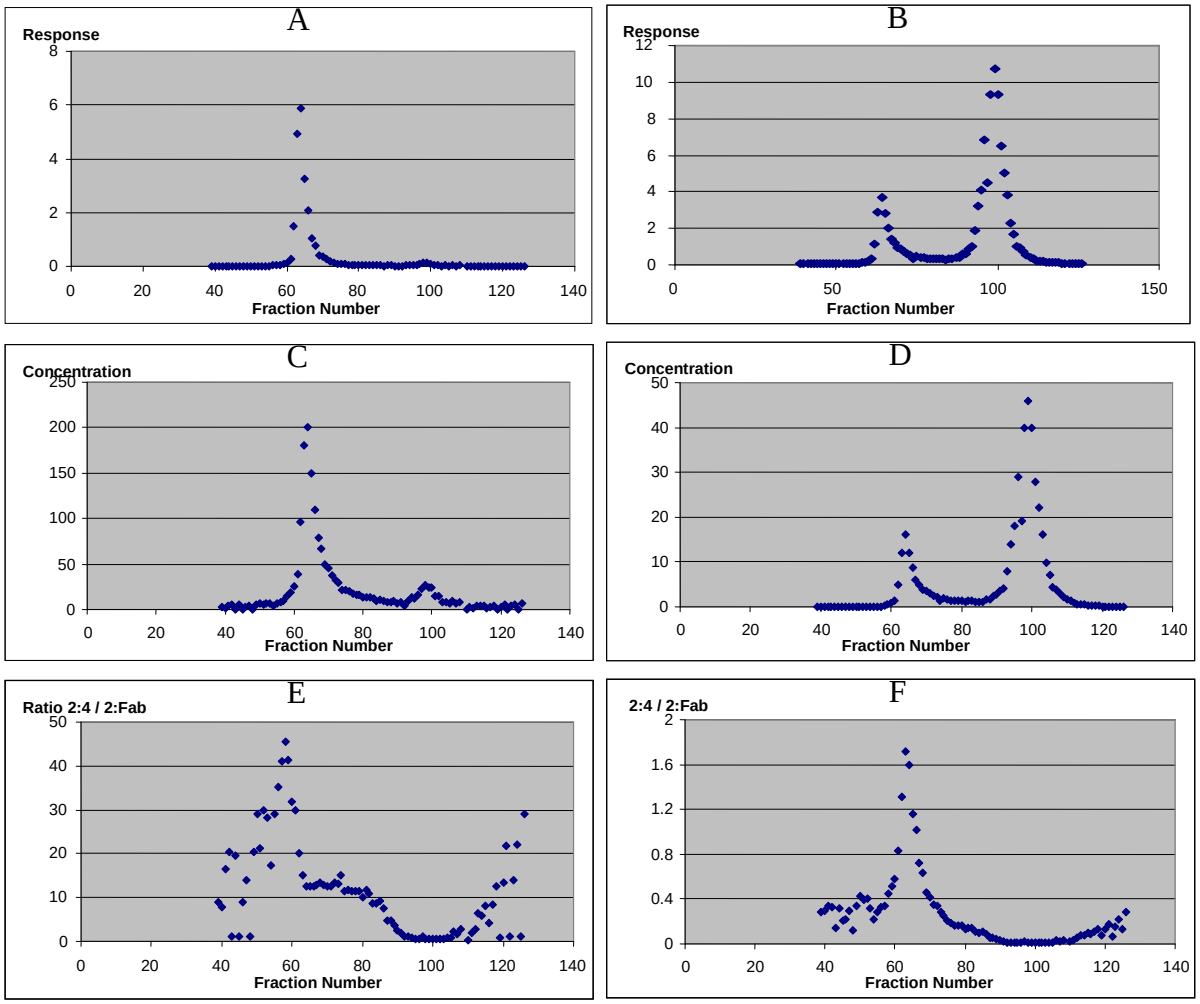

Figure 12. *Heat treated monoclonal IgG containing 37% aggregates was separated using size exclusion chromatography and fractioned in microtiter plates, 100 µl in each well, followed by analysis for IgG aggregates using two different assays. In graph A and C dimeric anti-IgG Affibody is used as capturing reagent and the tetramer form as detecting reagent. In graph B and D dimeric anti-IgG Affibody was used as capturing reagent and a Fab specific Fab'$_2$ fragment was used as detecting reagent. In graph E the ratios of the concentrations are shown vs the fraction number, and in graph F the ratios of the responses vs the fraction numbers are shown.*

4.4.4 Detection limit for proportion of aggregated IgG

The assay setup chosen as the setup best suited for detection of IgG is using two assays for analysis, both with dimeric anti-IgG Affibody as capturing reagent and the tetramer as detecting reagent in one assay and a Fab reactive Fab'$_2$ fragment of IgG as the detecting reagent in the other assay. The working interval for this assay setup seems to be from around 2 µg/ml to around 200-300 µg/ml. This can be deduced from inspection of Figure 10. To get an understanding of the detection limit of the system four separate experiments were performed. In the first two experiments heat treated IgG was separated using size exclusion chromatography. The aggregated and the monomer peak were pooled separately and monomeric IgG samples were spiked with various amounts of aggregates and analyzed using the two assays. In the first experiment a total IgG concentration of 50 µg/ml was used, 20 µg/ml was used in the second experiment. In the other two experiments a total of eight different monoclonal IgG's were heat treated, analyzed for aggregates content using size exclusion chromatography and analyzed with the two assays in Bioaffy 20 HC microlaboratories. The two latter experiments were conducted to see how this method behaved using different monoclonal IgG$_1$'s, one to get a value for the detection limit as well.

