STELLINGEN

1. De vorm van een IgG molecuul kan gebruikt worden om zijn oriëntatie op een oppervlak te sturen.

   Dit proefschrift.

2. Gedeeltelijk voorcoaten van een oppervlak is een effectieve manier om de conformatie van geadsorbeerd IgG te controleren.

   Dit proefschrift.

3. De grote herseninhoud van mensen heeft niet alleen te maken met denkvermogen maar duidt vooral op het vermogen om in een ingewikkelde sociale structuur te leven.

   Barton, R.A., Proc. R. Soc. Lond. B (1998) 265, 1933.

4. Als de opheldering van de structuur van DNA door Watson, Crick, Wilkins en Franklin een mijlpaal is in de wetenschap, dan is het in kaart brengen van het menselijk genoom slechts een hectometerpaal op weg naar een volgende mijlpaal.

5. Een land dat ouderen die in een sociaal isolement verkeren niets anders biedt dan de pil van Drion leeft in een enorme armoede.

6. De daklozenkrant is een volwaardig tijdschrift en zou door meer mensen op zijn juiste waarde geschat worden indien het blad in de boekhandel verkocht zou worden.

Stellingen behorende bij het proefschrift
"Immunoglobulin adsorption on modified surfaces".
M.G.E.G. Bremer, Wageningen, 25 juni 2001.

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IMMUNOGLOBULIN ADSORPTION ON MODIFIED SURFACES

M.G.E.G. Bremer

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Chapter 7 - Oriented IgG adsorption induced by a 'molecular sieve' in a microplate assay

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Chapter 5 - A.W.P. Vermeer, M.G.E.G. Bremer and W. Norde
Biochimica et Biophysica Acta (1998) 1425, 1

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Chapter 7 - submitted to *Analytical Chemistry*

Chapter 1

GENERAL INTRODUCTION

Abstract

Monoclonal antibodies are highly selective molecules that are widely used as the sensing elements in a range of technical and medical applications. In many of these, antibodies are physically adsorbed onto solid surfaces. The interactions involved in physical adsorption of proteins are described. Physical adsorption often results in an undesired loss of biological activity. In this thesis, a novel approach for controlling the physical adsorption of antibodies, and consequently their biological activity is introduced. This method is based only on the non-spherical shape of the immunoglobulins. Finally, an outline of this thesis is given.

1.1 Introduction

1.1.1 The immune system, antibodies and antigens

The immune system, which is specific to vertebrates, is a sophisticated defensive mechanism which offers protection against foreign substances like microorganisms and viruses. An important role in this system is played by a special kind of soluble proteins, immunoglobulins, which function as antibodies. Antibodies are produced by differentiated blood cells in response to the presence of foreign material. Foreign substances that induce the formation of antibodies are called antigens. Antibodies have the unique feature of recognizing and specifically binding the antigen that initially caused its synthesis. In this way, antibodies act as the recognition elements of the humoral immune response. The binding of the antigen by the matching antibody initiates processes that inactivate and eliminate the antigen.

Each antibody-producing cell is capable of making only one unique type of antibody. Since many different antibody-producing cells are involved in the immune response, serum (the aqueous part of blood) contains a mixture of various types of antibodies, i.e. polyclonal antibodies. From a biological point of view, polyclonal antibodies increase the probability of binding intruding antigens and thus improve the protection against infections. However, in research and industrial applications the using of polyclonal antibodies shows several disadvantages, e.g. a lack of specificity and variable composition of the antibody mixture. In 1975, Milstein and Köhler [1] developed the hybridoma technique, which enables the production of large amounts of antibodies of one single molecular species (monoclonal antibodies) by fusing a single antibody-producing cell with a single tumor cell. In this way, series of monoclonal antibodies directed against one particular antigen can be produced, and for each particular application those antibodies that are most suited can be selected.

Due to their high specificity and sensitivity, monoclonal antibodies have proven to be excellent probes for the recognition of, among others, proteins, peptides, lipids, and hormones. Nowadays, they are widely used in a range of technical and medical applications including affinity columns for preparative purification of protein mixtures or for separating different molecular forms of a protein, and in consumer goods like toothpaste and shampoo to inactivate the microorganisms that cause caries and dandruff, respectively. In medical practice, monoclonal antibodies are used to locate and possibly destroy tumor cells. Another group of health care applications is the use of monoclonal antibodies in in-vitro diagnostic tests. Here, one of the large-scale applications is that of screening blood, e.g. in blood banks,

for the presence of diseases like HIV and hepatitis. Another, less life-threatening application is the detection of the human pregnancy hormone [2,3].
It may be concluded that monoclonal antibodies play an increasingly important role in many disciplines, including biomedical engineering, biotechnology and environmental science.

1.1.2 Applications, immobilisation and biological activity

Many applications of antibodies require these to be attached onto solid surfaces in order to facilitate the concentration and visualisation of antibody-antigen complexes. Applications mainly involve immunoglobulin G (IgG) because this is the principal antibody in serum. In immunoassays such as Enzyme-Linked ImmunoSorbent Assay (ELISA) [4,5] and latex agglutination assays, [6,7] IgG is often immobilized (attached) to polystyrene microtiter wells or -particles. To achieve high sensitivity in applications maximized biological activity of the immobilized immunoproteins is a necessity. The biological activity of IgG is defined as the number of molecules of antigen bound per molecule of IgG. As the structure of IgG molecules is such that one molecule of IgG can bind two molecules of antigen the maximum value for the biological activity is two.

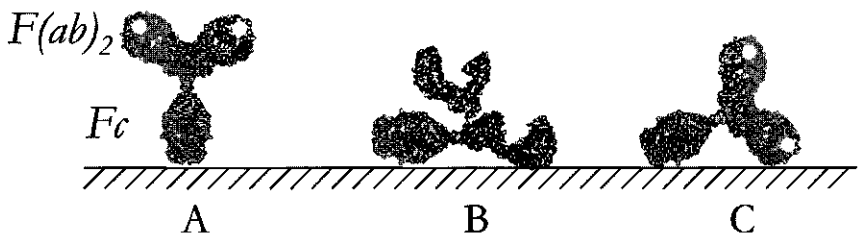

Figure 1.1: The effect of physical adsorption on the biological activity of IgG (schematic). (A) Optimal orientation and conformation and, hence, maximal biological activity. (B) Reduced activity caused by conformational changes or (C) reduced accessibility of antigen binding sites. The antigen binding sites are represented by the white dots at the top of the F(ab) parts.

The predominant immobilization procedure in industrial applications is physical adsorption, mainly because it is relatively easy to perform. Physical adsorption, however, often results in an undesired loss of biological activity [5,8]. This loss of activity may be caused by changes in the specific folding (the conformation) of the antibody or by a reduced accessibility of the antigen binding sites by blocking (figure 1.1). The antigen binding sites become inaccessible for antigens when they

are too close to the sorbent surface, for instance when the IgG molecules are adsorbed with their antigen binding sites oriented towards the sorbent surface. To overcome these problems several alternative immobilization techniques for IgG have been developed. Thereby it is tried to force the antigen binding parts towards the solution and hence, keeping them accessible to bind antigens. These techniques include covalent coupling [8] of the IgG molecules to the solid surface, streptavidine bridging [5], immobilisation using a previously adsorbed antiglobulin [5] or protein A [9]. Although some of these techniques have been reported to increase the average biological activity of IgG molecules, they may also introduce new complications: more costly manufacturing procedures, the need for expensive chemicals and dedicated solid surfaces, to mention some.

This leads us to the **central theme** of this thesis:

Controlling the orientation and conformation of adsorbing IgG molecules to achieve optimum biological activity of the adsorbed layer avoiding chemical modification of the IgG molecule or the solid surface. In this thesis we present a new method to achieve oriented physical adsorption of IgG. This concept is based on the anisodimensionality of IgG molecules and resembles 'molecular sieving' on the sorbent surface.

1.2 Factors determining physical adsorption of IgG

In order to control and manipulate the adsorption process, the characteristics of the IgG molecules and the relevant interactions between the different components in the system (i.e. the IgG molecules, the solvent, the solutes, pre-adsorbed molecules and the solid surface) must be understood.

1.2.1 Immunoglobulin G

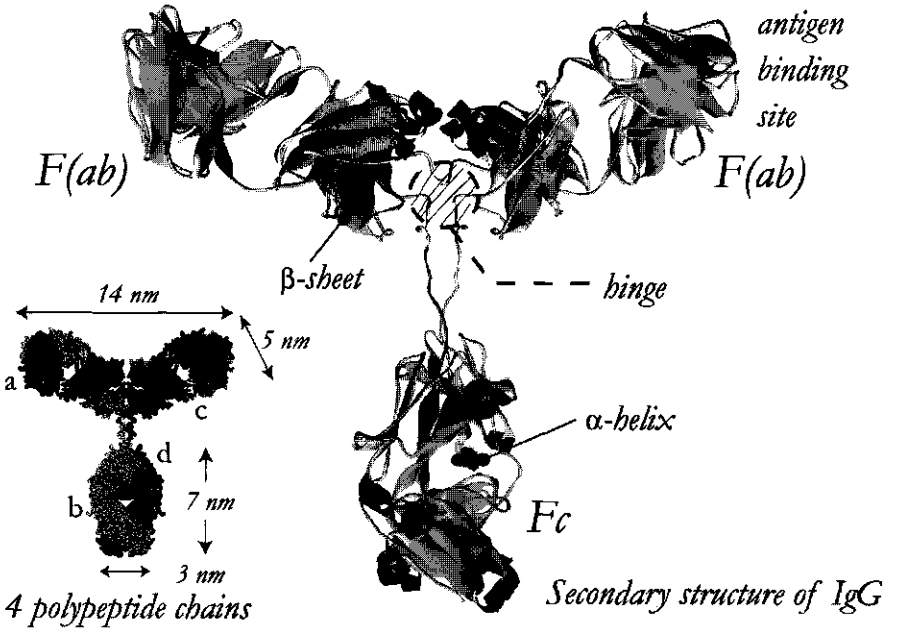

Figure 1.2: Secondary structure and space filling model of IgG.

An IgG molecule is composed of four polypeptide chains: two identical ones each containing ca. 450 amino acids (figure 1.2 chain b and d) and two identical ones each containing ca. 220 amino acids [10] (figure 1.2a and c), which are called heavy and light chains, respectively. The two heavy chains are joined by disulphide bonds and one light chain is connected by a disulphide bond to each heavy one. Interactions between the amino acids induce a specific folding of the chains and the formation of compact globular domains, four in each heavy chain and two in each light chain. These domains have a predominantly hydrophobic interior in which the antiparallel beta sheet conformation is the main structure element [11,12] (figure 1.2). On the exterior of the domains mainly the more polar and ionogenic amino acids are situated giving the molecule its polyelectrolyte character and its surface charge.

IgG molecules are asymmetric and Y-shaped. Traditionally IgG molecules are divided into three functional parts. Two of these parts are identical and contain an antigen binding site at their far end and are called F(ab) (ab stands for antigen-

binding, F for fragment). The antigen binding site is formed by combined regions in the N-terminal domains on the light and heavy chains. The third part, called Fc (because it crystallises readily) does not bind antigens but has other biological functions such as attaching the molecule to receptors on foreign cell walls. The three fragments are joined by a hinge. This hinge allows variation in the angle between the two F(ab) units [10]. The whole IgG molecule, the top part of the molecule (i.e., the two linked F(ab) fragments), and the bottom part (i.e., the Fc part) have a molecular weight of 150 kDa, 100 kDa and 50 kDa, respectively.

1.2.2 Interactions involved in physical adsorption of proteins

The Derjaguin-Landau-Verwey-Overbeek (DLVO) theory [13,14] has often been used to describe interactions between biological colloids [15,16]. Had the long-range electrostatic double layer forces and van der Waals forces been the sole forces between proteins and surfaces, such an application would have been appropriate. However, protein-surface interactions are complicated by additional non-DLVO contributions such as solvent structure mediated forces [16,17] and structural rearrangements [18,19]. Although these forces are generally short-range, i.e. acting within separations of a few nm, they can have a dominating impact. Therefore, we will use DLVO theory to describe only the long-range interactions between an IgG molecule and a sorbent surface.

In general, interactions between proteins and surfaces involve the following major contributions:[20-22] (a) electrostatic interactions between the protein and the sorbent, (b) London-van der Waals interactions, (c) dehydration of (hydrophobic) parts of the adsorbent and/or protein molecules and (d) structural rearrangements in the protein molecule.

Protein adsorption is promoted by those interactions that lead to a decrease in the Gibbs energy of the system upon adsorption. This means that interactions that decrease the enthalpy and/or increase the entropy of the system (at constant temperature and pressure) promote protein adsorption. A brief description of electrostatic interactions, van der Waals interactions, hydrophobic dehydration and structural rearrangements will be given below.

Electric double layer interactions

In most systems both the protein molecule and the sorbent surface are electrically charged. These surface charges are fully compensated by an excess of counterions and a deficit of co-ions in the adjoining solution. As a rule, a fraction of the counter ions and co-ions is specifically adsorbed on the surface. The surface

charge, together with its countercharge, forms an electric double layer and as a whole the system is electroneutral. Upon adsorption of the IgG molecule the double layers of the molecule and the sorbent surface overlap, giving rise to electrostatic interaction. Yet, accumulation of net charge in the protein-sorbent contact region is highly unfavourable due to the non-aqueous character of this region. The charge density in this region may be regulated by charge adjustment on the protein [23] and/or the sorbents surface or by the uptake of low molecular weight ions in this region [24]. However, this uptake of ions from an aqueous environment into the non-aqueous region is chemically unfavorable. In general, electrostatic interactions stimulate adsorption when the protein and the surface have opposite charge sign and when the charge density on the protein just matches that on the sorbent surface [20].

London-Van der Waals interaction

For macrobodies (except for highly polar materials) the London-van der Waals interaction is the most dominant term in the van der Waals interaction [25]. In gases, London-van der Waals, or dispersion, forces originate from the correlation between time dependent dipoles in atoms or molecules and depends only on the polarisabilities of the interacting molecules. The London-van der Waals interaction between two atoms is relatively weak and short ranged. For macroscopic bodies, however, the dispersion forces are to a large extent additive and therefore longer ranged [26]. The London-van der Waals interaction between two macrobodies depends on the radii of the interacting particles, their distance of separation and the so called Hamaker constant. In the Hamaker constant the molecular properties of the materials involved are accounted for, i.e., the polarisabilities and densities of the interacting particles. The value of the Hamaker constant is hard to assess and often an approximation is used.

In aqueous media usually the Hamaker constant has a positive value and this implies attraction between the interacting particles.

Water structure-mediated interaction

Surfaces immersed in aqueous solutions are covered by a thin layer of water molecules that are restricted in their orientation and position [25]. These structurised adjacent water layers give rise to an interaction when two surfaces approach each other. These water structure-mediated forces are sometimes called hydrophobic forces when they are attractive and hydration forces when they are repulsive.

Water molecules, adjoining non-polar groups at the exterior of proteins and hydrophobic surfaces, posses a higher degree of ordering than those in the bulk and hence their molar entropy is lower. Dehydration of such hydrophobic patches upon interaction results in increasing the entropy of the water molecules and therefore this phenomenon promotes protein adsorption at hydrophobic surfaces [20].
On the other hand, adsorption of proteins at a hydrophilic surface may be hindered by the hydration layers of ionic or polar functional groups at both surfaces [27]. When such surfaces approach each other partial dehydration of the ions and the polar surface would have to occur leading to an increase in the Gibbs energy and, hence, to repulsion.

Structural rearrangements in the protein
Upon adsorption changes in the environment of a protein molecule occurs, i.e. the protein molecule is not longer solely exposed to water but also to the sorbent surface. Therefore, the apolar parts that originally were situated at the interior of the protein may now become exposed to (hydrophobic parts of) the sorbent surface without making unfavourable contact with the water. This means that the intra-molecular interactions, that form the basis of the secondary structure of proteins, may be exchanged by interactions between the molecule and the surface. If such protein-surface interactions are profitable this will result in a decrease in secondary structure (and a favourable increase in conformational entropy of the protein) [21]. On the other hand, if favourable interactions between peptide units and the surface are not possible this may induce the formation of extra intra-molecular interactions, and in this way promote the formation of ordered structure [28]. It is clear, that structural rearrangements are determined by an intricate interplay of all the components in the system.

All the interactions between proteins and surfaces mentioned above may play important roles in the adsorption process. These interactions have been extensively studied [20-22,25] and many attempts have been made to apply the insight into these interactions to controlling the orientation of IgG molecules upon and after adsorption. However, in attempting to let the IgG molecules adsorb upright one aspect of these molecules, viz. their 'Y'-shape, has not yet been exploited. In this thesis we present a new method to achieve oriented physical adsorption of IgG. Our method is based on a further consideration of the molecular dimensions and it resembles 'molecular sieving' on a sorbent surface. We will call it size-exclusion adsorption and it will be explained below. Based on

this principle, a very simple procedure for the oriented immobilization of IgG will be elaborated rendering complicated chemical or biochemical modifications of the surface or the immunoglobulins redundant.

1.3 Size-exclusion adsorption

Physical adsorption of proteins can take place only when there is sufficient free space available on the surface and when the individual areas of uncovered adsorbate are sufficiently large to accommodate an individual protein molecule. By pre-coating a surface with polymers, proteins or other molecules the surface becomes partially blocked for further adsorption. At a properly selected surface coverage, only relatively small uncovered areas remain available to subsequently adsorbing proteins. Only those molecules that fit in these uncovered areas are able to adsorb when the size distribution of individual areas excludes larger molecules. In other words, a molecular sieve is created which induces a size-exclusion adsorption.

Such size-exclusion adsorption may also be extended to protein molecules of one and the same type that are anisodimensional, i.e. consisting of parts having different properties: the smaller part may attach whereas accommodation at the sorbent surface of the larger part may be obstructed (figure 1.3a). Under these circumstances, size-exclusion adsorption essentially becomes shape-exclusion adsorption. As mentioned above, a typical example of such an asymmetrical molecule is the Y-shaped IgG; the larger part ($F(ab)_2$) consists of two similar domains that bind antigens whereas the smaller part (Fc) only mediates effector functions. As a result of the difference in the biological function of the two parts, size-exclusion adsorption of IgG may lead to a more advantageously oriented and correspondingly to a biologically more active layer.

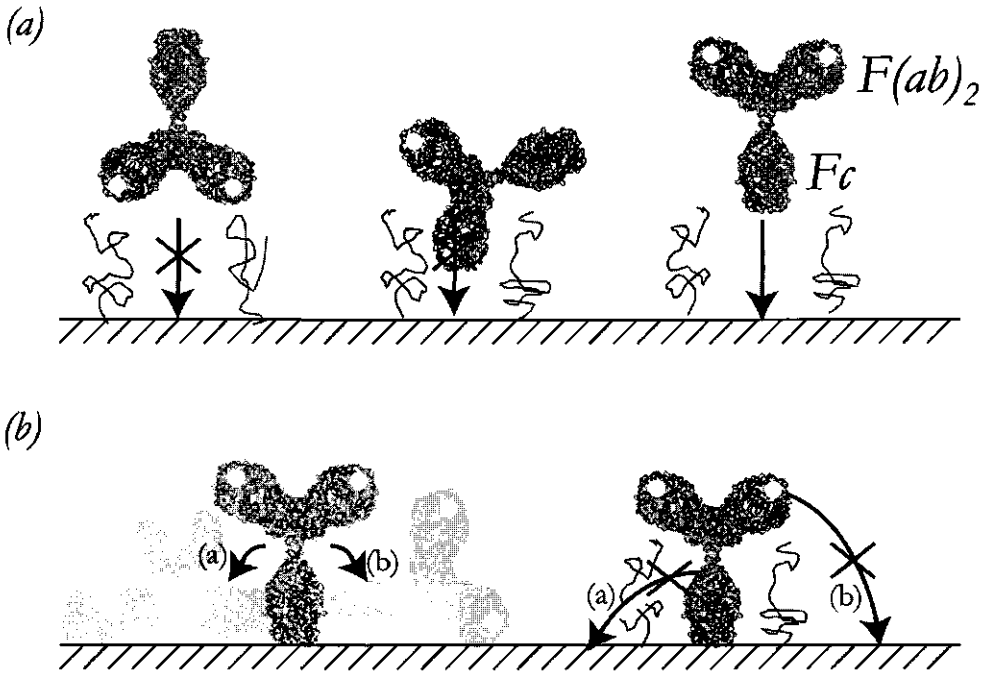

Figure 1.3: (A) Oriented adsorption of IgG on a precoated surface, leading to a biologically more active layer. (B) Prevention of (a) spreading and (b) tilting of adsorbed IgG by pre-adsorbed molecules. The antigen binding sites are represented by the white dots at the top of the F(ab) parts.

As an additional effect, the precoated layer may suppress changes in the orientation and conformation of protein molecules after being adsorbed. Pre-adsorbed molecules reduce the fraction of available surface area and may form a steric barrier that prevents undesirable tilting and spreading of the protein (figure 1.3b).

1.4 Outline of this thesis

In this thesis the effects of size-exclusion adsorption on the orientation and conformation of adsorbed IgG are studied within the purpose of maximizing their biological activity without chemical modification of either the molecule or the solid surface. The influence of electrostatic interactions, hydrophobic dehydration and structural rearrangements on the adsorption process are studied to complete the insight into the mechanism of (size-exclusion) adsorption.

The monoclonal IgG molecules used in this study are directed against human chorionic gonadotrophin (hCG). hCG is a hormone produced by the placenta during pregnancy. This hormone is found in pregnant woman's urine and pregnancy tests are designed to detect it [2,3]. The use of monoclonal IgG offers the advantage of studying a system that has well-defined physical properties.

In **chapter 2** we study the prospect of using the dipolar character of IgG molecules to control their orientation upon adsorption. The influence of electrostatic interactions on the adsorption of IgG is examined both theoretically and experimentally. The long range interaction between IgG and the sorbent surface is treated in terms of the DLVO theory. Reflectometry experiments for adsorption are performed on hydrophilic surfaces and the electrostatic interactions are investigated using pH and ionic strength as the experimental variables.

In **chapter 3** we describe the theoretical aspects of size-exclusion adsorption and report a set of experiments demonstrating the influence of the non-spherical molecular shape of IgG on its adsorption on surfaces partially covered with pre-adsorbed layers of either IgG or triblock copolymers of poly(ethylene oxide), PEO, and poly(propylene oxide), PPO, of the type PEO-PPO-PEO. The effect of these layers on the biological activity of adsorbed IgG is studied by choosing the surface coverage of the sorbent by the pre-adsorbed layer as the experimental variable. We monitored the adsorption process by reflectometry which allows us to study the effect of adsorption kinetics on the biological activity of adsorbed IgG.