These experiments are described in more detail below and the results are shown in Figures 13-17 and Tables 4-7.

The concentrations achieved from each assay in the first experiment are showed in Figure 13 vs. the proportion of aggregated IgG present. The ratios of concentrations from the first assay over the latter assay are also shown vs. the proportion of IgG aggregates present. The graph showing the concentration achieved from the first assay leads us to believe that the presence of 8% aggregates in an IgG sample containing 50 µg/ml IgG is detectable with this assay setup. The sample containing 4% aggregates gives concentrations a bit higher than when no aggregates are present, but very close to the response variation in the samples containing 1% and 2% aggregated IgG, which are inseparable from samples containing monomeric IgG only, which implies that a smaller proportion of IgG's might be detectable. Inspecting the graph in Figure 13 showing the ratio of concentrations as determined using the two assays, a ratio of 1.2 seems to be equivalent with a significant level of aggregates present. This number is only exceeded by samples containing 4% aggregates and more, thus giving a lower detection limit than using only one assay.

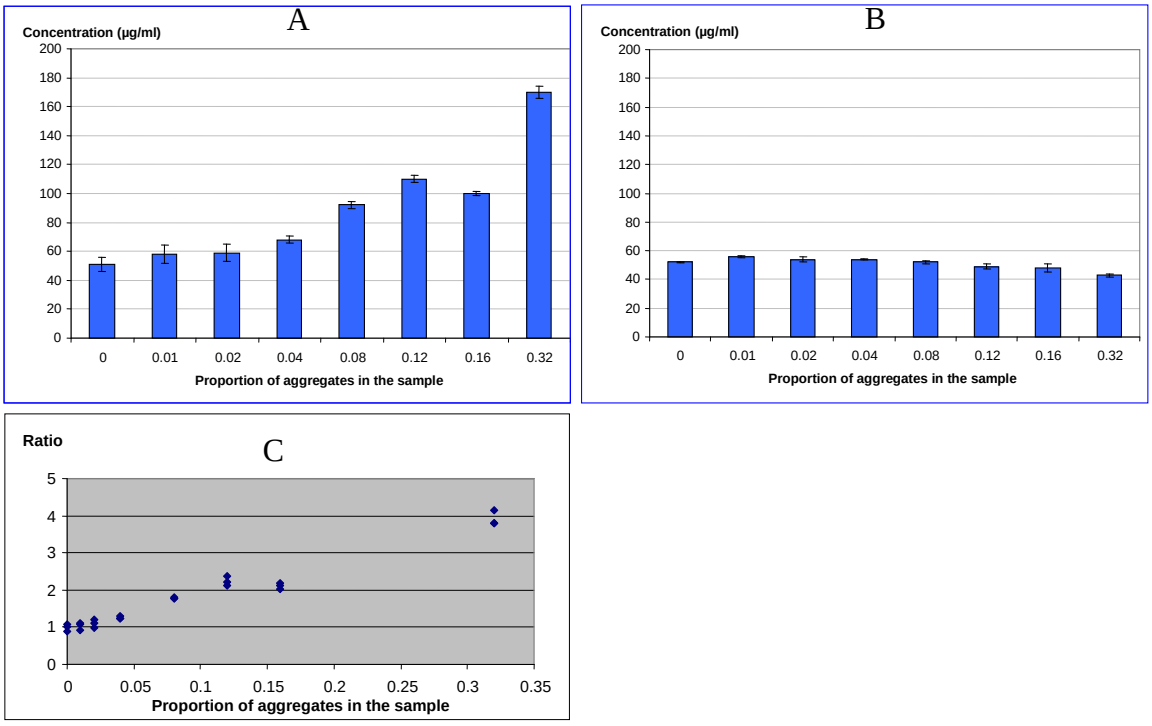

Figure 13. *Monomeric IgG sample containing a defined proportion of aggregated IgG and a total IgG concentration of 50 µg/ml has been analyzed using the two assays. Dimeric anti-IgG Affibody as capturing reagent in both assays and the tetramer form of the molecule as detecting reagent in graph A and a Fab reactive Fab'$_2$ fragment as detecting reagent in graph B. In graph C the ratio between the concentrations achieved using the different assays vs. the proportion of aggregates present in the sample are seen.*

The results from the second experiment are seen in Figure 14. Here a total protein concentration of 20 µg/ml was used. A ratio implying a significant amount of aggregates present in the sample deduced from this experiment is 1.3 and is only exceeded by the sample containing 16% aggregates.

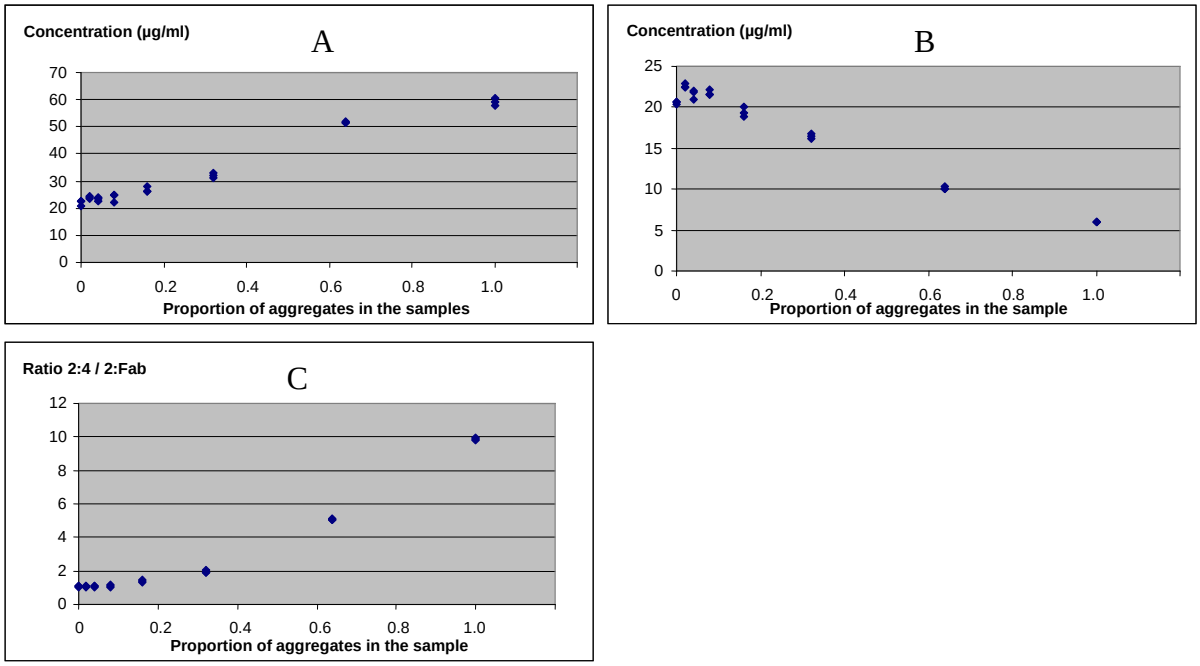

Figure 14. *A repeat of the experiment shown in Figure 13, here with a total IgG concentration of 20 µg/ml and an aggregate content ranging from 0 to 100%. Graph A shows the concentration calculated using dimeric anti-IgG Affibody as capturing reagent and the tetramer form as detecting reagent. Graph B shows the concentration calculated using dimeric anti-IgG Affibody as capturing reagent and a Fab specific Fab'$_2$ fragment of IgG as detecting reagent. Graph C shows the ratio of the concentrations calculated in graph A over the concentrations calculated in graph B.*

In experiment three and four, eight immunoglobulin G_1's with human myeloma origin were made available, originally used by Mats Inganäs during his doctoral studies in the early 80's[28]. These immunoglobulins were stored in –80°C until thawed for this experiment. In experiment three, two of the eight available IgG_1's were heated for 5, 10, 20 and 30 minutes in a water bath at 63°C. The different samples were analyzed using size exclusion chromatography on a Superdex 200 HR 10/30 column in an ÄKTApurifier system to detect the amount of aggregates formed. The results are summarized in Table 4. The different samples were analyzed using the two assays as well, and the results are seen in Figure 15 and 16.

Table 4. *Two human myeloma IgG's were heat treated for different times at 63°C, where after the samples were analyzed using gel filtration on a Superdex 200 HR 10/30 column connected to an ÄKTApurifier system.*

Sample Identity	IgG type	Time for heat treatment	Amount of aggregates present
IgG 12	IgG_1 κ	0 min	0 %
IgG 12	IgG_1 κ	5 min	5 %
IgG 12	IgG_1 κ	10 min	9 %
IgG 12	IgG_1 κ	21 min	15 %
IgG 12	IgG_1 κ	30 min	18 %
IgG 12	IgG_1 κ	40 min	22 %
IgG 13	IgG_1 λ	0 min	1 %
IgG 13	IgG_1 λ	5 min	3 %
IgG 13	IgG_1 λ	10 min	7 %
IgG 13	IgG_1 λ	21 min	12 %
IgG 13	IgG_1 λ	30 min	14 %
IgG 13	IgG_1 λ	40 min	17 %