In chapters 4, 5 and 6 conformational changes in IgG are studied. In **chapter 4** a structural analysis of one monoclonal IgG adsorbed on different silica surfaces (hydrophilic, hydrophobic, hydrophobic with pre-adsorbed triblock copolymers) using ATR-FTIR spectroscopy is given. These experiments were carried out using a cylindrical internally reflecting silicon element that has been covered with a silica surface layer. For comparison purposes, experiments were also carried out with bovine serum albumin (BSA).

In chapters 5 and 6, Circular Dichroism (CD) is used as a spectroscopic technique for studying protein structure. **Chapter 5** gives information on the structural changes of IgG molecules induced by heat treatment and compares these changes with those induced by adsorption on a hydrophobic surface. Prior to discussing the CD measurements, the thermal stability of IgG, as determined by differential scanning calorimetry (DSC), is presented. In **chapter 6** the effect of pre-adsorbed

layers on the secondary structure of adsorbed IgG is studied by choosing the surface coverage of the sorbent by the pre-adsorbed layer as the experimental variable. In addition, the effect of adsorption conditions, such as pH and adsorption time on the secondary structure is studied.

In **chapter 7** we examine whether the favourable results of size-exclusion adsorption obtained in our reflectometry model system (chapter 3) also apply to industrial diagnostic methods. We implemented the triblock copolymer pre-adsorption procedure in an assay designed for the early diagnosis of pregnancy. Radioactively labeled IgG and hCG molecules made it possible to monitor the adsorption of IgG and the subsequent binding of hCG. The data obtained are in agreement with our earlier model studies and demonstrate that sieving of the IgG by the polymer does takes place resulting in the creation of a more favourably oriented IgG layer.

1.5 References

1. Köhler, G., and Milstein, C. (1975) *Nature 256*, 495.
2. Frances, R., and Batzer, M.D. (1980) *Fertil. Steril. 34*, 1.
3. Konings, B.L.J.C., Pelssers, E.G.M., Verhoeven, A.J.C.M., and Kamps, K.M.P. (1993) *Colloids Surf. B 1*, 69.
4. Engvall, E., and Perlmann, P. (1971) *Immunochemistry 8*, 871.
5. Butler, J.E., Ni, L., Nessler, R., Joshi, K.S., Suter, M., Rosenberg, B., Chang, J., Brown, W.R., and Cantarero, L.A. (1992) *J. Immunol. Methods 150*, 77.
6. Singer, J.M., and Plotz, C.M., (1956) *Am. J. Med. 21*, 888.
7. Ortega-Vinuesa, J.L., Hidalgo-Alvarez, R., de las Nieves, F.J., Davey, C.L., Newman, D.J., and Price, C.P (1998) *J. Colloid Interf. Sci. 204*, 300.
8. Molina-Bolivar, J.A., Galisteo-Gonzalez, F., and Hidalgo-Alvarez, R. (1998) *J. Biomater. Sci. Polymer Edn. 9 (10)*, 1089.
9. Murata, M., Arakawa, M., Yoshida, T., and Hato, M. (1998) *Colloids and Surf. B 12*, 35.
10. Bagchi, P., and Birnbaum, S.M. (1981) *J. Colloid Interf. Sci. 83*, 460.
11. Amzel, L. M., and Poljak, R. J. (1979) *Annu. Rev. Biochem. 48*, 961.
12. Padlan, E. A. (1994) *Mol. Immunol. 31*, 169.
13. Derjaguin, B.V., and Landau, L.D. (1941) *Acta Physicochim. USSR 11*, 633.
14. Verwey, E.J.W., and Overbeek, J.T.G., *Theory of the Stability of Lyophobic Colloids*, (Elsevier, 1948).
15. Molina-Bolivar, J.A, and Ortega-Vinuesa, J.L (1999) *Langmuir 15*, 2644.
16. Leckband, D., and Sivasankar, S. (1999) *Colloids Surf. B 14*, 83.
17. Molina-Bolivar, J.A., Galisteo-Gonzalez, F. and Hidalgo-Alvarez, R. (1999) *Colloids Surf. B 14*, 3.
18. Vermeer, A.W.P., Bremer, M.G.E.G., and Norde, W. (1998) *Biochim. Biophys. Acta 1425*, 1.
19. Geddes, N.J., Furlong, D.N., Zientek, P., Than, K.A., and Edgar, J.A. (1993) *J. Colloid Interf. Sci. 157*, 491.
20. Norde, W. (1986) *Adv. Colloid Interf. Sci. 25*, 26.
21. Norde, W. (1998) *Biopolymers at interfaces, Driving forces for protein adsorption at solid surfaces*, Vol. 75, Marcel Dekker, Inc., New York.
22. Hayes, C.A., and Norde, W. (1994) *Colloids Surf. B 2*, 517.
23. Hayes, C.A., Sliwinski, E., and Norde, W. (1994) *J. Colloid Interf. Sci. 164*, 394.
24. Van Dulm, P., Norde, W., and Lyklema, J. (1981) *J. Colloid Interf. Sci. 82*, 77.
25. Israelachvili, J.N., *Intermolecular and Surface forces*, (2nd ed., Academic Press, New York, 1992).

26. Lyklema, J. *Fundamentals of Interface and Colloid Science, Vol I Fundamentals* (Academic Press, London, 1991).
27. Pashley, R.M. (1981) *J. Colloid Interf. Sci. 83*, 531.
28. Zoungrana, T., Findenegg, G.H., and Norde, W. (1997) *J. Colloid Interf. Sci. 190*, 437.

Chapter 2

ELECTROSTATIC INTERACTIONS BETWEEN IgG MOLECULES AND A CHARGED SORBENT SURFACE

Abstract

In this chapter we study the prospect of using the dipolar character of IgG molecules to control their orientation upon adsorption. The influence of electrostatic interactions on the adsorption of IgG is examined theoretically and experimentally. The long range interaction between IgG and the sorbent surface is treated in terms of the DLVO theory. Experiments are performed on hydrophilic surfaces, namely negatively charged silica and positively charged amine-functionalised silica. It is concluded that electrostatic interactions have a strong influence on the adsorption behaviour of IgG molecules on hydrophilic charged surfaces. The relevant electrostatic interactions are a combination of interactions between IgG and the sorbent surface and lateral interactions between adsorbed IgG molecules. The electrostatic interactions between the IgG molecules and the surfaces are relatively weak and cannot ensure permanent attachment of the molecules. Due to extensive desorption of IgG from both surfaces, electric field-induced orientation of IgG could not be established unambiguously.

2.1 Introduction

The adsorption of proteins at interfaces is a widespread phenomenon in both natural and man-made systems and it plays an important role in many disciplines, including biomedical engineering, biotechnology and environmental science. For instance, in various diagnostic test systems, such as ELISA [1,2], RIA [3,4] and latex agglutination assays [5,6], immunoproteins are adsorbed at solid surfaces. The use of immunoglobulin G (IgG) is of primary interest; it constitutes an important part of the immune system because it recognises and specifically binds antigens. The adsorption of immunoglobulins onto solid surfaces often results in a loss of antigen binding specifity and/or capacity [2,7]. For optimal biological activity the orientation and conformation of the adsorbed IgG molecule should allow the antigen-binding sites to be accessible to antigens [8]. In order to control IgG adsorption the interactions between proteins and interfaces must be understood.

Interactions between proteins and surfaces may be divided into the following major contributions: [9,10] (a) electrostatic interaction between protein and sorbent, (b) hydrophobic dehydration of parts of the adsorbent and/or protein molecules, (c) structural rearrangements in the protein molecule and (d) van der Waals interactions.

In this chapter we will focus on the electrostatic interactions between IgG molecules and a charged sorbent surface. It should be realised that the IgG molecules used in this study have a (large) dipole moment as the iso electric point of its antigen binding parts (Fab) differs from that of its Fc part. We study the prospect of using the dipolar character of the IgG molecules to control their orientation upon adsorption. A molecular dipole placed in the electric field of a sorbent surface at an angle relative to the field experiences a torque, which tends to align the dipole along the field direction [11]. For such alignment to occur, the complete dipole should be located within the field. This is the case provided the penetration depth of the sorbent's electric field exceeds the dimensions of the molecule. This penetration depth, however, strongly depends on the ionic strength of the solution and may vary from values much larger than the size of an IgG molecule down to values in the same order of magnitude. We will examine the influence of electrostatic interactions on the adsorption behaviour of IgG both theoretically and experimentally, following a colloid-chemical approach. The interaction between the IgG molecule and the sorbent surface will be treated in terms of the DLVO theory [12,13] for the interaction between a charged sphere and a charged flat surface.

Obtaining unambiguous experimental evidence on the contribution of electric field-induced orientation of adsorbed molecules is far from trivial. In earlier work [8,14-16] in this field, where charged dispersed hydrophobic surfaces were applied, all possible interactions between protein molecules and a surface acted simultaneously. It is generally found [9,17] that hydrophobicity of the sorbent surface promotes structural changes in the adsorbed protein molecule. Therefore, with hydrophilic surfaces electrostatic interactions are expected to play a dominant role. This lead us to perform experiments using hydrophilic surfaces, namely negatively charged silica and positively charged amine-functionalised silica. By selecting appropriate pH and ionic strength values, we may be able to monitor the role of electrostatic interactions. The adsorption process itself is followed in real time by reflectometry [18]. The use of monoclonal IgG offers the advantage of studying a system that has well-defined physical properties.

2.1.1 DLVO theory

The DLVO theory [12,13], developed to explain the roles of electric and van der Waals contributions in colloidal stability, has often been used to describe the interaction between biological colloids [8,19-21]. This theory describes the interaction Gibbs energy between two macroscopic bodies as a function of their separation. According to the DLVO theory the total interaction Gibbs energy is the sum of the London-van der Waals interaction and the interaction resulting from double layer overlap.

The DLVO theory has received considerable theoretical and experimental attention. Agreement between theory and experimental data has been shown at separations beyond a few nanometers. At shorter separations, however, interactions are found to be complicated by non-DLVO interactions such as solvent structure-mediated forces [19,22-24]. Therefore, we will use the DLVO theory to describe only the long-range interactions between an IgG molecule and a sorbent surface. Our experimental system will be modelled as a charged dipolar sphere interacting with a charged flat surface.

London-van der Waals interaction

For macrobodies (except for highly polar materials) the dispersion interaction is the most dominant term in the van der Waals interaction [23]. In gases, London-van der Waals, or dispersion, forces originate from the correlation between time-dependent dipoles in atoms or molecules and depends only, on the polarisabilities of the interacting molecules. The London-van der Waals interaction between two atoms is relatively weak and short ranged. For macroscopic bodies, however, the

dispersion forces are to a large extent additive and therefore longer ranged. Based on additivity, according to the Hamaker-de Boer approximation [25] (pairwise summation of the interaction energies between all interparticle molecule pairs) some simple equations can be derived. For the interaction between a sphere and a macroscopically flat surface interacting across a medium, the following approximation has been established [25]:

$$G_{vdW}(x) = -\frac{A_{132}}{6}\left[\frac{2R(x+R)}{x(x+2R)} - \ln\left(\frac{x+2R}{x}\right)\right] \qquad (2.1)$$

Where x is the distance of separation between the sphere and the surface and R the radius of the sphere. A_{132} is the Hamaker constant for the system, where the subscripts indicate the phases, with 1 the solid, 2 the particle and 3 the medium.
The Hamaker constant depends on the polarisabilities and densities of the interacting bodies. The value of the Hamaker constant is hard to assess and often an approximation is used. Tables of the values are listed in several references [25,26]. The Hamaker constant for a more-phase system can be derived from the individual Hamaker constants of the materials involved [25].

Electric double layer interaction
In most systems both the protein molecule and the sorbent surface are electrically charged. These surface charges are fully compensated by an excess of counterions and a deficit of co-ions in the adjoining solution. As a rule, a fraction of the counter ions and co-ions is specifically adsorbed on the surface. The surface charge, together with its countercharge, forms an electric double layer and as a whole the system is electroneutral. When two charged species approach each other, the electric double layers overlap. This results in repulsion if the particle and the surface have the same charge sign and in attraction if the signs are opposite.
The simplest description of the diffuse part of the double layer is given by the Gouy-Chapman theory [27,28]. In this theory it is assumed that the surface is flat and uniformly charged, the ions in solution are point charges, distributed according to Boltzmann and that the solvent is a structureless continuum.
In calculating the contribution of double layer interactions to the total interaction energy one has to assume that either the electric potential or the electric charge density at the interacting surfaces remains constant. Because of the relatively large dimensions of the protein its diffusion rate towards the sorbent surface will be

slow as compared to the relaxation rate of the electrical double layer. Therefore, the assumption of constant potential seems appropriate. Double layer overlap is governed by the diffuse part of the double layer; therefore the potentials needed in the equations are the diffuse double layer potentials, which are usually approximated by the experimentally accessible electrokinetic or ζ potential [29]. For surface potentials < 50 mV, the following equation has been derived for double layer interaction between a sphere and a flat surface [30]:

$$G_e(x) = \pi\varepsilon\varepsilon_0 R(\psi_{13}^2 + \psi_{23}^2)\cdot\left(\frac{2\psi_{13}\psi_{23}}{\psi_{13}^2+\psi_{23}^2}\cdot\ln\left[\frac{1+\exp(-\kappa x)}{1-\exp(-\kappa x)}\right]+\ln[1-\exp(-2\kappa x)]\right) \quad (2.2)$$

Here, ψ is the potential at the phase boundary indicated by the subscript between the phases as given under eq. 2.1. $\varepsilon\varepsilon_0$ is the dielectric permittivity of the medium and κ^{-1} the reciprocal Debye length. The Debye length, also called the double layer thickness, is a measure of the separation distance over which charges interact and is given by [29]:

$$\kappa^2 = \frac{F^2\sum_j C_j Z_j^2}{\varepsilon\varepsilon_0 RT} \quad (2.3)$$

where F is the Faraday constant, R the gas constant, T the absolute temperature and c_j and z_j the concentration and the valency, respectively of ionic species j.

2.2 Experimental

2.2.1 Proteins

The mouse monoclonal antibodies used in this study were directed against the human pregnancy hormone (hCG, Human Chorionic Gonadotropin) and were of isotype IgG 7B. The monoclonal antibodies, having different isoelectric points (table 2.1), and highly purified hCG were gifts from Organon Teknika (Boxtel, The Netherlands). Concentrated IgG solutions were stored at -20°C and thawed only once and shortly before use. Prior to every experiment IgG solutions were filtered through an 0.2 μm Acrodisc filter to remove aggregates. The extinctions at 280 nm were converted into protein concentrations using an extinction coefficient of 1.45 cm^2 mg^{-1}. The antigen hCG was received freeze-dried in sealed flasks

containing 550 IU (1 mg roughly equals 8000 IU) and stored at 7°C. Bovine serum albumin (BSA) was purchased from Sigma Chemical Co.

Table 2.1: Isoelectric points of the monoclonals IgG 7B, 1C and 9A and their fragments.

IgG	7B	1C	9A
complete	6.0	6.4	6.5
i.e.p $F(ab)_2$	5.9	6.7	6.9
i.e.p Fc		6.1	

The Fc parts of different monoclonal IgG molecules are generally considered to be identical for all IgG molecules which is reflected in the similar i.e.p. for the Fc fragments of the different monoclonals.

2.2.2 Chemicals

Amine-functionalised surfaces were prepared by modifying silica surfaces using 3 aminopropyl-triethoxysilane (Petrarch, United Chemical Technologies, USA). All adsorption experiments were carried out in 5 mM phosphate buffer solution. Ionic strengths higher than 5 mM were obtained by adding NaCl. Chemicals used for buffer preparation, Na_2HPO_4, NaH_2PO_4 and NaCl, were of analytical grade and used without further purification. Water was purified by reverse osmosis and passed subsequently through a superQ system (Millipore).

2.2.3 Surfaces

Silicon wafers (Wacker Chemitronic GmbH, Munchen, Germany) covered with a thin layer of SiO_2, formed by thermal oxidation at 1000° C for 90 minutes, were used as hydrophilic negatively charged substrates. A silica layer thickness of approximately 100 nm is essential for obtaining a high sensitivity in reflectometry experiments. By ellipsometry it was verified that this condition was satisfied. Amine-functionalised silica surfaces, prepared by the method described by Giesbers [31], were used as hydrophilic positively charged substrates. The contact angles of a drop of water on the silica and amine-functionalised silica were around 5° and 15°, respectively.

2.2.4 Reflectometry

Protein adsorption was measured using a reflectometer equipped with a stagnation-point flow-cell [18]. If the perfect sink boundary condition applies (c=0

at the interface) in the absence of a barrier, then the initial molecular limiting flux towards the surface depends on the geometry of the cell, the diffusion coefficient of the molecule, the flow rate and the concentration in solution as described by the following equation [18]:

$$J_0 = 0.776 \nu^{1/3} R^{-1} D^{2/3} (\bar{\alpha}\,\mathrm{Re})^{1/3} c \qquad (2.4)$$

where ν is the kinematic viscosity of the solution, R the radius of the inlet tube, D the diffusion coefficient of the solute, $\bar{\alpha}$ a stream intensity parameter which depends on the dimensions of the cell and the Reynolds number Re and c the solute concentration.

The effect of the flux on the adsorption of the immunoglobulins is studied by varying the concentration of the protein molecules, keeping all other experimental and material variables constant. A constant pulse-free flux is realised by using a microdosing pump (CAT, Staufen, Germany). The flow rates of the protein solutions were 1 ml per minute.

2.2.5 Biological activity

The biological activity of adsorbed IgG is determined by measuring the amount of hCG that can be bound by saturating adsorbed IgG layers. The average biological activity, defined as the number of moles hCG bound per mol IgG, is calculated by:

$$\frac{mol\ hCG}{mol\ IgG} = \frac{\Gamma_{hCG} \cdot M_{IgG}}{\Gamma_{IgG} \cdot M_{hCG}} \qquad (2.5)$$

Non-specific binding of hCG to the part of the surface not covered by IgG is minimised by post-coating the surface with BSA from a 1 mg ml^{-1} solution; this method is often applied in immunological tests. Blocking is important since otherwise non-specific binding of hCG would lead to an overestimation of the biological activity of adsorbed IgG. From experiments in which radio-active labelled hCG was used we concluded that BSA is a satisfactory blocking agent [32]. In between the different steps of the protocol, i.e. the IgG adsorption, the BSA post-coating and the hCG binding, the surface was rinsed for 10 minutes with phosphate buffer.

2.3 Results and discussion

2.3.1 DLVO calculations

When a molecular dipole enters an electric field at an angle relative to the field it experiences a torque which tends to align the dipole along the field direction. The thermal motion involving an energy of kT per molecule, however, tends to randomise the molecular orientation distribution. Furthermore, the molecule will be further aligned only if it can be accommodated completely within the field. Hence, to achieve alignment the interaction energy with the electric field must exceed kT and the penetration depth of the electrical field should exceed the dimensions of the molecule.

In 0.005 M and 0.1 M phosphate buffer the double layer thickness at the surfaces (equation 2.3) is around 3 nm and 1 nm, respectively. As the dimensions of the IgG molecule are 15*6*3 nm^3 it is evident that only a small part of the molecule can enter the sorbent's electric field so that no further alignment of the dipolar IgG molecule will occur.

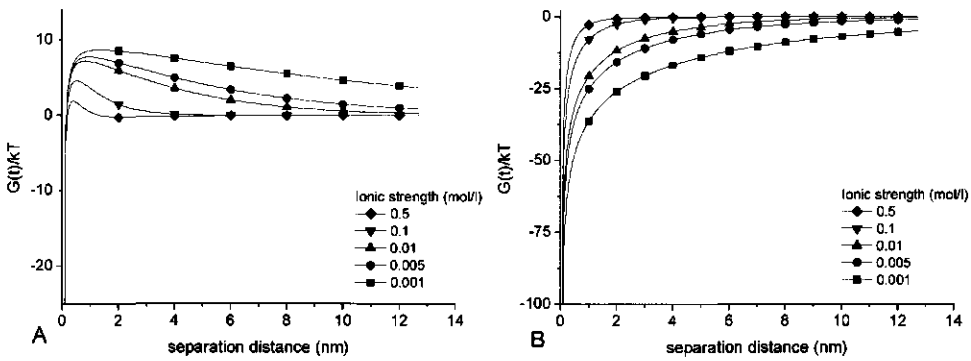

Figure 2.1: Interaction energy curves for a particle and surface with (A) same charge sign ($\psi_{13} = \psi_{23} = 30$ mV) and (B) the opposite charge sign ($\psi_{13} = -\psi_{23} = 30$ mV).

Figure 2.1 gives curves for the total Gibbs energies of interaction between a charged sphere of radius 7.5 10^{-9} m and a charged flat surface, as calculated according to (eq. 2.1) and (eq. 2.2). By convention, negative values for G correspond to attraction and positive ones to repulsion. From equation 2.1 it is clear that the London-van der Waals interaction depends on the radius of the particle, the particle-surface separation and the Hamaker constant. Electrostatic

interactions (eq. 2.2) depend on the radius, the separation, the surface potentials and κ. As κ (eq. 2.3) and the surface potentials are sensitive to the ionic strength of the medium, so is the total interaction energy. Therefore, calculations are performed for various values of ionic strength. We used a value of 5.10^{-21} J for the Hamaker constant in agreement with the value reported by others [8,20,22,33].

Figure 2.1a shows that attractive van der Waals interaction dominates at small separations. At intermediate distances, however, the repulsive double layer interactions dominate and the potential energy shows a maximum having a positive value. At low ionic strength, the Gibbs energy barrier opposing attachment to the surface is about 7 kT for IgG molecules arriving with a side that has the same charge sign as the surface. At the outer boundary of the electric field IgG molecules are randomly oriented with a thermal energy of 0.5 kT normal to the sorbent surface. It follows that the molecules that arrive with the part that has the same charge sign as the sorbent directed towards the sorbent will not be able to surpass the energy barrier and adsorption is prevented. Conversely, there is no energy barrier for the dipoles that arrive in an electrostatically favourable orientation (fig. 2.1b) and each particle-surface collision results in attachment. At high ionic strength, both electrostatic repulsion and attraction (fig. 2.1a and b) are decreased and hence, the influence of the electrostatic interactions on the adsorption will be suppressed.

Based on these calculations, we expect at low ionic strength a sort of 'on/off' adsorption mechanism: under conditions of electrostatic attraction the molecules will adsorb in the orientation in which they entered the field, whereas under conditions of electrostatic repulsion the molecules cannot adsorb. The extent to which electrostatic interactions control the adsorption process can be varied by adjusting the ionic strength of the solution. At high ionic strength the influence of electrostatic interaction on the adsorption behaviour is largely suppressed.

2.3.2 IgG adsorption

Prior to examining the effect of the surface charge on the antigen binding capacity of adsorbed IgG (indicative of the orientation of IgG molecules at the surface), we will study more general aspects of the adsorption of IgG. The various IgG molecules used in this study show similar adsorption characteristics and therefore we give, by way of example, only those for IgG 7B.