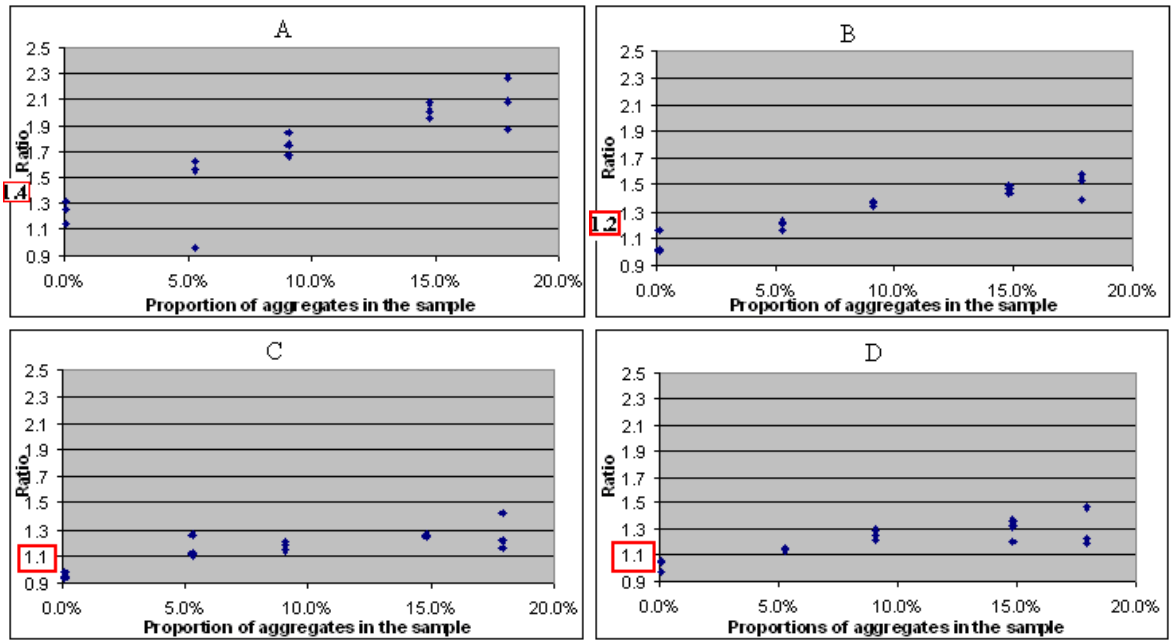

Figure 15. *IgG samples, IgG 12, with different amounts of aggregates and different total IgG amounts were analyzed. In graph A a total IgG amount of 20 µg/ml was used, the amount was 40 µg/ml in graph B, 80 µg/ml in graph C, and 160 µg/ml in graph D. The assay where dimeric anti-IgG Affibody was used as capturing and the tetramer form as detecting reagent, and the assay using the same capturing reagent and Fab reactive Fab´$_2$ fragment from IgG as detecting reagents were used for analysis. The ratio of the concentrations calculated using the different assays is plotted vs. the amount of aggregates as calculated from the chromatographic analysis. The ratio deduced as implying a significant amount of aggregates is circled in red in each graph.*