2.3.3 Initial adsorption rate

Figure 2.2 shows the adsorption of IgG 7B on silica as a function of time, at pH 6.0 and 0.005 M and 0.1 M ionic strength, for two protein concentrations, c_p.

At pH 6 (the i.e.p. of the IgG) and low ionic strength, the initial rate of adsorption, reflected by the slope of the curve at t=0, equals about 90% of the theoretical flux. The deviation between the initial rate of adsorption and the theoretical flux may be explained by an overestimation of the theoretical flux due to the finite thickness of the laser beam [18]. Also, when the actual alignment of the reflection spot is somewhat off-centre, the flux is again overestimated [18]. Figure 2.2 also shows that the normalised initial adsorption rate is independent of c_p. This means that essentially all molecules that arrive at the surface do adsorb and that the adsorption is transport-controlled.

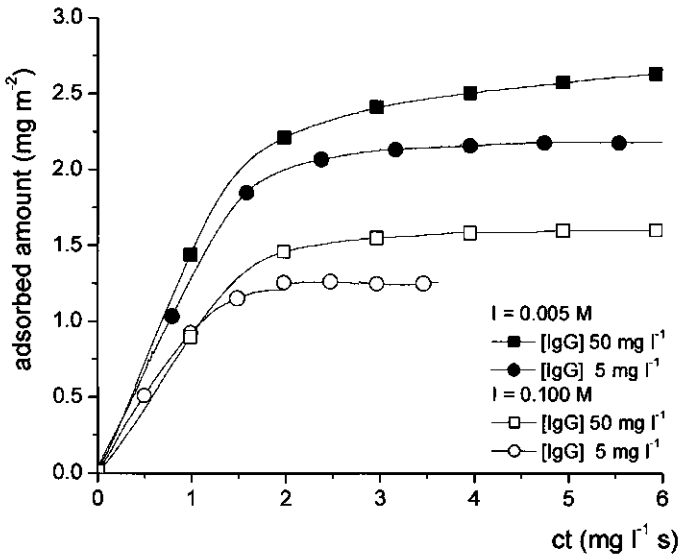

Figure 2.2: Adsorption of IgG 7B on silica (pH6) for two IgG concentrations at a ionic strength of 5 mM and 100 mM.

The pH influence on the initial adsorption rate of IgG 7B on silica and amine-layers is graphically presented in fig. 2.3. At pH values below the i.e.p, the IgG molecules are electrostatically attracted to the silica surface but at pH values above the i.e.p they are repelled. This repulsion leads to strongly reduced initial adsorption rates. For pH values higher than 7, the adsorption rate is extremely low. For IgG adsorption on the amine-layers a similar behaviour is observed: under conditions of electrostatic attraction initially all molecules arriving at the surface

adsorb, whereas this rate is strongly reduced under conditions of electrostatic repulsion. This is in line with the 'on/off' adsorption mechanism predicted on the basis of our DLVO calculations and with adsorption behaviour reported for immunoglobulins [8,15,17] and different proteins [8,34].

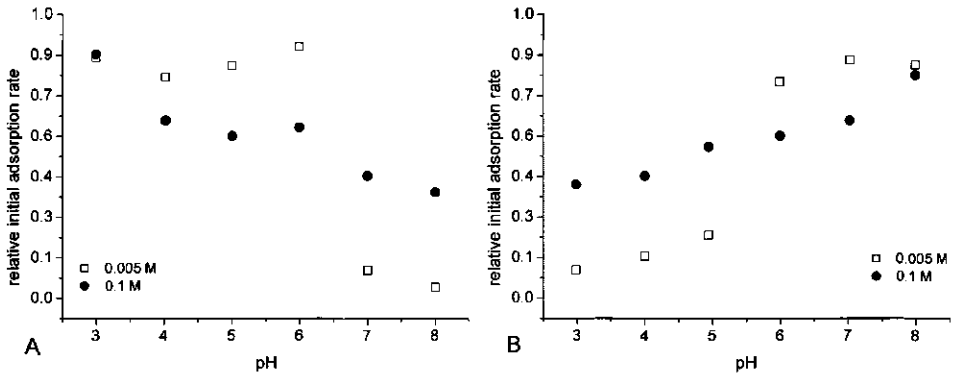

Figure 2.3: Relative initial adsorption rate of IgG 7B as a function of pH on (A) silica and (B) amine-functionalised silica.

The effect of screening of electrostatic interactions (both attraction and repulsion) by low molecular weight ions, as predicted by the DLVO theory, is clearly reflected in figs 2.3a and b, where the variation of the initial adsorption rate with pH is much less pronounced at higher ionic strength. At high ionic strength the initial adsorption rate is significantly lower than the theoretical flux. From IR experiments at similar conditions [35], we concluded that IgG adsorption on silica hardly induces structural rearrangements, if any, in the protein. From all these observations we conclude that electrostatic attraction is governing IgG adsorption on our hydrophilic surfaces and that no major additional driving force for adsorption (besides the van der Waals force) is present.

2.3.4 Maximum adsorbed amount

The maximum adsorbed amount is determined by a combination of the orientation (distribution) of the molecules on the surface and the surface coverage. IgG molecules are asymmetric and Y shaped. Therefore, assuming no or minor structural rearrangements, the maximum adsorbed amount is indicative of the average orientation of the IgG molecules at the surface. Molecules deposited at the surface in an end-on orientation may relax to optimise their interaction with the surface. This usually leads to a more side-on orientation and hence to a lower

maximum adsorbed amount. A reorientation can only take place when there is sufficient time and space. Consequently, when the initial rate of adsorption is high relative to the relaxation rate the possibilities for reorientation are minimised.
Electrostatic interactions between the arriving protein and the adsorbent surface play a role, as well as those between two adjacently adsorbed molecules. A change in electrostatic interactions between the surface and the adsorbing protein causes a change in initial adsorption rate and, as discussed above, a different maximum adsorbed amount. Lateral repulsion between two adsorbed charged protein molecules results in a lower surface coverage and therefore a smaller adsorbed amount.

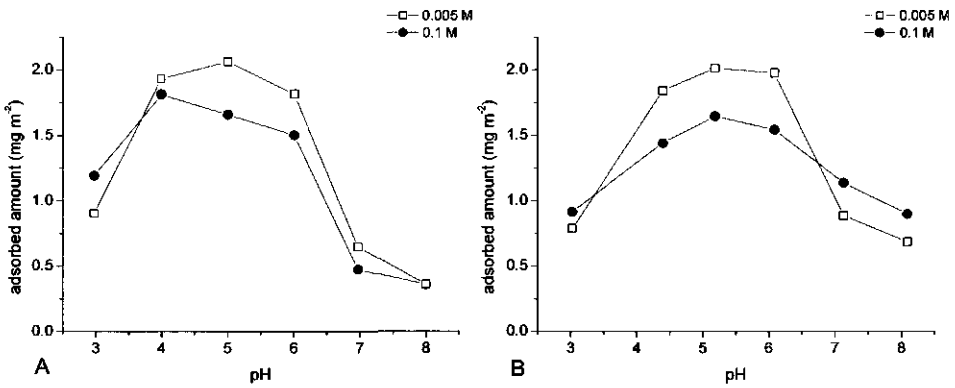

Figure 2.4: Maximum adsorbed amount of IgG 7B as a function of pH on (a) silica and (b) amine-functionalised silica.

The maximum adsorbed amount as a function of pH is plotted in fig. 2.4. At both surfaces, the plateau adsorption decreases towards the acid and alkaline sides of the isoelectric region.
At the silica surface at low pH, where the protein and the sorbent surface attract each other, the adsorption rate is high (fig. 2.3), resulting in very little time for reorientation. For this reason one would expect a high plateau adsorption. However, at these pH values the IgG has a high net charge and the molecules strongly repel each other electrostatically; this leads to reduced adsorbed amounts. Figure 2.3 shows that at low pH an increased ionic strength gives a lower adsorption rate which, in turn, is expected to result in lower adsorbed amounts. However, lateral repulsion between adsorbed IgG is screened as well by ions, and this would cause a higher adsorption. In figure 2.4 we see that the net effect of increasing the ionic strength causes a higher adsorbed amount. The conclusion is

that lateral repulsion, rather than protein-sorbent attraction, dominates the electrostatic interaction in this pH regime.

At intermediate pH values, around the i.e.p., the net charge on the protein molecules is essentially zero and the lateral repulsion is a minimum. The rate of adsorption is high, hence reorientation cannot take place. These two features allow high maximum adsorbed amounts, and this is observed for many different proteins [8,15,34]. Around the i.e.p. of the IgG the adsorption rate is lower at the higher ionic strength (see fig. 2.3), leaving more time for reorientation. This results in the smaller adsorbed amount shown in figure 2.4. In this pH regime there is no significant lateral repulsion so that the electrostatic interaction between the surface and the arriving molecule is dominant.

At high pH values the IgG molecules are electrostatically repelled from the silica surface, causing a very low adsorption rate. Moreover, the lateral repulsion is strong, resulting in a low adsorption saturation. With increasing ionic strength the repulsion between the surface and the protein as well as the lateral repulsion are decreased. This should result in a higher adsorbed amount, but no significant effect of the ionic strength is observed experimentally. It could be that, even at the higher ionic strength the adsorption rate is already sufficiently low to allow orientational relaxation of the adsorbed protein molecules.

The adsorption of IgG as a function of pH on the amine-functionalised surfaces (fig. 2.3b and 2.4b) displays similar features as on the charged silica but is mirrored with respect to the i.e.p. of the protein because the sorbent surface is oppositely charged.

It can be seen from fig. 2.2 that the IgG concentration has a profound effect on the final adsorbed amount. Plateau adsorption decreases with decreasing c_p. This effect may be caused by (re)orientation of the adsorbed molecules at a rate that is comparable with the rate of deposition at the sorbent surface, the latter being decreased with decreasing c_p.

2.3.5 Desorption

IgG desorption from both surfaces is studied by rinsing the adsorbed IgG layers with buffer. The extent of desorption reflects the average strength of the protein-sorbent interactions. On hydrophilic surfaces considerable desorption of proteins is observed, in particular for proteins adsorbed around their i.e.p. [36].

We measured the desorption of our immunoglobulins from both silica and amine-functionalised silica surfaces for periods of more than an hour. At both surfaces after 30 minutes of rinsing with buffer already 50% of the previously adsorbed molecules were desorbed. Furthermore, desorption still proceeded after 1 hour, although the desorption rate decreased in time. The extensive desorption from both positively and negatively charged surfaces contradicts the proposed selective desorption of the Fc fragment, as proposed by Buijs [36]. Furthermore, Ortega Vinuesa et al [16] found that removing electrostatic attractive interactions resulted in an almost complete desorption of antigen binding fragments ($F(ab)_2$) from a hydrophilic surface.
Because the adsorption of IgG on a hydrophilic surface is driven by electrostatic (and van der Waals) interactions only, we must conclude that these interactions are relatively weak and cannot ensure permanent attachment of the IgG molecules.

2.3.6 Biological Activity

The biological activity of the immunoglobulins is measured at their i.e.p. on both surfaces. At this pH the $F(ab)_2$ and the Fc parts have opposite charge signs, and therefore the influence of electrostatic interactions on the orientation of adsorbing IgG molecules may be maximal. In addition, the biological activity of IgG 7B is measured at various pH of adsorption on the silica surface.
If there were no directional forces involved in the adsorption process a more or less randomly organised adsorbed layer would result. However, at the negatively charged silica we expect a certain degree of preferential adsorption of the (slightly) more positively charged Fc parts, thereby forcing the antigen-binding parts directed towards the solution. Therefore, we expected that the antigen-binding sites would be at least partially accessible for binding hCG. Unexpectedly, we did not observe any biological activity of the adsorbed IgG at any pH value. Neither were IgG 9A and IgG 1C biologically active at the silica surface. The results for the two last mentioned IgG molecules is not surprising because the antigen-binding parts of these molecules have a charge sign opposite to that of the surface and unfavourable orientation for binding antigens was therefore anticipated. Upon supplying hCG to the adsorbed IgG layers we even did observe some protein desorption.
For IgG 7B on the positively charged amine-functionalised silica we expected an unfavourable orientation of the binding sites. For the other IgGs, however, a favourable orientation was expected. With IgG 9A and IgG 1C layers, after supplying hCG we initially detected a very small amount of bound hCG. However, on further hCG supply to the surface this binding was immediately overshadowed

by desorption of proteins, either IgG or BSA (used as blocking agent). For this reason, no accurate estimation of the biological activity of the adsorbed immunoglobulins could be made.

2.4 Conclusion

It is concluded that electrostatic interactions have a strong influence on the adsorption behaviour of IgG molecules on hydrophilic, charged surfaces. DLVO theory showed that only IgG molecules arriving at the periphery of the electrical field with a side that is charged opposite to the sorbent surface can further approach the surface. During this approach no alignment of the dipolar IgG molecules will occur.

The relevant electrostatic interactions are a combination of (a) interactions between IgG and the sorbent surface and (b) lateral interaction between adsorbed IgG molecules. The latter dominates at the acid and alkaline sides of the i.e.p.. The rate of deposition at the sorbent surface relative to the relaxation rate of adsorbed molecules is an additional important parameter: it influences the orientation of adsorbed IgG molecules.

The extensive desorption of the imunoglobulins from both positively and negatively charged surfaces showed that the electrostatic interactions between the IgG molecules and the surfaces are relatively weak and cannot ensure permanent attachment of the molecules. Unfortunately, this IgG desorption obstructed determination of its biological activity. Hence, electric-field induced orientation of IgG could not be established unambiguously. In conclusion, in the production of immunoassays hydrophobic surfaces are preferred to hydrophilic surfaces because of their stronger binding of immunoglobulins.

2.5 References

1. Engvall, E. and Perlmann, P. (1971) *Immunochemistry 8*, 871.
2. Butler, J.E., Ni, L., Nessler, R., Joshi, K.S., Suter, M., Rosenberg, B., Chang, J., Brown, W.R. and Cantarero, L.A. (1992) *J. Immunol. Methods 150*, 77.
3. Yalow, R.S. and Berson, S.A. (1960) *J. Clin. Invest. 39*, 1157.
4. Benesch, J., Askendal, A., Tengvall, P. (2000) *Colloids Surf. B 18*, 71.
5. Singer, J.M. and Plotz, C.M., (1956) *Am. J. Med. 21*, 888.
6. Ortega-Vinuesa, J.L., Hidalgo-Alvarez, R., de las Nieves, F.J., Davey, C.L., Newman, D.J. and Price, C.P (1998) *J. Colloid Interf. Sci. 204*, 300.
7. Molina-Bolivar, J.A., Galisteo-Gonzalez, F. and Hidalgo-Alvarez, R. (1998) *J. Biomater. Sci. Polymer Edn. Vol 9 (10)*, 1089.
8. Molina-Bolivar, J.A., and Ortega-Vinuesa, J.L. (1999) *Langmuir 15*, 2644.
9. Norde, W. (1986) *Adv. Colloid Interf. Sci. 25*, 267.
10. Haynes, C.A., Norde, W. (1994) *Colloids Surf. B 2*, 5170.
11. Worley, C.G., Linton, W.R., and Samulski, E.T. (1995) *Langmuir 11*, 3805.
12. Derjaguin, B.V. and Landau, L.D., (1941) *Acta Physicochim. USSR*, 11, 633.
13. Verwey, E.J.W. and Overbeek, J.T.G., *Theory of the Stability of Lyophobic Colloids*, (Elsevier, 1948).
14. Galisteo-Gonzalez, F., Puig, J., Martin-Rodriguez, A., Serra-Domenech, J., F. and Hidalgo-Alvarez, R. (1994) *Colloids Surf. B 2*, 435.
15. Galisteo-Gonzalez, F., Martin-Rodriguez, A., Hidalgo-Alvarez, R. (1994) *Colloid Polym. Sci. 272*, 352.
16. Ortega-Vinuesa, J.L., Galvez Ruiz, M.J., and Hidalgo-Alvarez, R. (1996) *Langmuir 12*, 3211.
17. Buijs, J., Norde, W. and Lichtenbelt, J.W.Th. (1996) *Langmuir 12*, 1605.
18. Dijt, J.C., Cohen Stuart, M.A., and Fleer, G.J. (1994) *Adv. Colloid Interf. Sci. 50*, 79.
19. Leckband, D., and Sivasankar, S. (1999) *Colloids Surf. B* 14, 83.
20. Norde, W., and Lyklema, J. (1989) *Colloids Surf. 38*, 1.
21. Leckband, D., Schmitt, F.-J., and Israelachvili, J.N. (1994) *Biochemistry* 4611.
22. Molina-Bolivar, J.A., Galisteo-Gonzalez, F. and Hidalgo-Alvarez, R. (1999) *Colloids Surf. B 14*, 3.
23. Israelachvili, J.N., *Intermolecular and Surface forces*, (2nd ed., Academic Press, New York, 1992).
24. Pashley, R.M., (1981) *J. Colloid Interf. Sci. 83*, 531
25. Lyklema, J. *Fundamentals of Interface and Colloid Science, Vol I Fundamentals* (Academic Press, London, 1991).
26. Visser, J. (1972)) *Adv. Colloid Interf. Sci. 2*, 331.

27. Gouy, M. (1910) *J. Phys. Paris, 9*, 457.
28. Chapmann, D.L., (1913) *Philos. Mag. 25*, 475.
29. Lyklema, J. *Fundamentals of Interface and Colloid Science, Vol II Solid-Liquid Interfaces* (Academic Press, London, 1995).
30. Hogg, R., Healy, T.W., and Fuerstenau, D.W. (1966) *Trans. Faraday Soc. 62*, 1638.
31. Giesbers, M., Ph.D. Thesis, Wageningen University, 2001.
32. Chapter 7, this thesis.
33. Roth, C.M. and Lenhoff, A.M. (1995) *Langmuir 11*, 3500.
34. Ortega-Vinuesa, J.L., Tengvall, P., and Lundstrom, I. (1998) *Thin Solid films 324*, 257.
35. Giacomelli, C.E., Bremer, M.G.E.G., and Norde, W. (1999) *J. Colloid Interf. Sci. 220*, 13.
36. Buijs, J., Ph.D. Thesis, Wageningen Agricultural University, 1995.

Chapter 3

'MOLECULAR SIEVING': ORIENTED IGG ADSORPTION ON (BLOCK COPOLYMER) PRECOATED SURFACES

Abstract

A method to achieve oriented physical adsorption of Immunoglobulin G (IgG) is presented which is based only on the non-spherical shape of IgG molecules. This concept resembles 'molecular sieving' on the sorbent surface in that binding of the smaller part of non-spherical molecules, in the case of IgG the Fc part, is favoured. Thus, the larger, antigen binding, parts become directed towards the solution and, hence, accessible to bind antigens. The mechanism of 'Molecular sieving' is first theoretically demonstrated using a Random Sequential Adsorption (RSA) model. This is followed by a set of reflectometer experiments. A 'sieve' formed either with pre-adsorbed IgG molecules or with triblock copolymers was proven to yield a high specific biological activity of the subsequently adsorbing IgG molecules.

The mass flux towards the surface also has a profound effect on the adsorbed amount and, consequently on the orientation of IgG. This indicates that the rate of reorientation of adsorbed IgG molecules was comparable to the rate of adsorption. We estimated a reorientation time of more than 2000 seconds for IgG molecules adsorbed on a hydrophobic surface. A specific feature of the 'molecular sieve' is that the pre-adsorbed molecules prevent this undesirable reorientation of adsorbed IgG.

3.1 Introduction

The preservation of biological functioning of proteins upon immobilisation is of special interest in various biomedical and biotechnical applications. In industry, including the manufacturing of capture immunoassays [1,2], physical adsorption of immunoglobulins onto solid surfaces is still the predominant immobilisation procedure. The immobilisation technique, however, often leads to an undesired loss of biological activity [3,4]. This loss may be caused by a reduced accessibility of the antigen binding sites for instance when they are in the vicinity of the sorbent surface or by conformational changes in the antibodies [5,6], the latter especially when hydrophobic surfaces are used. To overcome these intrinsic problems several alternative, mostly chemical, immobilisation techniques have been developed [3,4,7]. Thereby it is tried to force the antigen binding parts towards the solution and hence, keeping them accessible to bind antigens. Although some of these techniques have been reported to increase average biological activity of IgGs, they have given rise to new complications: more costly manufacturing procedures, the need for expensive chemicals and dedicated solid surfaces to name some.

So, rather than applying chemical modifications to orient IgG molecules on a solid surface, we propose a fresh view on the physical adsorption of molecules which is based on only one intrinsic physical property of the adsorbing molecules: viz. their anisodimensionality. Our concept resembles molecular sieving on a sorbent surface. It leads to a very simple procedure for the oriented immobilisation of IgG and rendering complicated chemical or biochemical modifications to the surface or the immunoglobulins redundant.

The set-up of this paper is the following: first, we describe the theoretical aspects of size-excluded adsorption. Second we report a set of experiments that demonstrate the influence of the molecular anisodimensionality on the adsorption of IgG on pre-adsorbed layers of either IgG or triblock copolymers of the type PEO-PPO-PEO. The effect of these layers on the biological activity of later adsorbed IgG is studied by choosing the surface coverage of the sorbent by the pre-adsorbed layer as the experimental variable. Hydrophobised silica is used as the sorbent material and the adsorption process is monitored by reflectometry.

3.2 Size-and shape exclusion phenomena in IgG adsorption

3.2.1 Manipulating initial IgG orientation upon adsorption

When a mixture of proteins in aqueous solution is exposed to a bare solid surface,

it often results in adsorption [8,9], especially when the surface is hydrophobic. However, when such a surface is already partially covered by other molecules, additional binding can only take place when the remaining uncovered areas are sufficiently large to accommodate at least single protein molecules. In other words, only those proteins that fit in these uncovered areas are able to adsorb when the size distribution of individual areas excludes larger molecules. This concept resembles a molecular sieve mechanism which induces adsorption to depend on the molecular dimensions. A more advanced size-excluded adsorption applies to single protein molecules provided they have an orientation-dependent footprint. Considering, for instance an immunoglobulin G molecule, which is Y-shaped: the desired mode of adsorption has the smaller Fc-part, that only mediates effector functions, attached whereas accommodation at the sorbent surface of the larger $(F(ab)_2)$ part, that can bind antigens, is obstructed. As a result of the difference in the biological function of the two parts, this shape-excluded physical adsorption leads to a larger fraction of properly oriented molecules.

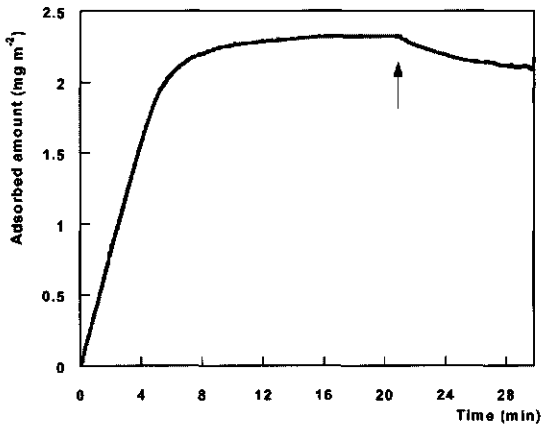

Figure 3.1: IgG adsorption process.