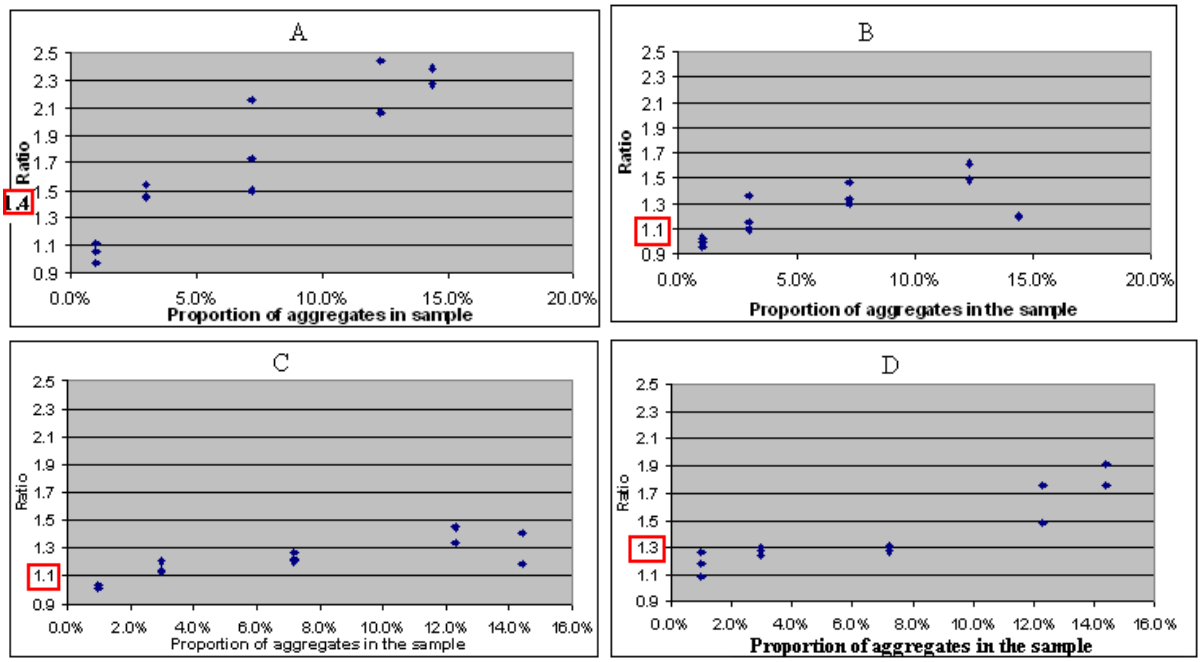

Figure 16. *IgG samples, IgG 13, with different amounts of aggregates and different total IgG amounts were analyzed. In graph A a total IgG amount of 20 µg/ml was used, the amount was 40 µg/ml in graph B, 80 µg/ml in graph C, and 160 µg/ml in graph D. The assay where dimeric anti-IgG Affibody was used as capturing and the tetramer form as detecting reagent, and the assay using the same capturing reagent and Fab reactive Fab´$_2$ fragment from IgG as detecting reagents were used for analysis. The ratio of the concentrations calculated using the different assays is plotted vs. the amount of aggregates as calculated from the chromatographic analysis. The ratio deduced as implying a significant amount of aggregates is circled in red in each graph.*

The ratios found as implying a significant amount of aggregates in the three above mentioned experiments are not the same. However, examining them more in detail one ratio was chosen for each concentration, see Table 5. Inspecting graph B in Figure 10 a different ratio for each concentration is expected since the curves with and without aggregates are nonparallel. A ratio implying a significant amount of aggregates when the total IgG concentration is 20 µg/ml is 1.4. Using this ratio on the values from the second experiment two out of three samples containing 16% aggregates are missed, using this ratio on the figures from the third experiment samples containing 3% aggregates are detected. When the total IgG concentration is 40 to 50 µg/ml a ratio of concentrations of 1.2 will detect samples containing 4% aggregates. When the total IgG concentration is 80 to 160 µg/ml a ratio of 1.1 will detect samples containing 3% aggregates.

Table 5. *A compilation of the detection limits and the corresponding ratios of concentrations from the three experiments shown above is seen here. The ratios corresponding to the detection limit is dependent on the concentration used. If the sample where 16% aggregates are missed is disregarded the detection limit is around 3% to 4% aggregates.*

Total IgG concentration	**Ratio**	**Detection limit**	**Notes**
20 µg/ml	1.4	16% aggregates	Samples containing 3% aggregates can be detected, but two out of three samples containing 16% aggregates are missed.
40-50 µg/ml	1.2	4% aggregates	
80-160 µg/ml	1.1	3% aggregates	Two out of three samples containing 1%. aggregates were detected.

In the fourth experiment six different myeloma IgG samples were heat treated for 40 minutes in 63°C. Both before and after heat treatment these samples were analyzed using size exclusion as well, the results are summarized in Table 6. It is obvious that the different IgG's reacted very differently to the heat treatment with the lowest degree of aggregate formation at 1% and the highest degree of aggregate formation 98%. 25 µl IgG at 2 mg/ml was applied to the column each time, thus the total area should be approximately the same. The somewhat smaller total area in heat treated IgG 9 indicates the presence of aggregates so large they are unable to penetrate the matrix, thus there might be even more than 98% aggregates in this sample.

Table 6. *Different IgG samples untreated and heat treated for 40 minutes in 63°C was analyzed to determine the amount of aggregates present using size exclusion chromatography in a Superdex 200 HR 10/30 column connected to an Äkta system.*

Sample Identity	**IgG Type**	**Aggregate amount - untreated sample**	**Aggregate amount - treated sample**	**Total area - heat treated sample [AU*min]**
IgG 1	IgG_1 λ	0 %	19 %	80
IgG 3	IgG_1 κ	0 %	59 %	87
IgG 5	IgG_1 κ	0 %	4 %	81
IgG 6	IgG_1 λ	0 %	1 %	87
IgG 8	IgG_1 λ	0 %	12 %	94
IgG 9	IgG_1 κ	0 %	98 %	68

The IgG samples were analyzed in a Bioaffy 20 HC microlaboratory using the two assays. Graphs of the responses vs. the known concentrations are shown in Figure 17. The pink curves using circles to depict the data points shows the response achieved from the first assay

and the blue curves using triangles to depict data points are achieved from an analysis using the second assay. The lighter shades of blue and pink having filled data points are used for heat treated samples, the others show the untreated samples. It is obvious that aggregate containing samples of the myeloma IgG_1 available were detected using the assays as well.

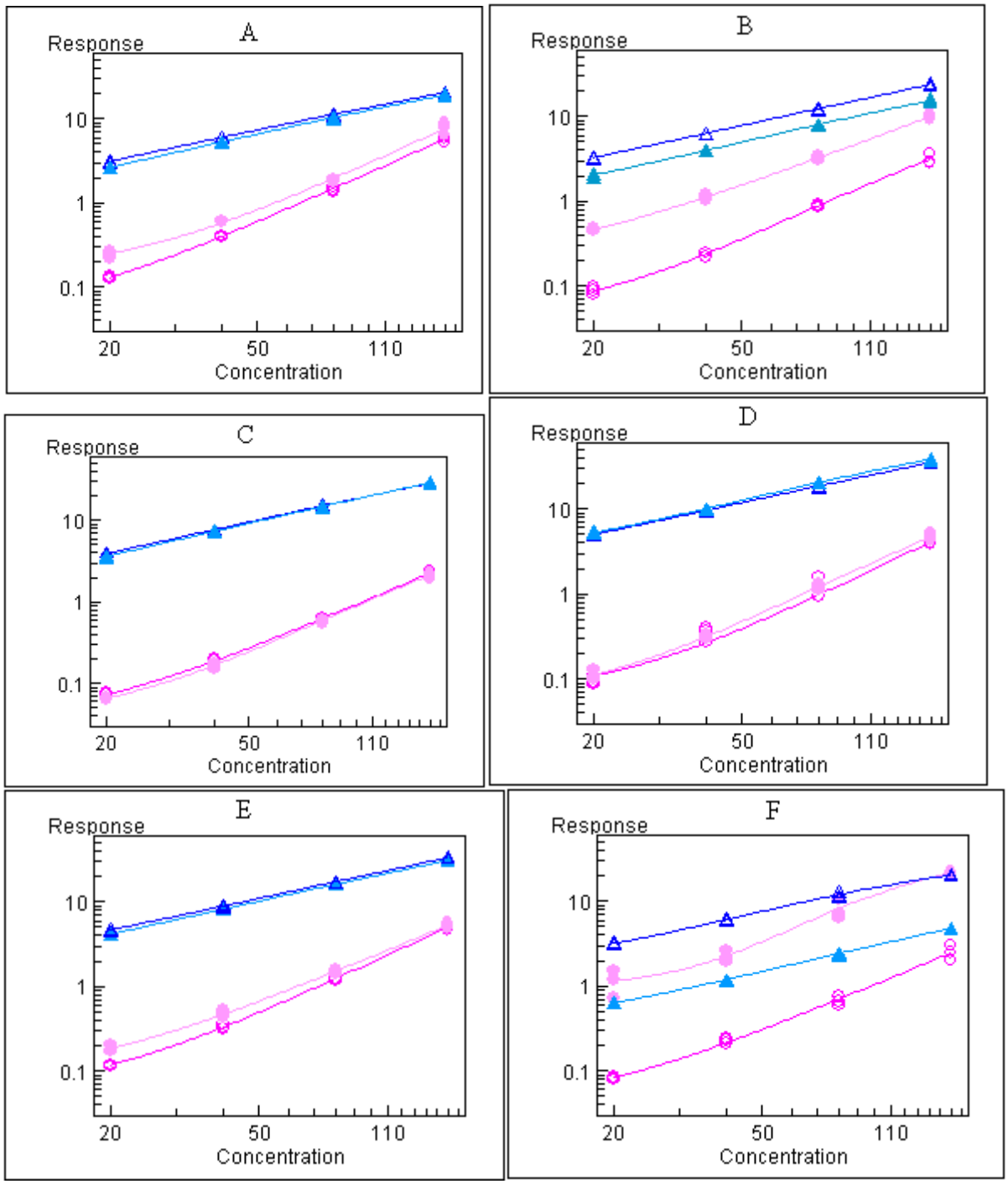

Figure 17. *Different human IgG myeloma proteins were analyzed using the assay where dimeric anti-IgG Affibody was used as capturing reagent and the tetramer form as detecting reagen, pink circles, and the assay using the same capturing reagent and a Fab´$_2$ fragment from IgG as detecting reagent, blue triangles. The curves in a lighter shade and with filled circles/triangles are the ones where the heat treated IgG were analyzed, and the curves with a darker shade and data points without fill show untreated IgG samples. The IgG's in the different graphs are as follows: IgG 1 in graph A, IgG 3 in graph B, IgG 5 in graph C, IgG 6 in graph D, IgG 8 in graph E, and IgG 9 in graph F. See Table 5 for the amount of aggregates present in the different heat treated samples.*

5. Discussion

5.1 Labeling

The labeling processes performed in this project are quite straight forward. Care should however be taken when selecting an appropriate purification method since the anti-IgG Affibody molecules used are quite small. The Nanosep centrifugal devices used here are suitable. When labeling the dimers of anti-IgG Affibody an appropriate time for reduction is vital. Biotinylated dimeric anti-IgG Affibody can be purchased through Affibody (10.0623.02.0005, Affibody, Bromma, Sweden), and the results when performing the above mentioned assays using this reagent are comparable to using dimeric anti-IgG Affibody labeled as described in section 3.3.4.