During the IgG adsorption process (fig. 3.1) the (charged) sorbent surface is gradually filled with adsorbed molecules. Initially, a constant rate of adsorption is observed. This indicates that a constant fraction of the molecules that arrive at the periphery of the sorbent's electric field adsorbs. At this outer boundary of the field the molecules have a random orientation. The electric field, however, may influence the orientation of adsorbing IgG molecules. When exposed to the field, the dipolar IgG molecules may experiences a torque, which tends to align the

dipole along the field direction. For such alignment to occur, the complete dipole should be located within the field. In general, the size of IgG molecules exceeds the extension of the field [10] and, hence, no alignment of the adsorbing IgG is expected. Thus, if the rate of adsorption equals the mass flux towards the surface, i.e., all molecules that arrive at the surface adsorb, the initial orientation distribution of the adsorbed IgG molecules is random.

In later stages of the process these initially adsorbed IgG molecules block a part of the surface, leaving patches of uncovered surface area in which newly arriving molecules may adsorb. With progressing adsorption, these uncovered areas will continuously decrease in number and size and eventually vanish. Then the adsorption rate also decreases; only a decreasing fraction of the molecules arriving in the vicinity of the surface can reach a free part of the binding surface and adsorb.
In the semi-plateau that arises, further attachment of IgG molecules is severely hampered, experimentally evidenced by the very small rate of adsorption. In this stage the influence of size on adsorption is maximal. Moreover, adsorbing IgG molecules are most probably oriented with their smaller Fc-part towards the surface and therefore they are expected to be intrinsically biologically more active. This effect will be referred to as 'auto-sieving'.

This concept is not restricted to molecules of the same type: a similar molecular sieving may also be created by precoating a surface with other, more protein-inert and certainly less expensive molecules. Pre-adsorption of triblock copolymers of poly(ethylene oxide), PEO, and poly(propylene oxide), PPO, is commonly used [11-14] to prevent subsequent protein adsorption. However, these polymers also are useful to create size-dependence in the adsorption of proteins on a hydrophobic surface. They adsorb through the water-insoluble PPO anchor part leaving the two water-soluble PEO buoy parts to protrude into the solution forming a steric barrier [15-17]. This is exactly the reason why these molecules have proven so successful in prohibiting further adsorption of proteins on a fully polymer covered surface. If however the amount of pre-adsorbed polymer is restricted, a rather open, low density polymeric layer is formed that contains uncovered hydrophobic areas. These areas vary in size depending on (1) the molecular characteristics of the polymer used, and (2) the degree of coverage of the sorbent surface by the polymer. It is our task to tune the open areas in the polymer layer in such a way that only the smaller Fc part of the IgG molecule can enter and adsorb onto the surface, thereby leaving the antigen binding parts

directed towards the solution and, hence, accessible to antigens.

3.2.2 Manipulating IgG orientation after initial adsorption

In order to optimise their interaction with the surface adsorbed molecules can do two things. First, they tend to change their orientation. For IgG this usually leads to a more side-on orientation. This reorientation will take a finite time. Second, especially on hydrophobic surfaces, proteins may change their internal structure [18,19]. After reorientation and reconformation an IgG molecule occupies a larger area. Therefore, these changes can only take place when there is sufficient space and time [18,20-22]. Proteins attaching in the initial stage find more area and time available for reorientation and reconformation than molecules that arrive when the surface is already partially filled. This makes one think that the orientation and the structure of later arriving molecules will deviate less from their original orientation and native biologically active structure. To be more precise, the degree of reorientation and reconformation after adsorption depends on the rates of these changes relative to the rate of adsorption. When reorientation and reconformation take place relatively fast and the incoming mass flux is relatively low the individual adsorbed molecules are fully relaxed before neighbouring sites become occupied by newly arriving molecules. If, however, the flux towards the surface increases, the adsorbed molecules will have less time for relaxation and may retain a more original orientation and a more native, compact conformation. In turn, this is reflected in a higher adsorbed mass per unit area. If the rate of relaxation is comparable to the flux incomplete relaxation of the molecules occurs and the adsorbed mass per unit area may vary with the flux.

By reducing the available surface area, precoated polymer molecules may not only force IgG to adsorb in a favourable orientation but also, as an extra advantage, suppress undesirable changes in the orientation and conformation of the proteins. If properly tailored, the pre-adsorbed polymers may form a steric barrier preventing undesirable tilting and unfolding of the protein.

3.3 Model calculations

The effect of 'auto-sieving' and a 'sieving precoated layer' on IgG adsorption can be demonstrated using a simple Random Sequential Adsorption (RSA) model [23-25]. In this model both protein and polymer molecules are approximated as hard disk-shaped particles that adsorb sequentially, irreversibly and at random positions onto a surface. Adsorption occurs only if the bare surface area encountered is sufficiently large to accommodate the adsorbing particle, once deposited, the particles can no longer move along the surface.

Polymer molecules are simulated by disks of radius 3 nm. A precoated surface is formed by the adsorption of these disks up to a certain surface coverage.
In our model an IgG molecule can adsorb in only two orientations:
with (a) its Fc part or (b) its $F(ab)_2$ part directed towards the surface.
A complete IgG molecule attached with its Fc part at the surface is simulated by an adsorbed disk of radius 6 nm (see figure 3.2). An IgG molecule oriented with its $F(ab)_2$ part directed towards the surface is simulated, in turn, by an adsorbing disk of radius 10 nm. The areas of the model disks are proportional to the projected areas of PEO chains used in the experiments and of crystallised Fc and $F(ab)_2$ fragments [28]. Adsorption of IgG molecules is simulated by the simultaneous adsorption of one 'Fc disk and one $F(ab)_2$ disk.

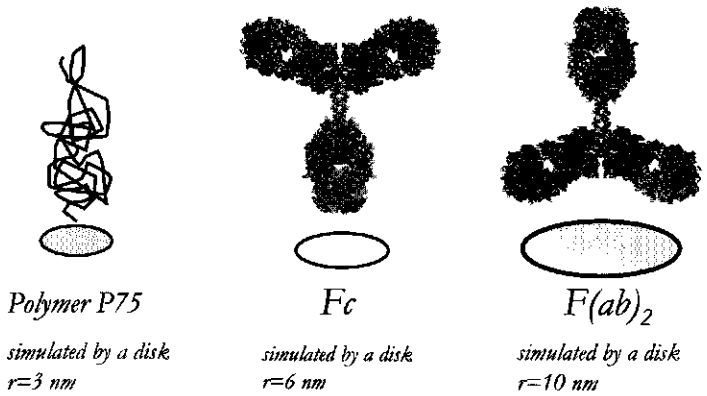

Figure 3.2: An IgG molecule can adsorb in only two orientations: with its Fc part or its $F(ab)_2$ part directed towards the surface.

In figure 3.3a, a simulated IgG adsorption on a bare, non precoated, surface is shown. The adsorbed amount, here represented by the number of particles attached to the surface, is plotted as a function of the mass flux towards the surface, here represented as the number of particles that has collided with the surface. From this graph we see that initially an equal number of Fc disks (i.e., IgG molecules attached with their Fc part to the surface) and $F(ab)_2$ disks adsorb (i.e., IgG molecules attached with their $F(ab)_2$ part). This means that the initial orientation distribution of the adsorbed IgG is random. After approximately 2000 particle-surface collisions the adsorption of the larger particles is inhibited, whereas adsorption of the small particles can still proceed. In other words, the concept of 'auto-sieving' is demonstrated: the pre-adsorbed IgG molecules force the newly

arriving IgG molecules to adsorb with their small Fc part down to the surface. In this way, a more oriented adsorbed IgG layer is created at higher surface coverages.

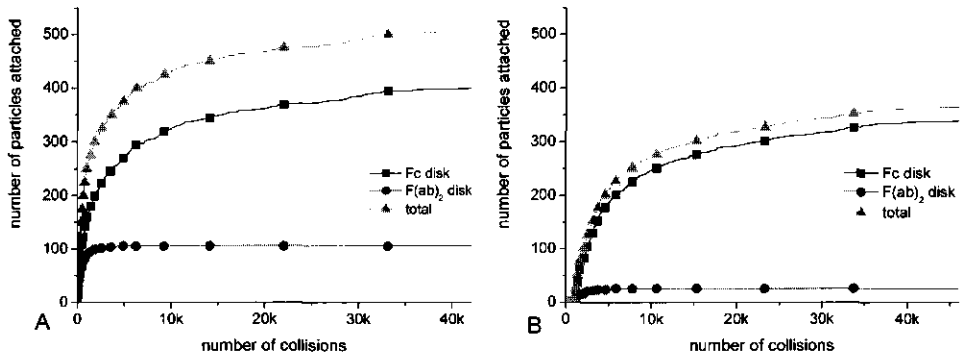

Figure 3.3: Simulated IgG adsorption on (A) a bare surface and (B) a surface precoated with polymer up to a surface coverage of 20%.

The adsorption of IgG on a surface precoated with polymer (about 20% surface coverage) is presented in figure 3.3b. As can be seen from this figure, the presence of the pre-adsorbed polymer molecules reduces both the total number of IgG adsorbed (Fc plus $F(ab)_2$ disks) and the initial rate of IgG adsorption (given by the number of particles attached per number of collisions at the start of the adsorption). The number of IgG molecules with their $F(ab)_2$ part adsorbed is extremely low in comparison with that on a bare surface. The number of IgG molecules with their Fc part adsorbed, however, does not decrease significantly.
A more detailed examination of the effect of the degree of surface coverage by the polymer on subsequently adsorbing IgG molecules is presented in figure 3.4. This figure shows that the total number of adsorbed IgG strongly decreases with increasing surface coverage of polymer (more polymer on the surface leaves less room for IgG molecules). However, the fraction of IgG molecules adsorbed with their Fc fragment increases with increasing P75 coverage. At a polymer surface coverage of 40% or higher only Fc disks are able to adsorb, this means that an oriented IgG layer is formed. When the surface coverage of the polymer is increased to 80% even the Fc disks can no longer adsorb and a protein repelling surface is created. Evidently, the degree of surface coverage of the sorbent surface by pre-adsorbed molecules is a very important parameter.
In brief, according to the random sequential adsorption model, precoating a surface with certain molecules can indeed induce oriented adsorption of IgG molecules.

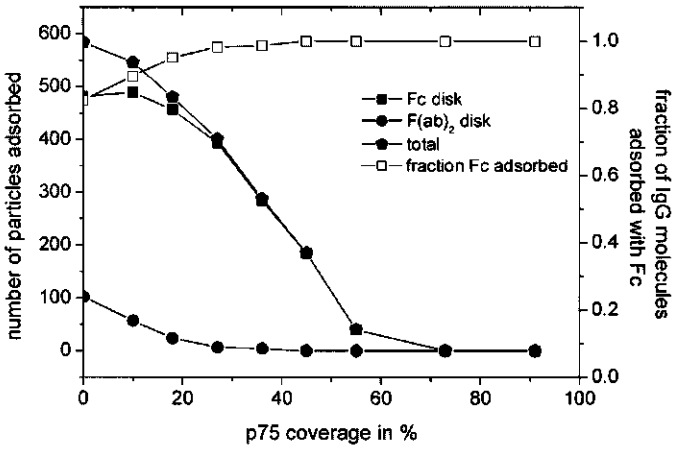

Figure 3.4: Simulated IgG adsorption as a function of polymer precoverage.

3.4 Materials and methods

3.4.1 Proteins

Mouse monoclonal immunoglobulin G (IgG 7B) from isotype 1 directed against the human pregnancy hormone (hCG, Human Chorionic Gonadotropin) and highly purified hCG were gifts from Organon Teknika (Boxtel, The Netherlands). Concentrated IgG solutions were stored at -20°C and thawed only once and shortly before use. Prior to every experiment IgG solutions were filtered through an 0.2 μm Acrodisc filter to remove large aggregates. The extinctions at 280 nm were converted into protein concentrations using an extinction coefficient of 1.45 $cm^2\ mg^{-1}$. The antigen hCG was received freeze-dried in sealed flasks containing 550 IU (1 mg roughly equals 8000 IU) and stored at 7°C. Bovine serum albumin (BSA) was purchased from Sigma Chemical Co.

3.4.2 Chemicals

Triblock copolymer of the type PEO-PPO-PEO (Synperonic P75), hereafter abbreviated as P75, was a gift from ICI (Rotterdam, The Netherlands). The two PEO (poly(ethylene oxide)) parts are water-soluble and the PPO (poly(propylene oxide)) part is water-insoluble. The average molecular weights of the whole polymer, its PEO, and PPO parts are 4150, 1075 Da (24 segments) and 2000 Da (34 segments) respectively.

Chemicals for buffer preparation, Na_2HPO_4, NaH_2PO_4, were of analytical grade and used without further purification. Water was purified by reverse osmosis and

passed subsequently through a superQ system (Millipore).

3.4.3 Surfaces

Silicon wafers (Wacker Chemitronic GmbH, Munich, Germany) covered with a thin layer of SiO_2, formed by thermal oxidation at 1000° C for 90 minutes, were used as the hydrophilic substrates. A silica layer thickness of approximately 100 nm is essential for obtaining a high sensitivity in reflectometry experiments. By ellipsometry it was verified that this condition was satisfied. Hydrophobic methylated silica surfaces were prepared by immersion of the silica surfaces in 1 % (v/v) dichlorodimethylsilane in toluene for 15 minutes. Afterwards the surfaces were extensively rinsed with ethanol and water. The contact angle of a drop of water on the hydrophilic and the hydrophobised silica surface were around 5° and 90°, respectively.

The surfaces were cleaned with a plasma cleaner prior to every individual adsorption experiment and before the surfaces were hydrophobised.

3.4.4 Reflectometry

All experiments were carried out in a 5 mM phosphate buffer solution of pH 6, which is the isoelectric point of the applied IgG.

Polymer and protein adsorption were measured using a reflectometer equipped with a stagnation-point flow-cell [26]. If the perfect sink boundary condition applies (c=0 at the interface) and in the absence of a barrier, then the initial molecular limiting flux towards the surface depends on the geometry of the cell, the diffusion coefficient of the molecule, the flow rate and the concentration in solution as described by the following equation [26]:

$$J_0 = 0.776 \nu^{1/3} R^{-1} D^{2/3} (\bar{\alpha}\,\mathrm{Re})^{1/3} c \qquad (3.1)$$

where ν is the kinematic viscosity of the solution, R the radius of the inlet tube, D the diffusion coefficient of the solute, $\bar{\alpha}$ a stream intensity parameter, which depends on the dimensions of the cell and the Reynolds number Re and c the solute concentration.

The effect of the flux on the adsorption of the polymers and proteins is studied by varying the concentration of the molecules, keeping all other experimental and material variables constant. A constant pulse-free flux is realised by using a microdosing pump (CAT, Staufen, Germany). The flow rates of the protein and

polymer solutions were 1 ml per minute and 0.5 ml per minute, respectively.

3.4.5 Biological activity

The biological activity of adsorbed IgG is determined by measuring the amount of hCG that can be bound by saturating adsorbed IgG layers. The average intrinsic biological activity, mol hCG bound per mol IgG, is calculated by:

$$\frac{mol\ hCG}{mol\ IgG} = \frac{\Gamma_{hCG} \cdot M_{IgG}}{\Gamma_{IgG} \cdot M_{hCG}} \tag{3.2}$$

Non-specific binding of hCG to the part of the surface not covered by IgG is minimised by post-coating the surface by adsorption of BSA from a 1 mg ml^{-1} solution; this method is often applied in immunological tests. This blocking is important since possible non-specific binding of hCG would lead to an overestimation of the biological activity of adsorbed IgG. From experiments in which radio-active labelled hCG was used we concluded that BSA is a satisfactory blocking agent [27]. In between the different steps of the protocol, i.e. the IgG adsorption, the BSA post-coating and the hCG binding, the surface was rinsed for 10 minutes with phosphate buffer.

3.5 Results and discussion

The effect of the presence of pre-adsorbed molecules on the adsorption behaviour of IgG molecules is studied. We examined whether this pre-adsorption leads to a more favourable average orientation of the adsorbed IgG and whether this results in a higher specific biological activity of the adsorbed IgG. First, the effect of pre-adsorbed IgG layers is studied, followed by the effect of pre-adsorbed triblock copolymer layers.

3.5.1 IgG layers

IgG adsorption on bare surfaces

A typical adsorption curve for IgG on methylated silica is shown in figure 3.5a. Figure 3.5b shows the relative rate of IgG adsorption as a function of time. When the adsorbed IgG layer is rinsed with a buffer solution only a small fraction of the IgG molecules desorbs. Hence, found before [28,29], the adsorption of IgG is essentially irreversible with respect to dilution.

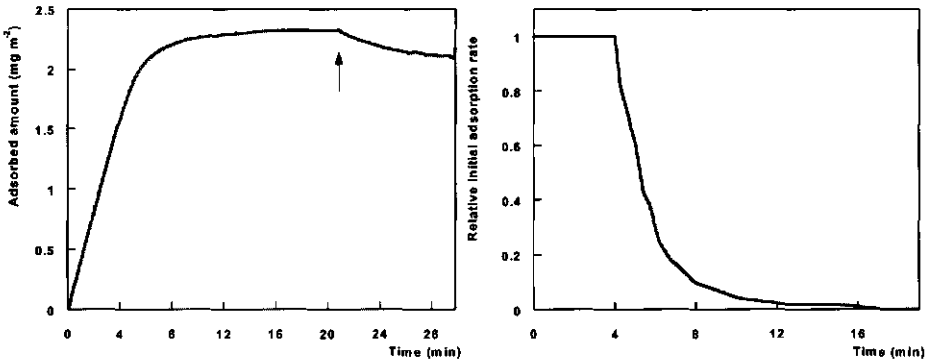

Figure 3.5: (A) IgG adsorption on methylated silica, rinse with buffer indicated by arrow. (B) Relative initial adsorption rate.

On the hydrophobic surface the initial rate of adsorption of IgG, reflected by the slope of the adsorption curve at t=0, equals about 85% to 90% of the theoretical flux (fig. 3.5a). This difference between the initial rate of adsorption and the theoretical flux may be explained by an overestimation of the theoretical flux due to the finite thickness of the laser beam [26]. Also, when the actual alignment of the reflection spot is somewhat off-centre, the flux is again overestimated [26]. Roughly, all IgG molecules that arrive at the surface do adsorb and this means that the adsorption is transport limited, which is also reported in literature [28]. The sticking probability of an IgG molecule on a freely accessible bare part of the surface approaches unity. In this case, binding occurs irrespective of the molecular orientation upon arrival. This is also observed for e.g. albumin adsorbed on a hydrophobic surface [21].

The range where the relative rate of IgG adsorption starts to deviate from unity marks the end of the transport-limited regime. At longer adsorption times the adsorption rate decreases gradually with surface coverage and time (fig. 3.5b). Such a gradual transition from the transport-limited region to the saturation value is also observed for e.g. albumin and fibrinogen adsorbing on a hydrophobic surface [21]. Due to the build-up of the adsorbed layer the attachment to the surface becomes more and more difficult. According to [21], unfolding (and to a lesser extent reorientation) of albumin and fibrinogen on the surface was the main reason for the restricted further attachment of protein molecules. Considering IgG, the rounded appearance of the adsorption curves may reflect both the need for a specific orientation of the later adsorbing molecules and spreading of the

molecules on the surface. Whether both processes contribute will be considered below.
Finally, after approximately 18 minutes from the first adsorption, the relative rate of IgG adsorption becomes extremely low.

The average specific biological activity (mol hCG bound per mol of IgG) of adsorbed IgG is measured in three stages in the adsorption process; (1) in the transport-limited range (1 minute), (2) in the transition range where the adsorbing molecules experience some hindrance of the pre-adsorbed molecules (8 minutes), and (3) in the final stage in which the orientation of the molecules is strongly restricted (14 hours). The results are presented in table 3.1. The biological activity of the IgG adsorbed between 1 and 8 minutes, and between 8 minutes and 14 hours is calculated assuming that the biological activity of the previously adsorbed IgG molecules remains constant with time. From this table it is deduced that the average biological activity has substantially increased for the IgG molecules that adsorb at a later stage.

Table 3.1: Biological activity of adsorbed IgG after different adsorption times.

adsorption time	adsorbed amount of IgG (mg m^{-2})	average biological activity (mol hCG/mol IgG)	average biological activity of *extra* adsorbed IgG (mol hCG/mol IgG)
1 minute	0.70	0.66	
			0.71
8 minutes	2.16	0.69	
			1.16
14 hours	2.74	0.79	

From other experiments that included infrared (IR) and circular dichroism (CD) measurements [30-31] we concluded that an increase in the adsorbed amount of IgG resulted in a more native-like structure of the adsorbed IgG. This means that in the later stages in the adsorption process adsorbed IgG retains a more native biologically active structure. However, the increase in biological activity of the later adsorbed IgG molecules (table 3.1) can not solely be attributed to this more native-like structure. In a random orientation of IgG molecules at a surface at most half of all molecules will have their antigen binding sites more or less directed towards the solution. In such an orientation distribution, assuming that all molecules have

their native activity, a maximum average biological activity of 1 mol hCG/mol IgG could be achieved. As our measured biological activity of the later adsorbed IgG is around 1.15 mol hCG/mol IgG, we conclude that both the sieving process and the prevention of reconformation of the molecules have contributed.

The adsorption of IgG molecules was further investigated as a function of the degree of relaxation, i.e., reorientation and reconformation. In figure 3.6a the influence of the IgG flux on adsorption is shown. The flux towards the surface was varied by changing the IgG concentration. The adsorbed amount is plotted as a function of the product of flux and time $J_0 \cdot t$. The normalised initial adsorption rates are only slightly lower than 1, as explained for figure 3.5, but they are independent of the IgG concentration. This indicates that initially the adsorption is transport-controlled, which in the case of stagnation point flow means diffusion-controlled.