5.2 Possible mechanisms behind the chosen assays

Anti-IgG Affibody binds to the Fc portion of IgG at the junction between constant domain two and three[13]. This means that an assay where dimeric anti-IgG Affibody is used as capturing and the tetramer form as detecting reagent will give a maximum signal equivalent to one detecting molecule per monomer. However when low amounts of essentially monomeric IgG is analyzed a very low signal is achieved, see Figure 10 B. This might be explained by both sites being occupied by capturing reagents when monomeric IgG is present in low concentrations. It has been shown that one anti-IgG molecule can not bind to both sites of IgG even though containing multiple domains capable of IgG binding[12], thus two capturing reagents are needed to bind each IgG molecule for both sites to be occupied before the detecting reagent is added. When IgG is present in higher concentrations both sites will not be occupied by capturing reagents in all molecules thus there will be a signal. IgG aggregates will give a higher signal than monomeric IgG since there are two binding sites for anti-IgG Affibody on every IgG monomer and in aggregates capturing reagents are unable to bind all. See Figure 18 and 19 for a schematic of this theory.

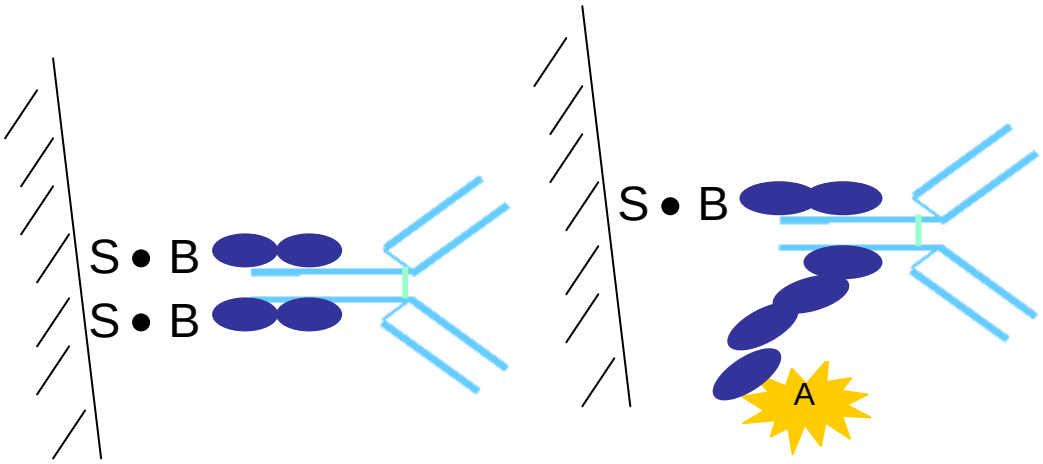

Figure 18. *A schematic picture of one hypothesis explaining the mechanism behind the assay utilizing dimeric anti-IgG Affibody as capturing reagent and the tetramer form as detecting reagent. Anti-IgG Affibody is depicted by blue circles, IgG by light blue Y shapes, Alexa 647 by the yellow polygon and streptavidin and biotin by an S and a B respectively. The left picture illustrates the hypothetical mechanism for low concentrations of IgG, yielding low or no signal, and the right picture for higher IgG concentration yielding higher signal.*

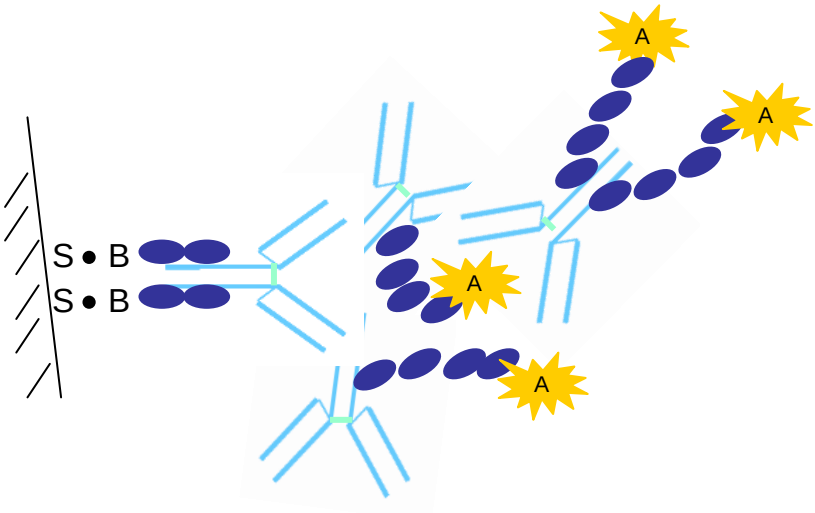

Figure 19. *A schematic picture of one hypothesis of the mechanism behind the assay utilizing dimeric anti-IgG Affibody as capturing reagent and the tetramer form as detecting reagent. Anti-IgG Affibody is depicted by blue circles, IgG by light blue Y shapes, Alexa 647 by yellow polygons and streptavidin and biotin by an S and a B respectively. This assay gives a quite high signal for aggregates.*

The assay used as reference assay here where dimeric anti-IgG is used as capturing reagent and a Fab reactive Fab'$_2$ fragment for detection will give a one to one signal for monomers. The signal for aggregates will be approximately one to one for each monomeric entity in the aggregate. See Figure 20 for a schematic of this theory. Large aggregates might give a lower signal than one to one, due to steric hindrance or aggregates too large to enter the pores of the matrix. This is seen in Figure 14 and 17. The signal from this assay is a bit lower than it could be due to unlabeled Fab'$_2$ being added to the detecting reagent, this was done to increase the working range of IgG concentrations detected by this assay. Calculating the ratio of the two assays however, is likely to favor detection of aggregates when present in the sample.