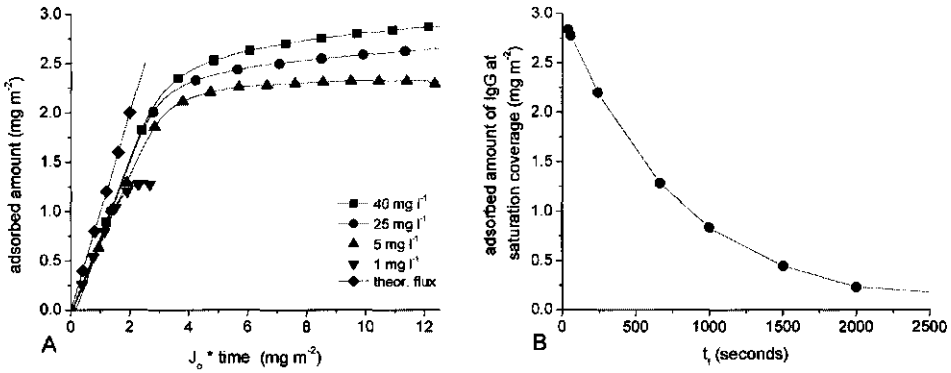

Figure 3.6: (A) IgG adsorption on methylated silica for several IgG concentrations. (B) IgG saturation adsorption as a function of the filling time of the surface.

The saturation adsorption increases with increasing IgG concentration (fig. 3.6a). It demonstrates that the IgG molecules change their orientation or conformation at the surface and that the rate of reorientation or reconformation and the rate of attachment are of the same order [ref]. From these experiments a relaxation time for the adsorbed IgG molecules can be roughly estimated in the following way. The time needed for completely filling up the surface, t_f, can be described by:

$$t_f = \frac{\Gamma_{sat}}{J} \qquad (3.3)$$

assuming transport control everywhere. Γ_{sat} is the adsorbed amount at saturation coverage. In figure 3.6b the saturation adsorption is plotted as a function t_f. After more than 2000 seconds the saturation adsorption becomes constant with time. This indicates that above that time the IgG molecules are fully relaxed with respect to orientation and configuration and that the relaxation time is shorter than the filling time. Accordingly, we estimate a relaxation time of more than 30 minutes. From IR and CD measurements [30,31] we concluded that the time scales over which structural changes were completed were less than 2 minutes. Therefore, an overall relaxation time of over 30 minutes implies a substantial contribution of orientation relaxation of the IgG molecules. Furthermore, these data show that pre-adsorbed IgG molecules can suppress the degree of orientational changes of adsorbed IgG molecules.
Wertz and Santore [21] estimated interfacial protein relaxation rates of 0.12 and 0.15 nm^2 $molecule^{-1}$ s^{-1} for albumin and fibrinogen, respectively. We estimate a relaxation rate of 0.3 nm^2 $molecule^{-1}$ s^{-1} for IgG (in about 2000 seconds IgG has spread from approximately 90 nm^2 per molecule (c. 2.8 mg m^{-2}) to 800 nm^2 per molecule (c. 0.3 mg m^{-2})). This value is comparable to those found by Wertz and Santore.

The biological activity of the adsorbed IgG layers shown in figure 3.6 was also measured. At high adsorbed amounts, when the molecules have a more end-on orientation, the specific biological activity of IgG is much higher. From this we conclude that reorientation leads to a loss in biological activity and that suppression of this phenomenon will lead to a biologically more active layer of IgG.

It is thus demonstrated that the orientation of later adsorbing IgG molecules can be improved by pre-adsorbed IgG molecules. In the next section the effect of other pre-adsorbed molecules, in our case triblock copolymers on subsequently adsorbing IgG molecules will be discussed.

3.5.2 Triblock copolymer layers

First the adsorption behaviour of triblock copolymers on the hydrophobised silica will be discussed and thereafter its effect on the adsorption and biological activity of IgG.

Triblock copolymer (p75) adsorption

Figure 3.7 shows the adsorption of P75 as a function of the flux towards the

surface. The flux is varied by changing the polymer concentration. In an aqueous solution the amphiphilic polymers start forming large micellar structures at the critical micellisation concentration (CMC). These micelles have a diffusion coefficient that is smaller than that of a single polymer. The CMC value for P75 is expected to be very high (12 mM - 34 mM) [32,33] therefore, P75 concentrations used in figure 3.7 are well below this CMC, hence in the calculations for the flux an estimated diffusion coefficient [34] of a single triblock copolymer molecule is used. In this figure the adsorbed amount is plotted as a function of the product of flux and time $J_0 \cdot t$. Again, the normalised initial adsorption rates are, within experimental error, independent of the P75 concentration. This rate amounts to about 65% of the theoretical flux, which is lower than the corresponding value for IgG. This lower adsorption rate likely results from the low affinity of small PEO chains for a hydrophobic surface: when the triblock copolymers arrive at the surface with their PEO chains directed towards the surface they do not adsorb. So, we are dealing with an orientation effect.

The P75 polymers are strongly bound to the surface. Although at higher polymer concentrations some desorption was observed upon rinsing the layers with buffer (fig. 3.7), the remaining molecules are strongly attached to the surface as judged from the reflectometer signal being constant for over an hour.

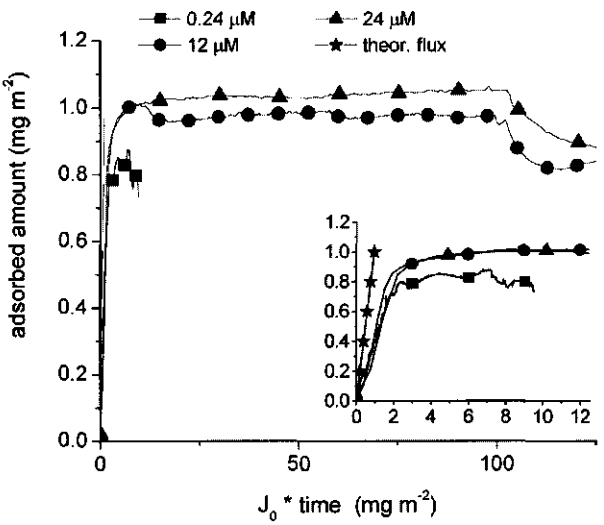

Figure 3.7: P75 adsorption for several P75 concentrations (mg l^{-1}).

Figure 3.7 shows that for these molecules the adsorption saturation is essentially invariant with the flux. Therefore, the conformation of the polymers at adsorption saturation is constant with time within the time scale of our experiments, which is expected as the spreading of flexible polymers is very fast. The amount adsorbed at saturation is in good agreement with values reported in literature [13]. The value of 0.8 mg m^{-2} indicates that no multilayers are formed and that micelles are not responsible for the adsorption, which is supported by the strong binding of the polymers to the surface. An adsorbed amount of 0.8 mg m^{-2} corresponds to about 9 nm^2 per polymer molecule i.e. per two PEO chains. The radius of gyration of one PEO containing 24 EO monomers is around 1.2 nm [34]. This corresponds to a projection area for two chains of about 9 nm^2. Apparently, at adsorption saturation, the surface area per PEO chain is determined by its radius of gyration.

3.5.3 IgG adsorption on pre-adsorbed P75 layers

On a polymer layer of about 0.70 mg m^{-2} and higher hardly any IgG adsorption was observed. In other words, pre-adsorption of polymer up to 0.70 mg m^{-2} creates a perfectly protein-repelling surface the more so because on the time scale of these experiments the polymers are not desorbed by IgG molecules. At lower coverage of pre-adsorbed polymer maximum IgG adsorption is only reached after very long times, e.g. at a pre-adsorbed amount of P75 of 0.60 mg m^{-2} the maximum adsorption requires about 10 hours. The plateau value of adsorbed IgG decreases with increasing adsorbed amount of P75. This decrease was about 1 mg m^{-2} when the pre-adsorbed amounts of P75 increased from about 0.15 mg m^{-2} to 0.6 mg m^{-2}.

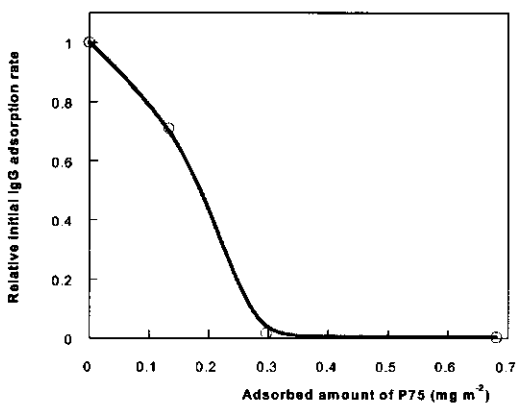

Figure 3.8: Relative initial adsorption rates of IgG on pre-adsorbed P75 layers.

Figure 3.8 shows the rate of IgG adsorption on polymer layers at different degrees of coverage of the sorbent surface. The initial adsorption rate strongly decreases with increased amount of adsorbed polymer, i.e. with increasing surface coverage. This again indicates that not all arriving IgG molecules are able to adsorb. Whether or not this also results a in preferential orientation of IgG molecules can be determined by measuring the biological activity.

As the maximum adsorption of IgG on polymer layers was reached after very long adsorption times and because it was different between the various polymer precoatings, we measured the biological activity at fixed IgG adsorption (namely 0.7 mg m^{-2}) but at different polymer surface coverages. At this adsorbed amount a random molecular orientation distribution of adsorbed IgG is formed on a non precoated surface.

Table 3.2: Biological activity of adsorbed IgG on precoated P75 layers. In column 4 the biological activity of IgG after 90 minutes of rinsing with phosphate buffer is presented.

adsorbed amount of p75 (mg m^{-2})	adsorbed amount of IgG (mg m^{-2})	average biological activity (mol hCG/mol IgG)	resultant average biological activity after 90 minutes (mol hCG/mol IgG)
0	0.70	0.66	0.47
0.13	0.70	1.11	1.06
0.19	0.70	1.23	1.17

From table 3.2 it can be seen that the specific biological activity of adsorbed IgG molecules on the polymer layer is almost twice as high as on the bare hydrophobic surface. This again demonstrates convincingly the molecular sieve effect inducing the IgG molecules to adsorb in a preferential orientation on the surface.

The time dependency of the biological activity of adsorbed IgG on the bare hydrophobic surface and on the pre-adsorbed polymer layer was studied by rinsing the adsorbed IgG layers for 90 minutes with buffer. On the bare surface the activity of the adsorbed IgG decreased by about 30% whereas for IgG molecules adsorbed in between the polymer layers it was only slightly lowered (table 3.2). In both cases no desorption of IgG was observed. Apparently, the pre-adsorbed polymer has a conserving effect on the biological activity of the adsorbed IgG molecules, at least within the time scale of our experiment. The long-term loss in

26. Dijt, J.C., Cohen Stuart, M.A., and Fleer, G.J. (1994) *Adv. Colloid Interf. Sci. 50*, 79.
27. Chapter 7, this thesis.
28. Buijs, J., Ph.D. Thesis, Wageningen Agricultural University, 1995.
29. Ortega-Vinuesa, J.L., Galvez Ruiz, M.J., and Hidalgo-Alvarez, R. (1996) *Langmuir 12*, 3211.
30. Chapter 6, this thesis.
31. Giacomelli, C.E., Bremer, M.G.E.G., and Norde, W. (1999) *J. Colloid Interf. Sci. 220*, 13.
32. McPherson, T., Kidane, A., Sleifer, I., and Park, K. (1998) *Langmuir 14*, 176.
33. Alexandridis, P., Holzwarth, J.F., and Hatton, T.A. (1994) *Macromolecules 27*, 2414.
34. Fleer, G.J., Cohen Stuart, M.A., Scheutjens, J.M.H.M., Cosgrove, T., Vincent, B. *Polymers at Interfaces*, Chapmann & Hall, London, 1993.

Chapter 4

ATR-FTIR STUDY OF IGG ADSORBED ON DIFFERENT SILICA SURFACES

Abstract

The secondary structure of adsorbed immunoglobulin G (IgG) on different silica surfaces (hydrophilic, hydrophobic, hydrophobic with pre-adsorbed triblock copolymers consisting of a polypropylene oxide buoy and two polyethylene oxide chains dangling in the solution) is studied by ATR-FTIR. Some results for adsorbed bovine serum albumin (BSA) are also presented. The secondary structure of adsorbed IgG was quantified using second derivative spectra for the input parameters of the curve-fitting analysis of the original spectra. The secondary structure of adsorbed IgG on hydrophilic silica surface resembles that of IgG in solution (about 60% of β-sheet and almost no α-helix content). There is some loss in the helix content of BSA after adsorption on the hydrophilic surface but this structural element is still the most important one in the adsorbed protein. The IR spectra of the adsorbed proteins on the hydrophobic silica surface can not be interpreted probably because of a large contribution to the IR signal of water molecules that are exchanged against the proteins during adsorption. The presence of pre-adsorbed triblock copolymers reduces the adsorbed amount and causes an effect on the adsorbed proteins similar to that exerted by ethylene glycol: a different type of β-sheet structure in IgG and a more ordered α-helix structure in BSA are provoked.

4.1 Introduction

Adsorption of monoclonal immunoglobulin G (IgG) to solid surfaces is of great interest for designing immuno-diagnostic test systems. In these systems IgG is immobilised onto particles (e.g. immunolatices) or onto flat surfaces (e.g. ELISA). To assure proper antigen binding it is essential that the IgG molecule retains sufficient structural integrity and that it adopts a right orientation on the sorbent surface. An IgG molecule is composed of four polypeptide chains that are connected by disulphide bonds and non-covalent forces. The four chains can be divided into two groups: two heavy and two light chains. All four polypeptide chains are grouped in different domains, two F(ab) segments and one Fc segment, together forming a Y-shaped conformation. The antigen-binding sites are located on the far ends of the F(ab) segments. It may be clear that the right orientation of the adsorbed IgG molecules is obtained when the Fc part is attached to the surface and its F(ab) parts are exposed to the antigen-containing solution.

Co-adsorption of proteins and copolymers may be applied to control the conformation and/or orientation of adsorbed IgG. The copolymer used anchor with one block to the sorbent surface, whereas the other part(s) extend into the solution. The adsorption of (co)polymers may reduce or even prevent the subsequent protein adsorption due to a steric repulsion. Furthermore, the adsorbed (co)polymers influence the sorbent surface characteristics as charge density, hydrophobicity, etc. Hence, the interaction between IgG and the sorbent surface modified by the pre-adsorbed copolymers is determined by a complex combination of steric, electrostatic, hydrophobic and van der Waals contribution. Whatever the exact mechanism of the interaction is, the subsequent IgG adsorption behavior, with respect to the adsorbed amount, conformation and orientation, strongly depends on the surface density of the pre-adsorbed copolymer and on the lengths of its parts that anchor to the surface and that extend into the solution, respectively [5,6]. In addition, the presence of pre-adsorbed components influences the sorbent surface characteristics as charge density, hydrophobicity, etc. In turn, these properties are known to affect the protein adsorption behaviour. Hence, even if the proper orientation is achieved, the pre-adsorbed copolymer or protein may affect the amount and the conformation of the subsequently adsorbed IgG.

The secondary structure of proteins in an environment relevant for immunological applications may be studied by spectroscopic methods. Infrared (IR) spectrometry has been used extensively to examine the structure of proteins in solution [7-9]

while IR in combination with attenuated total reflectance (ATR) has been used to obtain spectra of adsorbed protein [10-11]. The IR spectra of proteins exhibit a number of amide bands; the amide I band (1600-1700 cm^{-1}) is mostly used to extract information on the protein's secondary structure. Because each of the different secondary structural elements contributes to the IR spectrum, the observed amide bands are composed of several overlapping components. The fundamental difficulty encountered in the analysis of the contour of an amide band is that the individual component peaks can not be resolved and/or identified directly in the measured spectra. Derivation of structural information requires extensive mathematical manipulation [12]. Separation of the overlapping peaks can be increased by calculating the *n*th (usually second) derivative of the original absorption spectrum [10,13] or by using the computational procedure of Fourier deconvolution [7,14]. A quantitative analysis of the secondary structure of the protein can then be performed by using the information provided by the *n*th derivative to curve-fitting the original spectra or by curve-fitting the Fourier deconvolved spectra.

A structural analysis of a monoclonal IgG adsorbed on different silica surfaces (hydrophilic, hydrophobic, hydrophobic with pre-adsorbed triblock copolymers) was performed using ATR-FTIR spectroscopy. The experiments were carried out with a silicon cylindrical internal reflecting element which has a silica surface layer. Structural analysis was obtained from the amide I region and the area of the amide II region was used to asses the relative adsorbed amounts. A fitting procedure using second derivative spectra for the input parameters was applied to the ATR-FTIR original spectra in order to analyse the secondary structure elements. In addition, some experiments were carried out with bovine serum albumin (BSA) in order to compare the results between this protein and IgG.

4.2 Materials and Methods

4.2.1 Proteins

The monoclonal immunoglobulin, IgG, was obtained from Organon Teknika (Boxtel, The Netherlands); its characteristics were published elsewhere [10,15]. The IgG concentration was spectroscopically determined using an extinction coefficient of 1.45 cm^2 mg^{-1} at 280 nm. BSA was a commercial product (Sigma, A7906) and used without further purification. Protein solutions were prepared in a 5 mM phosphate buffer at pH 6 and the experiments were performed using 0.050 g l^{-1} solution.

4.2.2 *Surfaces*

The experiments were carried out with three different surfaces:

Hydrophilic silica, the cylindrical internal reflectance crystal. This silicon crystal has a silica surface layer. The contact angle of a drop of water on such an oxide layer is between 0° and 5°.

Hydrophobic methylated silica, obtained by immersion of the crystal in 9 % (v/v) dichlorodimethylsilane in toluene for 30 minutes, then shortly rinsed with ethanol and finally heated at 110 °C during 15 minutes. After this treatment the contact angle of a drop of H_2O on this surface was 90°.

Hydrophobic methylated silica with adsorbed triblock copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), i.e. PEO-PPO-PEO.

The crystal was cleaned with a plasma cleaner prior to IR measurements (hydrophilic silica surface) or before the surface was hydrophobised.

4.2.3 *Other solutions*

Buffer solutions were prepared by mixing the appropriate volumes of 5 mM Na_2HPO_4 and 5 mM NaH_2PO_4 solutions.

A 0.3 g l^{-1} stock solution of the P75 triblock copolymer (ICI, Rotterdam The Netherlands) was prepared in buffer, stored at 5 °C and diluted properly for the experiments. The molecular weight of the PPO and PEO segments in the P75 triblock copolymer is 2000 Da (34 monomers) and 1075 Da (24 monomers) respectively.

4.2.4 *FT-IR measurements*

Spectra were collected with a model 1725X FTIR spectrometer (Perkin-Elmer). The spectra were obtained with a N_2-cooled mercury cadmium telluride detector at a resolution of 1 cm^{-1}, using a normal Norton-Beer apodisation function. The FTIR spectrometer was continuously purged with air which was freed from H_2O and CO_2 vapour. The data acquisition and processing were performed as described by Buijs et al. [10]. Briefly, the protein spectra were obtained after subtracting the background (buffer solution) from the sample (buffer + protein solution) spectra, using a scaling factor that made the 1720-1800 cm^{-1} region flat [16]. A correction for the presence of water vapour was also carried out. Before taking the second derivative spectra, the original protein spectra were smoothed using a triangular smoothing procedure. The curve-fitting was performed on the original spectra by assuming a Lorentzian shape for the amide I band and for each individual peak.

4.3 Results and Discussion

4.3.1 Structural Analysis

Hydrophilic Silica

Representative spectra of IgG adsorbed on hydrophilic silica are shown in figure 4.1. In order to compare data obtained at different adsorption times and in separate experiments, the spectra were normalised between 0 and 1. Figure 4.1 compares the normalised spectra obtained in the same experiment at different adsorption times; the deviation between these curves is less than 2%. The spectra obtained at the same adsorption time but in different experiments (not shown) revealed that the reproducibility is within 5%. Table 4.1 lists the positions of the maximum of the amide I and amide II bands and their intensity and area ratios for different adsorption times (**Si**; IgG). These values are the average of, at least, two separate experiments. There is almost no variation with adsorption time neither in the positions of the bands nor in their intensity and area ratios.

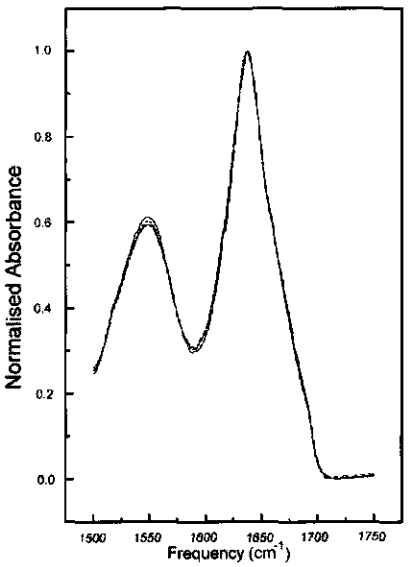

Figure 4.1: Normalised spectra of IgG adsorbed on hydrophilic silica surface at different adsorption times. The time scale is based on the sequence in which the spectra were taken, i.e., the first spectrum (t=0) was taken after 13 minutes of the first protein-surface contact (5 minutes for filling the cell and 8 minutes to perform the frequency scan).

Table 4.1: comparison of the amide I and II bands for IgG and BSA adsorbed on hydrophilic (Si) and hydrophobic + copolymer (Si+C) silica surfaces.

	Time (minutes)[a]		0	8	16	24	32	40
Position Amide I (cm^{-1})	Si	IgG	1637	1637	1637	1637	1637	1637
		BSA	1647	1647	1648	1647	1648	1649
	Si+C	IgG	1637	1637	1635	1635	1635	1635
		BSA	1650	1649	1649	1649	1649	1649
Position Amide II (cm^{-1})	Si	IgG	1548	1549	1548	1548	1549	1549
		BSA	1547	1546	1546	1547	1547	1547
	Si+C	IgG	1545	1546	1547	1545	1549	1549
		BSA	1547	1547	1547	1547	1547	1547
Intensity Ratio[2]	Si	IgG	1.56	1.61	1.65	1.63	1.69	1.67
		BSA	1.64	1.58	1.55	1.53	1.53	1.53
	Si+C	IgG	1.58	1.60	1.60	1.61	1.60	1.59
		BSA	1.53	1.56	1.50	1.53	1.52	1.49
Area Ratio[2]	Si	IgG	1.30	1.30	1.37	1.36	1.38	1.38
		BSA	1.86	1.73	1.67	1.66	1.64	1.61
	Si+C	IgG	1.33	1.33	1.46	1.44	1.34	1.43
		BSA	1.53	1.68	1.50	1.57	1.67	1.62

[a]The time scale is based on the sequence in which the spectra were taken, i.e., the first spectrum (t=0) was taken after 13 minutes of the first protein-surface contact (5 minutes for filling the cell and 8 minutes to perform the frequency scan). [2]Amide I / Amide II.

Table 4.2: comparison of the peak positions obtained with the second derivative spectra (SD) and after curve-fitting (CF) the original spectra of IgG adsorbed on different silica surfaces.

	Hydrophilic Silica		Hydrophobic Silica + Copolymer	
	SD	CF	SD	CF
β-sheet	1672 ± 3 cm^{-1}	1675 ± 2 cm^{-1}	1672 ±4 cm^{-1}	1674 ± 2 cm^{-1}
	1637 ± 2 cm^{-1}	1636 ± 1 cm^{-1}	1638 ± 1 cm^{-1}	1637 ± 1 cm^{-1}
	1624 ± 4 cm^{-1}	1625 ± 2 cm^{-1}	1622 ± 2 cm^{-1}	1622 ± 2 cm^{-1}
			1631 ± 1 cm^{-1}	1630 ± 1 cm^{-1}
β-turns	1692 ± 1 cm^{-1}	1688 ± 2 cm^{-1}	1693 ± 1 cm^{-1}	1688 ± 2 cm^{-1}
	1661 ± 3 cm^{-1}	1661 ± 1 cm^{-1}	1662 ± 1 cm^{-1}	1660 ± 1 cm^{-1}
α-helix+unordered	1652 ± 5 cm^{-1}	1647 ± 1 cm^{-1}	1650 ± 5 cm^{-1}	1647 ± 1 cm^{-1}
side chains	1615 ± 3 cm^{-1}	1613 ± 2 cm^{-1}	1612 ± 3 cm^{-1}	1613 ± 2 cm^{-1}

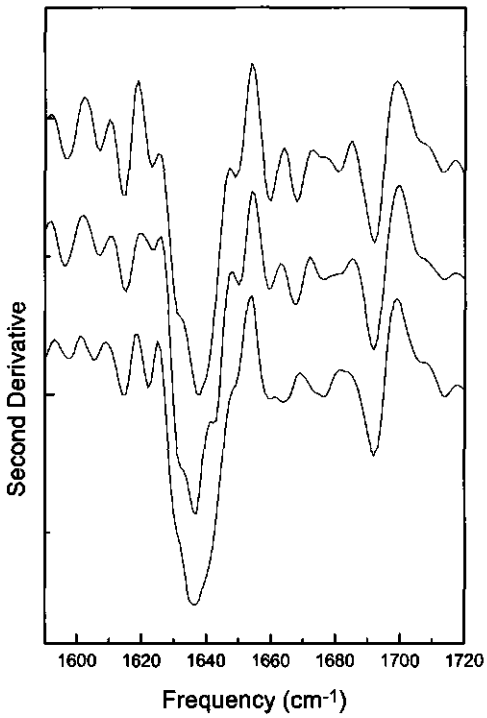

Figure 4.2: Second derivative (SD) spectra of IgG adsorbed on hydrophilic silica surface at different adsorption times after smoothing the original spectra over a range of 13 cm^{-1}. From top to bottom, t=8 minutes, t=24 minutes and t=40 minutes (see figure 4.1, for time scale).

All IR spectra obtained for IgG adsorbed on hydrophilic silica show the maximum of the amide I band at 1637 cm^{-1}, which is typical of proteins with high β-sheet content [7,17]. Several different experimental methods to determine the structural components of IgG in solution reveal that this protein largely consists of β-sheet structures while the α-helix content, if any, is small [7,15,18]. The amide I position at 1637 cm^{-1} found in the spectra shows that also in *adsorbed* IgG the β-sheet structure is the main structural component. Moreover, the fact that the normalised spectra of adsorbed IgG at various adsorption times show no differences in their positions and band widths strongly suggests that the protein does not change its structure after adsorption on the hydrophilic surface, or if there is any change it has occurred before the first spectrum is taken. This last possibility should not be ruled out, because before the first spectrum is taken there may have been enough time elapsed for the adsorbed protein to change its conformation: 5 minutes were

necessary to displace the buffer solution and fill the cell with protein solution and another 8 minutes were required to take the first spectrum.

Some studies suggest that the amide I/amide II intensity and area ratios may be associated with changes in the secondary structure of the protein [19,20]. The observation that for the adsorbed IgG these ratios do not significantly change with time reinforces the idea that the secondary structure of the protein is the same for the different adsorption times. Therefore the number of peaks in the second derivative (SD) spectra should be invariant with time as well, which implies that the input parameters for the curve-fitting (CF) procedure should be the same.
Figure 4.2 compares the SD spectra of the amide I band obtained at three adsorption times. Since small fluctuations in absorption intensity are magnified in the derivatives, noise must be suppressed before taking the SD in order to discriminate noise from real peaks. The spectra in Figure 4.2 were obtained after smoothing the original spectra over a range of 13 cm^{-1}. Table 4.2 lists the average positions of the peaks found in the SD spectra of the amide I band for different adsorption times and in different experiments. It shows that the deviation in the average of each characteristic frequency is in the worst case $\pm$ 5 cm^{-1}. These positions are in very good agreement with those reported in the literature for IgG using different IR methods (ATR and transmission) and using different mathematical manipulation of the data, such as SD, CF or Fourier self-deconvolution [7,10,21-23].

The CF procedure was performed on the normalised original spectra (without smoothing) using the seven peak positions found in the SD as input parameters. Figure 4.3 shows a representative CF result for IgG adsorbed on hydrophilic silica. Table 4.2 compares the peak positions of each structural element present in the SD spectra and those obtained after CF of the original spectra. A great deal of care must be taken with CF, because there are a large number of adjustable parameters (3n, where n is the number of peaks) and the solution may not be unique. Nevertheless, the aim of this fitting procedure is to compare the secondary structure of the IgG adsorbed on different surfaces and if the procedure is carried out always in the same way the results allow such a comparison. It is to be noted that the amide I band can be fitted using less than seven peaks but their positions are different from those obtained in the SD spectra (they are somewhat in the middle of two positions) and the widths of the peaks are larger than those reported as 'normal' for structural elements [7,10].

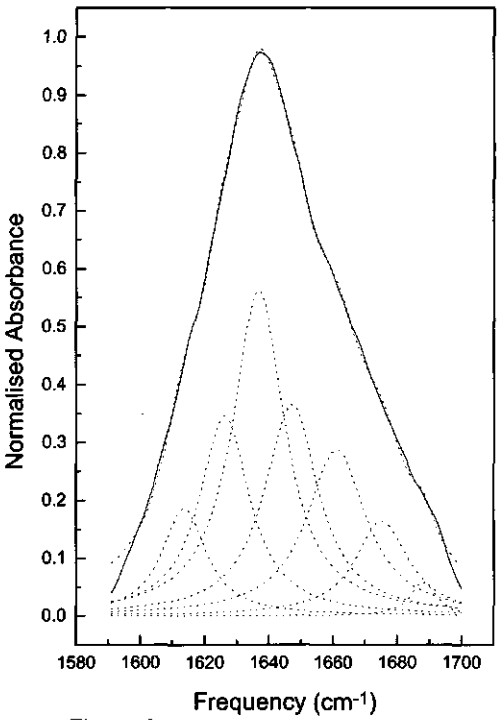

Figure 4.3: Experimental (solid curve) and calculated spectra (dashed curves) of IgG adsorbed on hydrophilic silica surface. The calculated spectrum was obtained by curve-fitting the experimental one using 7 peaks.

Perhaps the most critical step in the interpretation of IR spectra is the assignment of each peak to an individual structural element. However, the analysis becomes less ambiguous if the protein is known to possess a major secondary structural element. It is well known that the β-sheet is the main secondary structure element of IgG in solution. As it can be concluded from the amide I band position, from the lack of changes in its band width and in the intensity and area ratios and from the dominant peak in the SD spectra, the β-sheet is also the main structure in the adsorbed IgG molecules. The absorption peaks at 1637 and 1624 cm^{-1} are univocally related to β-sheet structure, and are usually called as the low frequency 'β-component'. This multiplicity between 1620 and 1640 cm^{-1} in 'β-peaks' has been frequently observed in β-sheet-containing proteins and reflects differences in the hydrogen bonding strength as well as differences in transition dipole coupling in different β-strands [12]. The high frequency 'β-component' may be overlapping with contributions from β-turns and unordered structures and it is not univocally related to a particular absorption peak: in the literature the peaks observed

between 1690 and 1660 cm^{-1} were assigned to either β-turns or β-sheet [7,10,21]. Based on the spectra of proteins with very high β-sheet content, Byler and Susi [7,13] assign the peaks observed in the region of 1680-1670 cm^{-1} to the high frequency component of β-sheet structure and suggested that the sum of all the integrated areas of the 'β-peaks', as a fraction of the total amide I band area, is closely related to the total 'β-content' of a given protein. On this basis, the β-sheet content of the adsorbed IgG was calculated by using the peaks centred at 1675, 1636 and 1625 cm^{-1}. The peaks at 1688 and 1661 cm^{-1} were assigned to β-turns. The peak centred at 1613 cm^{-1} was assigned to the absorption of the side chains of tyrosine and arginine [10,22] and it was therefore ignored in the quantification of the different structural elements. The largest problem in the assignment of the secondary structure of the IgG to a particular absorption peak arises with the α-helix and unordered structures because the positions of these structural elements differ only slightly [12]. It is well established that the presence of H_2O vapor gives rise to narrow adsorption peaks in the amide I region which are very weak in the original spectrum but become amplified upon derivation [12]. Then, H_2O vapor should be properly eliminated (by purging the spectrometer with dry air) and corrected for (by spectral subtraction) to remove as much as possible these artefacts appearing in the SD spectra. The presence of these peaks becomes critical in the assignment when the real component peak is produced by a structural element which overlaps with another component or is not the main element. Usually, peaks centred at around 1655 cm^{-1} are assigned to α-structures and those around 1646 cm^{-1} to unordered structures [7,24]. A comparison between positions obtained in the SD and in the CF procedure respectively, reveals that the peak obtained with the SD (1652 cm^{-1}) seems to be related with α-helix while that obtained after CF (1647 cm^{-1}) should rather be assigned to unordered structure. Therefore, the absorption peak centred at around 1650 cm^{-1} may reflect either one of the two elements or both. As it is known that IgG contains both structural elements, this peak was interpreted as representing the sum of α-helix and unordered structure.

The percentage of each individual structural element was calculated as the ratio between the area of the corresponding peak and the total amide I band area. This implies that the effective absorptivities of each structural element were assumed to be equal. Figure 4.4 shows the average percentages of the different structural elements as a function of adsorption time. The error bars were calculated from the differences between the percentages obtained in separate experiments at the same adsorption time. In order to check the certainty of the CF procedure different

sequences to arrive to the final result were followed. The conclusion was that β-sheet content in adsorbed IgG is between 55 and 63% (even when an extra peak was added to discriminate between α-helix and unordered structures), that of β-turns between 10 and 20% and of α-helix + unordered between 14 and 25%. The ratio of the areas of the β-peaks (1675 cm^{-1}, β_1, 1636 cm^{-1}, β_2, 1625 cm^{-1}, β_3,) is constant over the time period studied: $\beta_1/\beta_2=0.28\pm0.04$; $\beta_1/\beta_3=0.6\pm0.1$; $\beta_2/\beta_3=2.0\pm0.1$, which further indicates the reliability of the used curve-fitting procedure.

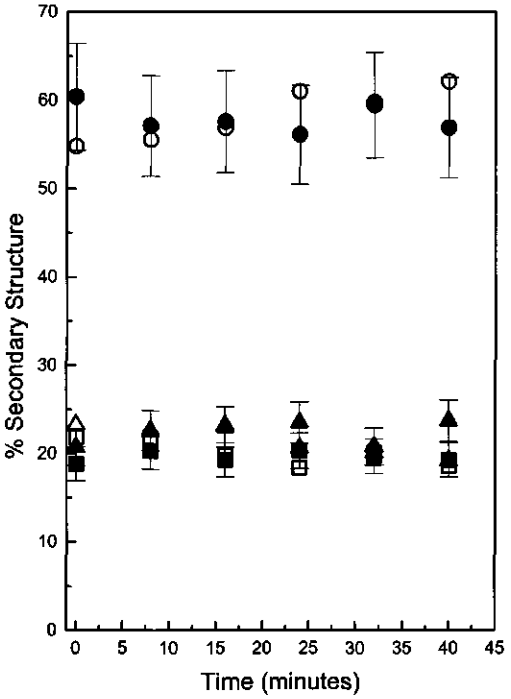

Figure 4.4: Total percentages of the different structural elements as a function of adsorption time for IgG adsorbed on (solid symbols) hydrophilic and on (open symbols) hydrophobic + copolymers silica surfaces: circles, β-sheet; triangles, unordered + α-helix; squares, β-turns. Time scale as in figure 4.1.

Figure 4.4 also shows that there is no variation of the secondary structural composition of the IgG adsorbed on hydrophilic surface with adsorption time (t>13 minutes). A comparison of the secondary structure of the adsorbed IgG on hydrophilic silica surface with data reported in the literature [7,15] for the IgG in solution shows that their structures are rather similar. Comparing the amide I band positions and the intensity and area ratios of the amide I and amide II bands

already points to that conclusion. However, SD and CF analyses are required to estimate the secondary structural composition of the adsorbed protein in a quantitative way.

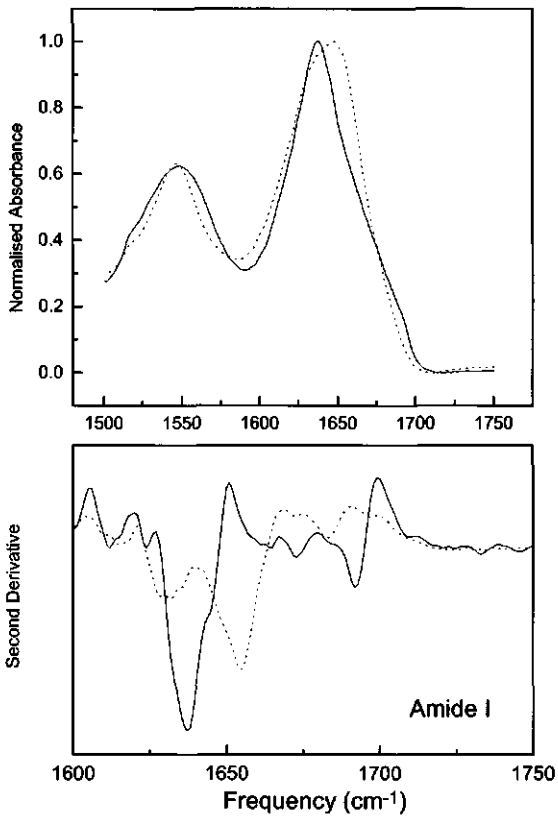

Figure 4.5: Normalised spectra of (solid curves) IgG and 9 (dashed curves) BSA adsorbed on hydrophilic silica surface and their second derivative (SD) spectra in the amide I region.

Before presenting the results obtained for IgG adsorbed on a modified silica surface, some results obtained for BSA adsorbed on hydrophilic silica under the same experimental conditions will be discussed. Figure 4.5 compares the normalised spectra (and their SD in the amide I region) of IgG with those of BSA, both at the same adsorption time (24 minutes). The figure shows a shift in the position of the amide I maximum when the protein is changed while the amide II position is almost the same. This is not surprising because the secondary structures of BSA and IgG are rather different. It is reported that, in contrast to IgG, BSA in

solution contains only a small amount of β-sheet whereas the α-helix content is about 55% [25,26]. Moreover, the SD spectra of BSA adsorbed on hydrophilic silica surface show the strongest peak centred at around 1654±1 cm^{-1}, related to the amide I vibration due to the α-helix structure [24]. This peak is not significantly present in the SD spectra of IgG adsorbed on hydrophilic silica surface, reinforcing the idea that the helix content in adsorbed IgG is small, if any.
Table 4.1 compares some characteristics of the amide I and amide II bands obtained with IgG and BSA (**Si**; IgG and **Si**; BSA, respectively). There are some differences in the amide I/amide II intensity ratios between the two proteins, but they are not large enough to account for the different secondary structures of the two proteins. Hence, the intensity ratio is not very sensitive to variation in the secondary structure. However, the area ratio seems to be more sensitive to differences in the secondary structure of the protein: the area ratio increases as the α-helix content of the protein becomes greater.
Regarding structural changes upon adsorption, BSA shows a different behaviour than IgG. Where for IgG both amide I/amide II intensity and area ratios are nearly invariant with time, for BSA these ratios decrease with increasing adsorption time. A transmittance IR study of BSA in solution shows that this ratio decreases as the α-helix content diminishes, it varies from 1.82 for the native BSA (55 % α-helix) to 1.07 for the thermally denatured protein (5 % α-helix) [20]. Hence, the observed decrease in the area ratio can be related to some loss of α-helix in BSA after adsorption. This result is in agreement with other studies that show a reduction of α-helix in BSA upon adsorption on hydrophilic surfaces [27]. There is no significant shift in the position of the amide I maximum as the amide I/amide II area ratio changes, suggesting that even if there is some loss in the helix content, this structure is still the most important one in the adsorbed BSA.

Hydrophobic Silica

Unfortunately it was not possible to obtain reliable data for IgG adsorbed on hydrophobic silica. In this case the sample spectrum is lower in absorbance than the background one. At the hydrophobic surface proteins displace low-entropy water from the hydration layer which provides a major contribution to the driving force for the adsorption of protein on such surfaces [28]. It seems that the IR spectra taken during IgG adsorption is dominated by this hydrophobic dehydration. The problem arises because there is not a 'background' spectrum for this water release and the contribution of the water is much larger than that of the protein because several water molecules are exchanged against one protein molecule. It is to be noted that almost the same result is observed when triblock

copolymers are adsorbed on the hydrophobic silica surface. Therefore, this effect is related to dehydration of the hydrophobic sorbent surface rather than to the adsorbed protein or copolymer molecules.

Hydrophobic Silica + Copolymer

The experimental procedure was slightly different when triblock copolymers were firstly adsorbed on the surface. First, a spectrum was measured in the presence of buffer solution, then the flow cell was filled with a triblock copolymer solution (0.3 mg l^{-1}) and a copolymer spectrum was taken. After that, the cell was rinsed with buffer solution and the background spectrum was taken. Finally, the cell was filled with protein and the sample spectra were taken following the same experimental procedure as described above.

The silica surface was hydrophobised before copolymer adsorption in order to have the copolymer adsorbed through its PPO parts (approximately 12 nm) whereas its PEO parts (approximately 6 nm) protrude into the solution. The result of this adsorption is a brush conformation of the adsorbed copolymers [29]. It is well known that such a coating, under well-chosen conditions regarding the length and the adsorption density of the PEO chains, diminishes or even prevents protein adsorption [29]. However, the adsorption density and length of the chains chosen in this study are lower than those that prevent protein adsorption. The adsorption of the P75 copolymer on hydrophobic surfaces, as determined by streaming potential measurements, leads to a layer thickness ranging between 1.5 and 2.5 nm, which is smaller than the radius of gyration expected from the dimensions of the PEO blocks (3.6 nm). The maximum adsorbed amount of the PEO-PPO-PEO copolymers on hydrophobic silica was measured to be 1 mg m^{-2} [29]. In the present study the adsorbed amount is lower than the maximum in order not to fully block the surface for subsequent IgG adsorption but rather to direct the arriving IgG molecules to attach in a preferred orientation on the surface. Therefore, the modified silica surface contains an incomplete monolayer of PEO-PPO-PEO block copolymers in a brush conformation. The hydrophobicity of the surface is expected to be intermediate between that of the hydrophilic unmodified silica surface (0°-5°) and that of the methylated silica surface (90°). However, the water wettability of surfaces modified with PEO show substantial hysteresis between the advancing and receding contact angles [30]. Consequently, contact angle measurements on such surfaces do not provide unambiguous information on its hydrophobicity.

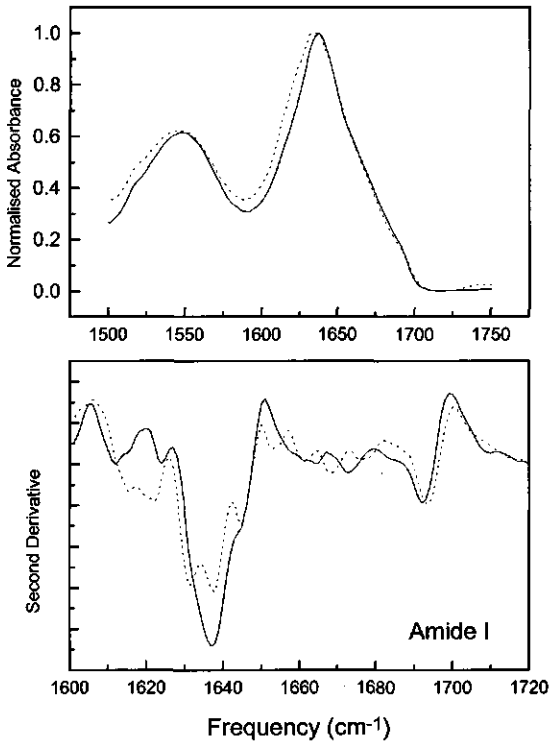

Figure 4.6: Normalised spectra (and their second derivative spectra) of IgG adsorbed on different silica surfaces: (solid curves) hydrophilic; (dashed curves) hydrophobic + copolymer.

Figure 4.6 compares the spectra obtained for IgG adsorbed on hydrophilic and hydrophobic + copolymer silica surfaces (and their SD in the amide I region) and Table 4.1 gives the positions of the maximum of the amide bands and their area and intensity ratios (**Si**; IgG and **Si+C**; IgG). The main difference between these spectra is that the amide I band is a little broader when IgG is adsorbed on the hydrophobic + copolymer surface. It is known that the widths of the IR amide bands depend strongly on the interaction of the polypeptide with its environment [31,32]. On the other hand, the position of the amide I band is almost independent of the copolymer presence suggesting that the secondary structures of the adsorbed protein on hydrophilic and on hydrophobic + copolymer surfaces are rather similar. This conclusion is supported by the intensity and area ratios which are the same (within experimental error) for both hydrophilic surface and hydrophobic surface covered with triblock copolymers.

The SD spectra of IgG adsorbed on hydrophobic + copolymer silica surface were analysed in the same way as those obtained with the hydrophilic surface. There are no major differences between the SD spectra obtained for adsorbed IgG on both surfaces (Figure 4.6 and Table 4.2). The only remarkable difference is the appearance of an extra peak, partly overlapping with the dominant peak, centred at 1631 cm^{-1}, when IgG is adsorbed on the hydrophobic + copolymer surface. Wasacz et al. [21] reported a shift of the strongest amide I peak from 1638 cm^{-1} to 1629 cm^{-1} leaving a weak shoulder near 1640 cm^{-1} when γ-globulins were transferred from aqueous solution to ethylene glycol. This shift was interpreted as a change in the β-sheet structure, i.e. the β-sheet type of γ-globulins exposed to ethylene glycol is different from that observed in aqueous solution. The authors assigned the shoulder to the original β-sheet structure (in aqueous solution) and the strongest peak to a different type of β-sheet structure (in ethylene glycol). The low frequency shift indicates The additional peak found in the presence of the pre-adsorbed copolymers (of which the PEO parts resemble the structure of ethylene glycol) could as well reflect a change in the type of β-sheet structure. Indeed, the change in the environment of the protein (from hydrophilic to hydrophobic + copolymer surface) is to some extent comparable with the change from water to ethylene glycol. This change is less pronounced in the system studied here because of the dimensions of the IgG molecule and the PEO chains, relative to each other. The IgG has a Y-shaped conformation with dimensions of 7.0x6.3x3.1 nm^3 for the Fc fragment and 8.25x5.0x3.8 nm^3 for each of the two F(ab) fragments [10,15], whereas the PEO parts of the copolymer protrude into the solution over shorter distances [29]. Hence, IgG can penetrate the PEO-brush only partly, leaving a part of its molecule in the aqueous environment. This could be the reason that the β-sheet peak appearing around 1631 cm^{-1} does not replace the original one at 1637 cm^{-1}. The low frequency shift indicates stronger hydrogen bonds [33] which could result from either intermolecular or intramolecular bonding. Wasacz et al. [21] suggested intermolecular β-sheet formation (related to some unfolding of the protein) when IgG is transferred from aqueous solution to ethylene glycol. However, as stated above, the presence of the copolymers on the adsorbed IgG has a smaller effect on the β-sheet structure than ethylene glycol. In the copolymer case the shift may be related to the difference in the strength of the hydrogen bonds within the β-sheet structure upon changing the protein to a less polar environment.