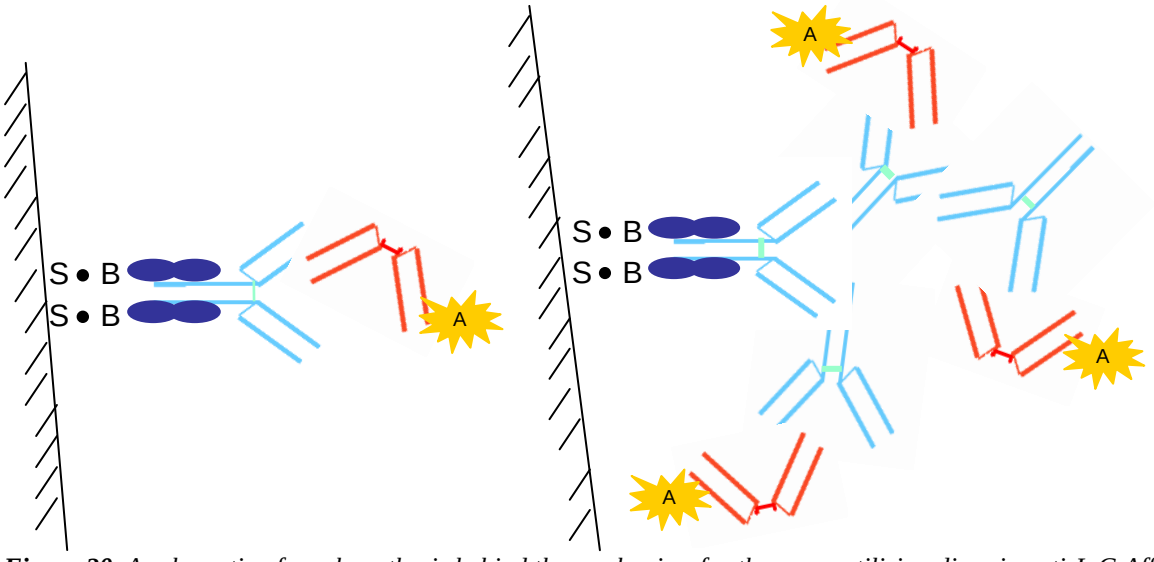

Figure 20. *A schematic of one hypothesis behind the mechanism for the assay utilizing dimeric anti-IgG Affibody as capturing reagent and a Fab specific Fab'$_2$ fragment as detecting reagent. Anti-IgG Affibody is depicted by the blue circles, IgG by the light blue Y shapes, the Fab'$_2$ fragment by the red V shapes, Alexa 647 by the yellow polygons, and streptavidin and biotin by the S and B respectively.*

6. Conclusion

6.1 Assay procedure

The assay setup most suitable for detecting the proportion of IgG aggregates in IgG samples of the setups examined here is using a combination of two assays, both using dimeric anti-IgG Affibody as capturing reagent, one using the tetramer form as detecting reagent and the other using Fab specific Fab'_2 fragment of IgG as detecting reagent. The ratio of the concentrations calculated using the first assay over the concentrations calculated using the latter assay shows the presence of aggregates when exceeding a certain proportion dependent on the total IgG concentration in the sample.

In this setup the IgG of interest should be analyzed as samples with concentrations calculated using standard curves of essentially monomeric IgG in both assays. The microlaboratory used is a Bioaffy 20 HC microlaboratory.

6.2 Assay performance

The total IgG concentration range wherein this assay setup can be used is from around 2 µg/ml to around 200 µg/ml. This concentration range is acceptable for this type of assay, but a wider range would be desired. The aggregate content detectable seems to be around 3% to 4%. However this method does not seems to correctly identify 100% of the aggregate containing samples with an aggregate content up to 16%. Therefore duplicate or triplicate determinations might be justified.

6.2 Future perspectives

Further tests of this procedure on nonpurified antibodies developed for therapeutical purposes should be performed to assure a good behavior of the assay setup for these antibodies as well. A few different antibodies have been tested for aggregate content using this method and this assay setup seems to be applicable for them all. However recombinant antibodies such as those developed for therapeutical purposes today have not been tested, neither in purified form nor in cell supernatants, due to difficulties in acquiring any from open sources. Further tests should give more statistics about the method which is needed before commercial use. However this method holds great promise as a fast and simple way to measure the aggregate content of therapeutic antibodies directly in cell supernatants. The method can easily be used to study the aggregate content in a great number of samples which is desirable when screening for different properties in the development of therapeutic antibodies. Therefore this method can be a great asset for companies developing therapeutic antibodies as well as for Gyros.

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