Ethylene glycol further causes an increase in the amount of helix in IgG; such a variation in helical content is not induced by the presence of copolymers because

the amide I maximum does not shift and the SD spectrum does not indicate an additional peak. Altogether, it seems that the pre-adsorbed copolymers have a similar, but smaller, effect on the β-sheet of IgG than that caused by ethylene glycol.

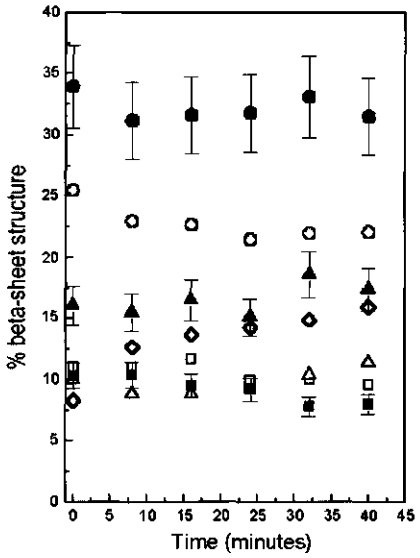

Figure 4.7: Percentages of the different structural elements in β-sheet as a function of adsorption time of IgG adsorbed on (solid symbols) hydrophilic and on (open symbols) hydrophobic + copolymer silica surfaces: circles, low frequency component; triangles, low frequency component; squares, high frequency component; , diamonds 'additional' component. Time scale as in figure 4.1.

The CF procedure was performed as with the hydrophilic surface except that, in view of the appearance of the peak at 1631 cm^{-1}, eight peaks (instead of seven) were used. Table 4.2 and Figure 4.4 compare the peak positions and the percentages of the different structural elements as a function of adsorption time for IgG on hydrophilic and on hydrophobic + copolymer surfaces, respectively. There are no significant differences in the total content of each element. Figure 4.7 compares the percentage of each β-sheet component (1672, 1637, 1625 and 1631 cm^{-1}) with and without copolymers on the surface. There is a decrease in the percentage of the two low frequency components when the copolymer is present on the surface while the high frequency one is unaffected. As it is the case with ethylene glycol, the new form of β-sheet structure is formed from the original β-sheet segments [21]. The ratio of the areas of the β-peaks are also constant for β_1,

β_2 and β_3 but their values are different from the ones calculated for the hydrophilic silica surface: $\beta_1/\beta_2=0.46\pm0.05$; $\beta_1/\beta_3=1.0\pm0.2$; $\beta_2/\beta_3=2.2\pm0.3$. On the other hand, since the percentage of the new peak increases with time (Figure 4.7), the ratios for this peak are not constant in time.

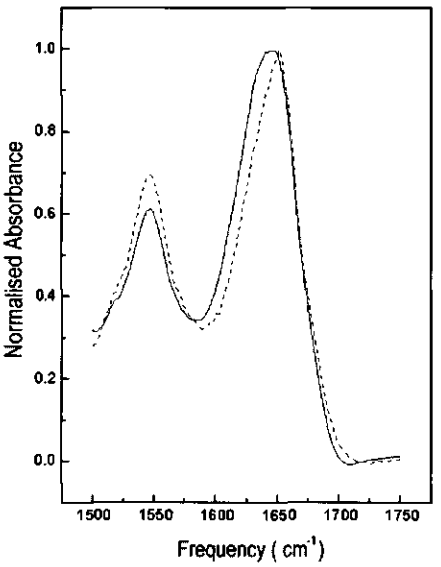

Figure 4.8: Normalised spectra of BSA adsorbed on different silica surfaces: (solid curves) hydrophilic; (dashed curves) hydrophobic + copolymer.

Figure 4.8 compares the ATR/FTIR spectra of BSA adsorbed on hydrophilic and hydrophobic + copolymer silica surfaces and Table 4.1 gives their maximum band positions and their intensity and area ratios (**Si**; BSA and **Si+C**; BSA). The presence of copolymer on the surface does not affect the position of the amide I band but it makes the band narrower. Chirgadze et al.[32] demonstrated that there is a correlation between the amide I bandwidth and the stability of the α-helix of polypeptides: the amide I bandwidth is directly connected to the degree of geometric distortion of the helical structure. As reported in the literature [17,21] changes in the solvent (from aqueous to non-aqueous) caused narrowing and shifting to higher frequencies of the major peak of the amide I band of BSA. These results were interpreted as an increase in the order and in the content of the helix structure of the protein. There is no evidence (neither from the amide I maximum nor from the intensity and area ratios) that the copolymer induces helix formation. Consequently, the pre-adsorbed copolymers cause the same effect as

ethylene glycol on both proteins except that they do not increase the α-helix content. The change is more pronounced in BSA than in IgG which can be understood because a BSA molecule is much smaller than an IgG molecule. The BSA molecule in its native state is heart-shaped of 8 nm of side and 3 nm of depth [26]. Then, assuming this size for the adsorbed molecule, it can be completely surrounded by a copolymer environment while, as mentioned before, IgG can only partially penetrate the pre-adsorbed copolymer layer.

Whereas the amide I/amide II area ratio for BSA at the bare silica surface decreases with increasing time (from 1.86 to 1.61), it has a rather constant value of 1.6±0.1 at the hydrophobic + copolymer silica surface. It suggests that at the copolymer-silica surface the (small) conformational change in BSA occurs quickly, i.e. before the first spectrum is taken, whereas it takes more time at the bare hydrophilic silica surface. It is worth to note that the structural change in BSA at the hydrophobic + copolymer silica surface does not seem to be larger than at the hydrophilic silica surface.

In summary, the pre-adsorbed triblock copolymers do not to a large extent influence the adsorption characteristics of IgG and BSA. This result is remarkable because varying the sorbent surface hydrophobicity usually provokes different extent of changes in the protein structure. The result obtained in this study suggests that the presence of copolymers on the surface, even though it increases the surface hydrophobicity, does not allow the protein to rearrange its structure extensively. Indeed, the PEO chains cause an additional lateral steric interaction that could prevent the protein from spreading over the surface. It is known that, as a rule, the extent of structural alterations in adsorbed protein molecules decreases with increasing surface coverage [27,34], in a similar way the precoated copolymers may restrict structural changes in the subsequent adsorbing protein molecules.

4.3.2 Adsorbed amount

The amide II band may be used to determine the adsorbed amount. The maximum of this band is commonly found around 1550 cm^{-1}, independently of the type of protein. The position of the amide II band for IgG and BSA adsorbed on both surfaces does not change neither with the type of protein nor with the type of surface (Table 4.1). Figure 4.9 compares the relative adsorbed amounts (amide II areas) at the two surfaces as a function of time.

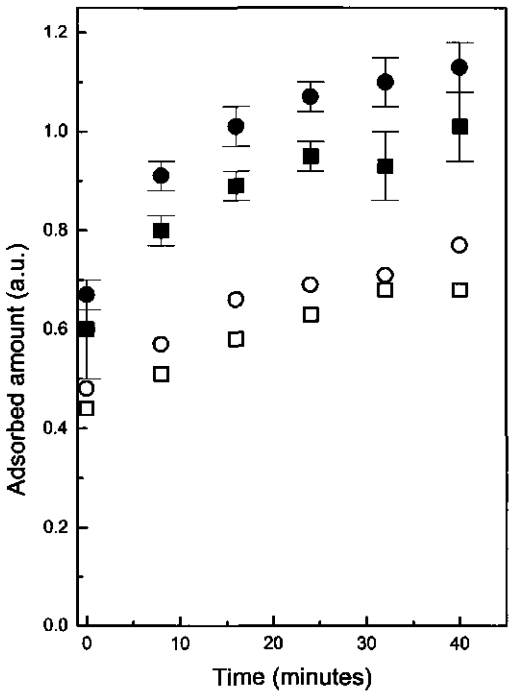

Figure 4.9: Relative adsorbed amounts (amide II areas) of squares, IgG and circles, BSA adsorbed on (solid symbol) hydrophilic and on (open symbol) hydrophobic + copolymer silica surfaces. Time scale as in figure 4.1.

Although the amide II position is almost the same for both proteins, its area is always larger for BSA than for IgG suggesting that BSA adsorbs in larger quantities on both surfaces. However, the comparison between the amide II areas of these two proteins may be erroneous since the proteins may have different extinction coefficients. Absorptivity values reported in the literature indicate that the extinction coefficient not only depends on experimental conditions (protein, pH) but also on the experimental IR set-up [19]. Nevertheless, it is evident that the presence of the copolymers on the surface reduces the adsorbed amount. It supports the hypothesis that a steric mechanism hinders protein adsorption: the proteins have to be accommodated between the PEO-chains causing a lower adsorbed amount and, as discussed above, preventing extensive conformational changes in the protein molecules.

4.4 Conclusions

The adsorption on hydrophilic silica surface has different consequences for IgG and BSA:

I. The secondary structure of adsorbed IgG is rather similar to that of the protein in solution: a major β-sheet content and a low, if any, α-helix content.

II. There is some loss in the α-helix content of adsorbed BSA, although this structural element is still the most important one.

The pre-adsorbed triblock copolymers (PEO-PPO-PEO) on the hydrophobic silica surface cause two significant effects on both IgG and BSA adsorption:

I. A change in the IR spectra, similar to that observed when the proteins are exposed to ethylene glycol: a different type of β-sheet in IgG and a more ordered α-helix in BSA are induced. The effect is more clear for BSA than for IgG probably due to the fact that BSA can be completely surrounded by the copolymers while IgG is located partly between the copolymers and partly in the aqueous solution.

II. A decrease of the adsorbed amount, probably due to a steric effect that, moreover, hampers severe structural rearrangements in the adsorbed protein molecules.

4.5 References

1 Lee, J., Martic, P. A., and Tan, J. S., *J. Colloid Interface Sci.* 131, 252 (1989).

2 Jeon, J. S., Sperline, R. P., and Raghavan, S., *Appl. Spectrosc.* 46, 1644 (1992).

3 Peula, J. M., Hidalgo-Alvarez, R., and De Las Nieves, F. J., *J. Biomater. Sci. Polymer Edn.* 7, 241 (1995).

4 Peula, J. M., Hidalgo-Alvarez, R., and De Las Nieves, F. J., *J. Biomater. Sci. Polymer Edn.* 7, 231 (1995)..

5 Jeon, S. I., Lee, J. H., Andrade, J. D., and De Gennes, P. G., *J. Colloid Interface Sci.* 142, 149 (1991).

6 Jeon, S. I., and Andrade, J. D., *J. Colloid Interface Sci.* 159, 149 (1991).

7 Byler, D. M., and Susi, H., *Biopolymers* 25, 469 (1986).

8 Bramanti, E., and Benedetti, E., *Biopolymers* 38, 639 (1996).

9 van Stokkum, I. H. M., Linsdell, H., Hadden, J. M., Haris, P. I., Chapman, D., and Bloemendal, B., *Biochemistry* 34, 10508 (1995).

10 Buijs, J., Norde, W., and Lichtenbelt, J. W. Th., *Langmuir* 12, 1605 (1996).

11 Ball, A., and Jones, R. A. L., *Langmuir* 11, 3542 (1995).

12 Surewicz, W. K., Mantsch, H. H., and Chapman, D., *Biochemistry* 32, 389 (1993).

13 Susi, H., and Byler, D. M., *Biochem. Biophys. Res. Commun.* 115, 391 (1983).

14 Singh, B. R., Fuller, M. P, and Schiavo, G., *Biophys. Chem.* 46, 155 (1990).

15 Vermeer, A. W. P., Bremer, M. G. E. G., and Norde, W., *Biochim. Biophys. Acta* 1425, 1 (1998).

16 Jakobsen, R. J., and Strand, S. W., *in* "Internal Reflection Spectroscopy. Theory and Applications", Practical Spectroscopy Series Vol. 15 (F. M. Mirabella, Jr, Ed.), p. 107. Marcel Dekker, Inc., New York, 1993.

17 Jakobsen, R. J., and Wasacz, F. M., *in* "Proteins at Interfaces. Physicochemical and Biochemical Studies" ACS Symposium Series 343 (J. L. Brash and T. A. Horbett, Eds.), p. 339. American Chemical Society, Washington DC, 1987.

18 Levitt, M., and Greer, J., *J. Mol. Biol.* 114, 181 (1977).

19 Ishida, K. P., and Griffiths, P. R., *Appl. Spectroscopy* 47, 584 (1993).

20 Kato, K., Matsui, T., and Tanaka, S., *Appl. Spectroscopy* 41, 861 (1987).

21 Wasacz, F. M., Olinger, J. M., and Jakobsen, R. J., *Biochemistry* 26, 1464 (1987).

22 Fu, F., DeOliveira, D. B., Trumble, W. R., Sarkar, H. K., and Singh, B. R., *Appl. Spectroscopy* 48, 1432 (1994).

23 Singh, B. R., and Fuller, M. P., *Appl. Spectroscopy* 45, 1017 (1991).

24 Jakobsen, R. J., and Wasacz, F.M., *Appl. Spectroscopy* 44, 1478 (1990).

25 Peters, T. Jr., *Adv. Protein Chem.* 37, 161 (1985).

26 Carter, D. C., and Ho, J. X., *Adv. Protein Chem.* 45, 153 (1993).

27 Norde, W., and Favier, J. P., *Colloids and Surfaces* 64, 87 (1992).
28 Haynes, C. A., and Norde, W., *J. Colloid Interface Sci.* 169, 313 (1995).
29 Schroën, C. G. P., Cohen Stuart, M. A., van der Voort Maarschalk, K., van der Padt, A., and van't Riet, K., *Langmuir* 11, 3068 (1995).
30 Freij-Larsson, C., Nylander, T., Jannasch, P., and Wesslen, B., *Biomaterials* 17, 2199 (1996).
31 Jakobsen, R. J., Wasacz, F. M., Brasch, J. W., and Smith, K. B., *Biopolymers* 25, 639 (1986).
32 Chirgadze, Yu. N., Brazhnikov, E. V., and Nevskaya, N.A., *J. Mol. Biol.* 102, 781 (1976).
33 Jackson, M., and Mantsch, H. H., *Can. J. Chem.* 69, 1639 (1991).
34 Kondo, A., Murakami, F., and Higashitani, K., *Biotechnol. Bioeng.* 40, 889 (1992).

Chapter 5

STRUCTURAL CHANGES OF IGG INDUCED BY HEAT TREATMENT AND BY ADSORPTION ONTO A HYDROPHOBIC SURFACE STUDIED BY CIRCULAR DICHROISM SPECTROSCOPY

Abstract

Thermal denaturation of immunoglobulin G, as well as structural rearrangements resulting from adsorption on a hydrophobic surface, are studied by circular dichroism spectroscopy. It is observed that both heat induced and adsorption induced denaturation do not lead to complete unfolding into an extended polypeptide chain but leave a significant part of the IgG molecule in a globular or corpuscular form. Heating dissolved IgG causes a decrease of the fractions of β-sheet and β-turn conformations, whereas those of random coil and, to a lesser extent, α-helix increase. Adsorption enhances the formation of α-helixes and random coils, but the β-sheet content is strongly reduced. Heating adsorbed IgG results in a gradual break-down of the α-helix and β-turn contents, and a concomitant formation of β-sheet structures. Thus, the structural changes in IgG caused by heating and by adsorption, respectively, are very different. However, after heating the structure of adsorbed IgG approaches the structure of thermally denatured IgG in solution.

5.1 Introduction

Since recently, immunoglobulin G (IgG) molecules, i.e., antibodies, are widely used as convenient and valuable tools for various immunochemical and biochemical analyses. Progress in monoclonal antibody technology has led to the production of substantial amounts of highly specific monoclonal antibodies. The availability of these monoclonal antibodies has resulted in the development of various diagnostic tests. An example of a typical diagnostic application taking full advantage of the specificity of monoclonal antibodies, their sensitivity and their ability to identify different epitopes, are immunoassays against Human Chorionic Gonadotropin, (hCG) [1], a glycoprotein hormone that is commonly used as a marker for the early detection and monitoring of pregnancy [2].

The main principle of such diagnostic tests is based on the agglutination or a colour reaction of the immunoassay, induced by the presence of antigen. To assure that these processes can be followed by eye, the monoclonal IgG molecules are adsorbed onto a particle or flat surface. It is evident that the orientation and structural integrity of the adsorbed antibody and the related activity of the antigen binding site are essential factors for the preparation of a reliable immunoassay.

The immobilisation of monoclonal antibodies against hCG (ahCG) onto polystyrene latex and on silica particles has been studied extensively [3,4]. An important mechanism for immobilisation of immunoglobulins on a solid surface is physical adsorption. It is generally accepted that protein adsorption is the net result of various interactions between and within the components of the system, including the sorbent surface, the protein molecules, the solvent (water) and other solutes (ions of low molecular weight). These interactions originate from electrostatic forces, Lifshits- van der Waals forces, hydrogen bonding and more entropically based effects such as hydrophobic interactions and internal conformational restrictions in the protein molecules. At apolar surfaces hydrophobic interactions dominate the overall protein-sorbent interaction, so that essentially all proteins adsorb on hydrophobic surfaces even under adverse electrostatic conditions. At polar surfaces proteins do adsorb when they are electrostatically attracted. However, in case adsorption-induced structural changes lead to a sufficient increase in the conformational entropy of the protein, adsorption may also occur on a like-charged, polar surface [5-7]. Hence, surface characteristics such as hydrophobicity [8,9], surface charge [10] and co-adsorption of, and exchange with surfactants, copolymers [11,12] or proteins [13,14] are key controlling factors for the adsorption of proteins and for the stability and specificity of adsorbed IgG in immunoassays in particular. From these studies it can be concluded that, as a trend, protein adsorption increases with increasing

hydrophobicity of the substrate, with a lower degree of competition with other surface active components, and with increasing steric hindrance. Further, it has been shown that for most proteins the rate of adsorption is enhanced by the hydrophobicity of the sorbent surface.

As mentioned above, in addition to the adsorbed amount, the orientation and conformation of the adsorbed immunoglobulin molecules are very important in relation to the performance of the assay. An IgG molecule is composed of four polypeptide chains that are connected by disulphide bonds and non-covalent forces. The four chains can be divided into two groups: two heavy and two light chains. All four polypeptide chains are grouped in different domains, two F(ab) segments and one Fc segment, together forming a Y-shaped conformation. The antigen binding sites are located on the far ends of the F(ab) segments. From this description it is clear that the antigen binding sites on the F(ab) segments must be accessible, for the antigen for the adsorbed IgG to be immunologically active. Buijs et al. [15] have mentioned that the orientation of IgG is the main factor that influences the adsorbed amount and the antigen binding capacity of the adsorbed molecules. However, in addition to the orientation effects, conformation of the adsorbed molecules may as well be an important factor that affects the performance of the assay.

The secondary structure of native immunoglobulins has been extensively studied, mostly by X-ray spectroscopy. The different domains of the IgG molecule form compact globular structures with a characteristic fold [16-18]. The predominant secondary structure elements in immunoglobulins are the anti-parallel β-sheet and random coil conformations. Further, short stretches of α-helices (that are found in some bends) and β-turns are present. These structure elements are partially stabilised by intramolecular hydrogen bonding. The amounts of the structural components in IgG fragments as determined by X-ray spectroscopy [19,20] are 45 % and 47 % of β-sheets and 7 % and 2 % of α-helices for the Fc and F(ab) segments, respectively. It should be realised that the X-ray data are taken from the IgG in its solid state and that no interactions with solvent are involved. It is, of course, desirable to monitor the protein structure in an environment that is of interest for the above described immunological applications. However, experimental studies on determining the structure of immunoglobulins in an aqueous environment are still in a relatively primitive stage.

It is well known that spectral vibrations and electronic transitions of the amide

linkages of the polypeptide chain reflect the presence of the different secondary structure elements. Thus, studying these vibrations by spectroscopic methods gives information about the structure surrounding these amide groups. FT-IR attenuated total reflection (ATR) spectroscopy [21-23] is one of the methods that may be used to study the conformation of proteins adsorbed from aqueous solution. Buijs et al. [23] reported FT-IR/ATR spectra of IgG adsorbed on both hydrophilic and hydrophobic surfaces. They mentioned that the structural changes induced by adsorption onto a hydrophobic surface are significant, whereas for hydrophilic surfaces such changes are much smaller. However, especially at hydrophobic surfaces determination of the protein structure becomes practically impossible [52]. The reason is that water, released from the hydrophobic surface, largely contributes to the IR-spectrum. The appearance of a water peak upon protein adsorption is far less with a hydrophilic surface, indicating that hydration water largely remains in the protein-sorbent contact region. A further problem is that the spectral bands for the random coil structure, the β-turns and to a lesser extent the α-helices are difficult to assign because of their low intensity and overlap with other bands [24]. Thus, infrared spectroscopy is, in the first place, suitable to detect β-sheets and can only be successfully be applied to proteins adsorbed on a hydrophilic surface.

Circular Dichroism (CD) [25] is another spectroscopic technique that has been used to study the secondary structure of proteins, including IgG [26-28]. A major advantage of this technique is that the spectroscopic signal is not affected by the presence of water and that well-defined procedures are available to elucidate the secondary structure based on reference spectra of the different structure elements. It is furthermore possible to study the protein both in solution and in the adsorbed state. To accomplish the latter, particles have to be used that neither absorb nor scatter in the far-UV region. In a previous study Maste et al. [29] have shown that hydrophobic perfluoro-alkoxy fluoro carbon Teflon particles can be used for this purpose. Finally, as compared to FT-IR, CD may be a suitable technique to study the protein structure at different temperatures since no correction spectrum has to be taken at each temperature. This enables us to compare the heat-induced denaturation of IgG in solution with the structural changes induced by adsorption onto a hydrophobic surface [30-32].

Structural rearrangements in proteins are not only induced by their adsorption on (hydrophobic) surfaces. The most common cause for structural rearrangements in proteins is heat treatment. McCarthy and Drake [33] suggested that heating IgG

induces partial unfolding of the F(ab) segment which, consequently, aggregate, leaving the Fc parts exposed to the aqueous environment.

The aim of this paper is to obtain information on the structural changes of IgG molecules induced by heat treatment and to compare these changes with those induced by adsorption on a hydrophobic surface. Furthermore, the effects of heating on the conformation of pre-adsorbed IgG is studied. In order to support the temperature dependence observed in the CD measurements, the thermal stability of IgG as determined by differential scanning calorimetry (DSC) is presented prior to the CD results.

5.2 Materials and methods

All chemicals used were of analytical grade and were used without further purification. The experiments were carried out in a 5 mM phosphate buffer, pH 6.0. The water used was purified by percolation through a mixed bed ion exchange column followed by an activated carbon column and a micro filter.

5.2.1 Immunoglobulin

The immunoglobulin studied is a monoclonal mouse immunoglobulin G (IgG) from isotype 1, specific for the binding of the human pregnancy hormone human Chorionic Gonadotropin (hCG). The antibody was kindly donated by Organon Teknika (Akzo Nobel, Boxtel, The Netherlands). Concentrated IgG solutions were stored at -20 °C and thawed shortly before use. The IgG samples were filtered through an 0.2 μm Acrodisc filter prior to the CD experiments to remove aggregates. The concentration of the IgG solutions was determined by UV-spectroscopy at 280 nm assuming an extinction coefficient of 1.45 cm^2 mg^{-1}. A sample of 3.3 mg ml^{-1} was, without further purification, used for Differential Scanning Calorimetry. The iso-electric point (i.e.p.) of the molecules was measured on a PhastSystem (Pharmacia LKB, Uppsala, Sweden), using a Phastgel, with a pH range of 3.5 to 8.65. The i.e.p. of the IgG molecules was about 6.

5.2.2 Teflon particles

In order to study the secondary structure of adsorbed IgG molecules by Circular Dichroism, colloidal particles, representing a sufficiently large surface area to volume ratio, are required with a refractive index nearly matching that of water [29]. The sorbent used in this study was a Teflon latex suspension. The latex is prepared by emulsion polymerisation of tetrafluorethylene and perfluorovinylether. The latex suspension was kindly donated by Du Pont de Nemours (Du Pont de

Nemours SA, Le Grand-Saconnex, Switzerland). The dispersion is charge-stabilised with sulphate groups originating from the polymerisation initiator potassium persulphate. The refractive index of these particles is 1.35, which is about equal to that of water, i.e. 1.33. The diameter of the Teflon particles is 215 nm yielding a specific surface area, as determined by N_2 adsorption (BET), of 12.5 $m^2 g^{-1}$.

5.2.3 Adsorption of IgG onto Teflon

The adsorption of IgG on the Teflon particles was accomplished by adding 262 µl of 0.20 mg ml^{-1} IgG in a 5 mM phosphate buffer, pH 6.0, to 238 µl 1 % Teflon suspension. Prior to further experiments the sample was incubated at room temperature for at least 30 minutes. Additional experiments have shown that after this period all IgG was adsorbed. These conditions result in an adsorbed amount of 1.68 mg m^{-2}.

5.2.4 Differential Scanning Calorimetry

DSC experiments were performed using a Setaram Micro-DSC III (Setaram, Caluire, France). The samples were placed in the calorimeter in a 1 ml sample cell against a 1 ml reference cell that was filled with the appropriate blank solution. The cells were stabilised for 1 hour at 20 °C inside the calorimeter before heating up, at a given heating rate, to the final temperature. Subsequent cycles of cooling and reheating of the samples were performed as indicated. The peak temperature and enthalpy were determined using the Setaram software (Setaram, Caluire, France, Version 1.3).

5.2.5 Circular Dichroism measurements

The far-UV CD spectra were measured with a JASCO spectropolarimeter, model J-715 (JASCO International Co., Tokyo, Japan). A quartz cuvette of 0.1 cm path length was used. Temperature regulation was carried out using a JASCO PTC-348WI (JASCO International Co., Tokyo, Japan) thermocouple. Comparison of the actual temperature in the cell with the temperature set by the Peltier element showed that the deviation of the actual temperature was less than 0.1 °C. In the 190-260 nm wavelength region (0.2 nm resolution) 64 scans were accumulated with a scan rate of 100 nm min^{-1} and a time constant of 0.125 sec. The final spectra are the average of these scans. Additionally, temperature scans at 206.5 nm were recorded. For these experiments the temperature is increased using the Peltier thermocouple at a given scan rate (°C min^{-1}) with a resolution of 0.2 °C and a time constant of 16 sec. Prior to the spectral analysis both wavelength and temperature

scans were corrected for the buffer background signal.

5.2.6 Circular Dichroism Spectral Analysis

The CD curves of poly-L-lysine containing varying amounts of α-helix, β-sheet, β-turn and randomly coiled conformations have been applied for determining the IgG conformation. The CD spectra of IgG are a superposition of the spectra of these four structure elements. The poly-L-lysine reference spectra used were described by Greenfield and Fasman [34] and Chang et al. [35]. Fitting of the measured spectra was performed by a non-linear regression procedure, making use of the Gauss-Newton algorithm [36]. The reference spectra (data from Chang et al. [35]) were fitted independently from 190 to 240 nm with 1 nm resolution. Fitting of an experimental IgG CD spectrum was performed with a 1 nm resolution. No constraints were used in the fit procedure. The quality of the fit was expressed using the definition of the normalised root-mean-square (RMS) error as described by Brahms and Brahms [37]. A fit was considered to be reliable when the RMS error is smaller than 10.

5.3 Results and Discussion

5.3.1 Thermal unfolding of IgG

The thermal stability of IgG was studied by differential scanning calorimetry. The DSC thermograms of IgG in a 5 mM phosphate buffer at a heating rate of 0.5 °C min^{-1} and 0.1 °C min^{-1} are shown in Fig. 5.1 and Fig. 5.2, respectively. In spite of the multi-domain character of IgG, only a single endothermic transition is observed. It is known from literature that thermal denaturation of IgG occurs in different domains [38-41]. Because of the appearance of only one single peak, it is impossible to distinguish, without further deconvolution analysis, such domains from the thermogram. Fig. 5.1 shows that, at a heating rate of 0.5 °C min^{-1}, an exothermic aggregation peak is present, whereas this peak disappears when the heating rate is lowered (Fig 5.2). After cooling down, the thermograms of the subsequent cycle do neither show a denaturation nor aggregation peak, indicating that the structural changes in the IgG are irreversible. It is generally accepted that the irreversibility results from aggregation succeeding a reversible unfolding step and that the enthalpy of aggregation is far less than that of unfolding [42]. The denaturation enthalpy, $\Delta_d H$, is evaluated as the peak area of the transition and the denaturation temperature, T_d, corresponds with the temperature at the half peak area. In the thermogram obtained at 0.5 °C min^{-1} the peak position and enthalpy could not be determined accurately because the aggregation peak disturbed the signal. After treatment in the calorimeter the sample was observed to be strongly

aggregated, containing small compact aggregates. For lower heating rates the aggregation peak diminishes strongly, resulting in a negligible enthalpy effect. At a heating rate of 0.1 °C min^{-1} the denaturation could be well characterised with a T_d of 74 °C and a $\Delta_d H$ of 12.7 J g^{-1}. Both peak position and enthalpy compare well with data reported in literature. After the heat treatment the sample showed large flocs with an open structure.

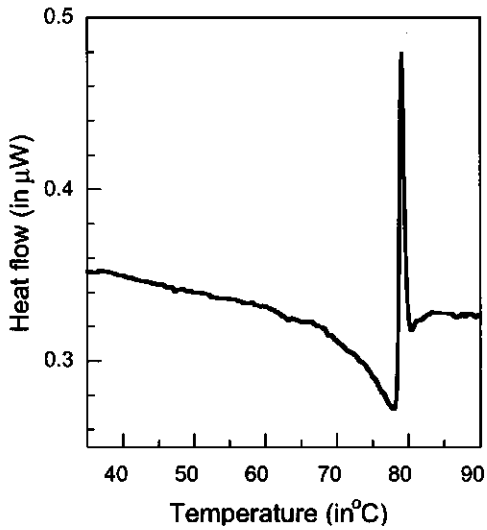

Figure. 5.1: DSC thermogram of IgG (3.3 mg ml^{-1}) in a 5 mM phosphate buffer at pH 6.0. Heating rate was 0.5 °C min^{-1}.

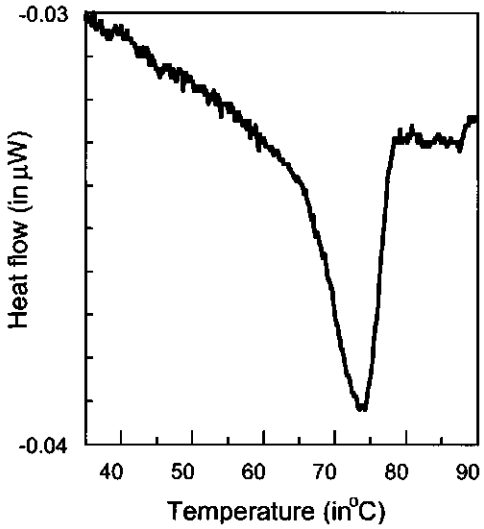

Figure 5.2: DSC thermogram of IgG (3.3 mg ml^{-1}) in a 5 mM phosphate buffer at pH 6.0. Heating rate was 0.1°C min^{-1}. Td = 74 °C and ΔdH = 12.7 J g-1.

Although the thermogram obtained for the lower heating rate does not show an aggregation peak, aggregates were well observed upon heat treatment in DSC. The absence of an aggregation peak when the protein is heated at a rate of 0.1 °C min^{-1} can be explained by assuming that at low heating rates at any time only a low concentration of denatured molecules is available for aggregation. This leads to the incoherent formation of less intensive contacts [43], resulting in the formation of fractal aggregates with an open structure. This sterically unhindered aggregation finally results in the formation of a gel [44], as was indeed observed experimentally. At the higher heating rate (0.5 °C min^{-1}), however, a much higher concentration of denatured molecules is available, resulting in the formation of small, very dense, aggregates.

5.3.2 Heat induced changes in the secondary structure of IgG

Having established the heat induced denaturation of immunoglobulin using a calorimetric method, information is obtained on the enthalpy effects involved in the structural transitions. The (endothermic) enthalpy change is related to the unfolding of the protein molecule. The information obtained by DSC is on a macroscopic level. Spectroscopy would be a complementary approach because it provides information about the protein structure at a sub-molecular level. To

investigate the effect of temperature on the average secondary structure of IgG, Circular Dichroism was used. The Far-UV wavelength spectra of IgG in a 5 mM phosphate buffer (pH 6.0) are, for different temperatures, given in the Fig. 5.3a and b. Fig. 5.3a shows that up to 65 °C the CD spectra are almost invariant with temperature. All spectra have an intensity that equals zero at a wavelength of 206 nm, show a minimum at 217 nm and a broad shoulder around 230 nm. It is further observed that the shoulder gradually increases in intensity with increasing temperature. At temperatures above 65 °C (Fig. 5.3b) the wavelength corresponding to zero-intensity shifts to a lower value, which indicates a loss of ordered structure. The influence of temperature on the structure of IgG as observed in the CD spectra corresponds with the DSC thermograms shown in Fig. 5.1 and Fig. 5.2, where only at temperatures higher than 65 °C an endothermic effect is observed.

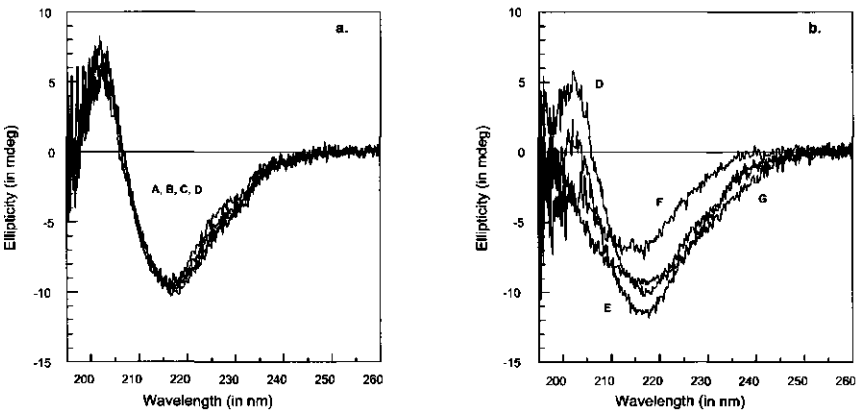

Figure 5.3: Far-UV CD spectra of IgG (0.2 mg ml^{-1}) in a 5 mM phosphate buffer at pH 6.0, for different temperature values. a: pictured from left to right; A = 20 °C, B =50 °C, C = 60 °C, D = 65 °C and b: D = 65 °C E = 70 °C, F = 75 °C, G = 80 °C.

The CD spectrum of IgG is a superposition of the signals of the four structure elements; α-helix, β-sheet, β-turn and randomly coiled conformations. Analysis of the CD spectra reveals that the β-sheet is the most dominant secondary structure element present in native IgG. Although the IgG remains essentially in its native state for temperatures up to 65 °C, some minor changes have been observed in the distribution of the structural components with increasing temperature. A typical result of the spectral analysis is shown in Fig 5.4, where the dashed lines represent

the contributions of the four structure elements to the fitted curve. To assure that the RMS error was lower than 10, the fit was performed in the wavelength range 203-240 nm. It is observed that the fit, for wavelengths higher than 205 nm, matches the experimental data points very well. At lower wavelengths the calculated value deviates significantly from the measured signal. An explanation for the overestimation of the fitted curve may be that at lower wavelengths the light scattering by the protein becomes too large, which was indeed indicated by a very high photomultiplier signal.

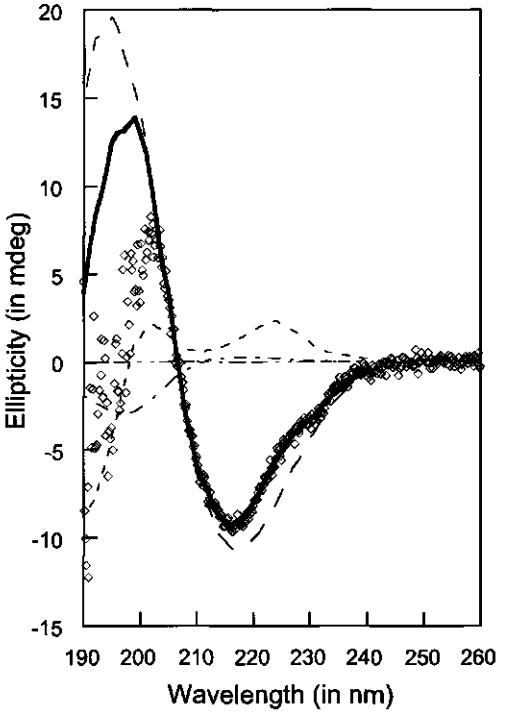

Figure 5.4: A result of the fitting procedure for a Far-UV CD spectra of IgG (0.2 mg ml^{-1}) in a 5 mM phosphate buffer at pH 6.0. Symbols, experimental data point at 20 °C; solid line, fitted curve; The different contributions of the four structure elements are shown by the dashed lines.

Table 5.1 summarises the content of the different structure elements as a function of temperature. For the native IgG about 75 % of the polypeptide chain was found to be in a β-sheet type conformation. The fractions of β-turn and random coil conformations are much lower and the α-helix structure was not observed at all. These results compare well with those reported in literature [23,45]. The gradual

decrease in ellipticity around 230 nm with increasing temperature, is ascribed to a transition of a small fraction of β-turn conformations into random coil structures. This transition may be due to the higher thermal motion of the polypeptide side-chains, which is not yet sufficient to unfold the protein but may cause these minor changes. Above 65 °C the fractions of β-sheet and β-turn decrease whereas those of random coil and, to a minor extent, α-helix conformations increase. Unfortunately, it was not possible to obtain a fit with a RMS error < 10 for the CD spectra at 75 and 80 °C, but these spectra also conform well to the clear general trend for the influence of temperature over the range 20 ° - 80 °C.

Table 5.1: Content (in %) of structure elements in IgG at different temperatures. Medium: 5 mM phosphate buffer, pH 6.0.

Temperature (in °C)	α-helix	β-sheet	β-turn	random coil	RMS error
20	0	76	14	10	6.9
50	0	76	13	11	8.1
60	0	76	9	15	7.8
65	0	76	7	17	7.1
70	0	64	3	33	6.9
75	0	46	11	43	12
80	8	52	0	40	10

In addition to these wavelength scans, a temperature scan at a given wavelength may be used to study the effect of temperature on the secondary structure of IgG. With such temperature scans a spectrum may be obtained under conditions that can be compared with those of the DSC thermograms, previously shown. A more thorough investigation of the CD reference spectra [35] reveals that at 206.5 nm the contribution from the β-sheet to the ellipticity is essentially zero, whereas those of the other structures are significant. Thus, by measuring the ellipticity at 206.5 nm as a function of temperature, one monitors the changes in β-turn, α-helix and random coil content. At this wavelength the ellipticity due to the fraction of β-turns is positive, whereas formation of α-helixes and random coil structures gives a negative contribution to the ellipticity.

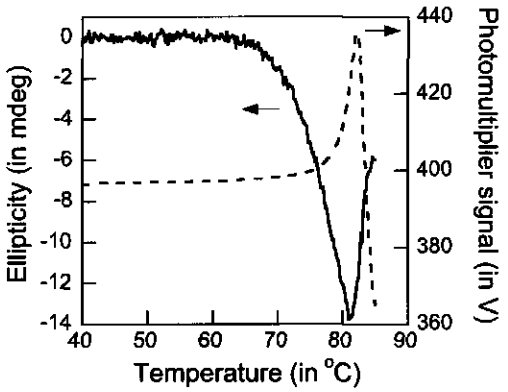

Figure 5.5: CD temperature scan (solid line) and the corresponding CD photomultiplier signal (dashed line) of IgG (0.2 mg ml^{-1}) in a 5 mM phosphate buffer at pH 6.0 and a heating rate of 0.5 °C min^{-1}.

Fig. 5.5 shows the ellipticity at 206.5 nm and the corresponding photomultiplier signal, as a function of temperature for a heating rate of 0.5 °C min^{-1}. This heating rate is the same as that of the DSC thermogram, shown in fig. 5.1. It is observed that the signal remains constant and zero up to a temperature of about 65 °C, after which it starts to decrease. The decrease corresponds to a loss in β-turn and/or formation of random coil and α-helix conformations. These trends correspond well with those reported in Table 1. Comparison with fig. 5.1 clearly shows that the temperature at which this transition occurs is the same as the denaturation temperature measured by DSC. Above 81 °C the CD signal strongly increases. This may be caused by aggregation of the IgG sample (cf. Fig. 5.1), after which the

aggregates settle and no longer contribute to the absorbance. It is further observed that above 75 °C the photomultiplier signal increases, most likely because of light scattering by the growing IgG aggregates. When the aggregates grow even further and settle, the signal decreases drastically, which illustrates that the protein aggregates disappear from the solution. The same phenomena may explain why at these high temperatures the fitting of the CD spectra was less successful.

Comparing the DSC and CD experiments clearly indicates that the transition observed in the thermograms corresponds with the change in secondary structure observed in the CD spectra. Dissolved IgG unfolds as a result of heat treatment, after which the molecules strongly aggregate causing the irreversibility of the denaturation process.

5.3.3 Adsorption characteristics of IgG

The adsorption is monitored by reflectometry and the adsorbed amount is calculated as described by Dijt et al. [46]. The adsorption of IgG from a 5 mM phosphate buffer, pH 6.0, onto a hydrophilic and a hydrophobic surface is shown in Fig. 5.6.

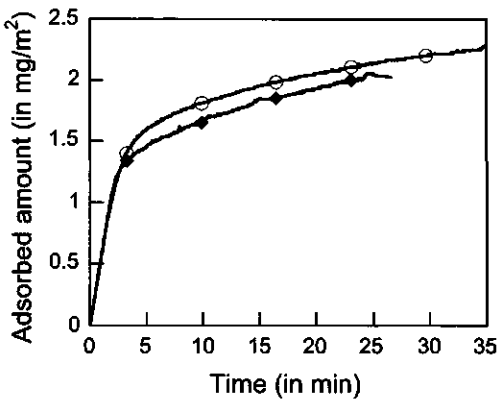

Figure 5.6: The adsorbed amount of IgG as a function of time in a 5 mM phosphate buffer, pH 6.0, on a hydrophobic surface (methylated silica) and a hydrophilic surface (silica). (open symbols = hydrophobic; solid symbols = hydrophilic).

It is observed that the adsorbed amount reached in about half an hour does not differ very much between the two surfaces, i.e. 2.2 mg m^{-2} at the hydrophilic

surface and 2.0 mg m^{-2} at the hydrophobic one. It is further shown that the initial adsorption is somewhat lower on the hydrophobic surface. Our results compare well with those previously reported by Buijs et al. [47]. Buijs et al. [48] also studied the antigen binding capacity of adsorbed IgG molecules. They found a reduced antigen binding capacity under conditions where the interaction between the F(ab) fragment and the surface was relatively strong. This result was primarily ascribed to unfavourable orientations of the IgG molecules on the hydrophobic surface. However, it seems likely that conformational changes in the protein also play an important role. Upon adsorption on a hydrophobic surface the IgG molecule may partly unfold, due to which it occupies more space which, in turn, leads to a decreasing capacity for antigen binding. To study the degree of denaturation and its effect on the secondary structure, we will now discuss some CD data for IgG molecules adsorbed on Teflon particles.

5.3.4 Heat induced changes in the secondary structure of adsorbed IgG

The CD spectra for different temperatures of IgG adsorbed onto Teflon are shown in Fig. 5.7. The CD spectra measured at intermediate temperatures changed gradually from the curve at 20 °C to that at 85 °C. For reasons of clarity only the two extremes are shown. In contrast to the effect of temperature on the secondary structure of dissolved IgG, the heat-induced changes in the structure of adsorbed IgG are only minimal. All spectra have zero intensity at 199 nm and show a minimum at 208 nm with a broad shoulder around 220 nm that gradually changes to a single minimum at 215 nm. In Fig. 5.8 the CD spectra of dissolved and adsorbed IgG are plotted for 20 and 75 °C. Comparison of the spectra of the dissolved and adsorbed IgG at 20 °C, which is much lower than the T_d observed by DSC, shows that adsorption at the hydrophobic surface has a strong effect on the secondary structure. It is further unambiguously demonstrated that the secondary structure of heat induced denatured IgG is different from that of adsorbed IgG; after heat treatment of the adsorbed molecules the difference is less pronounced.

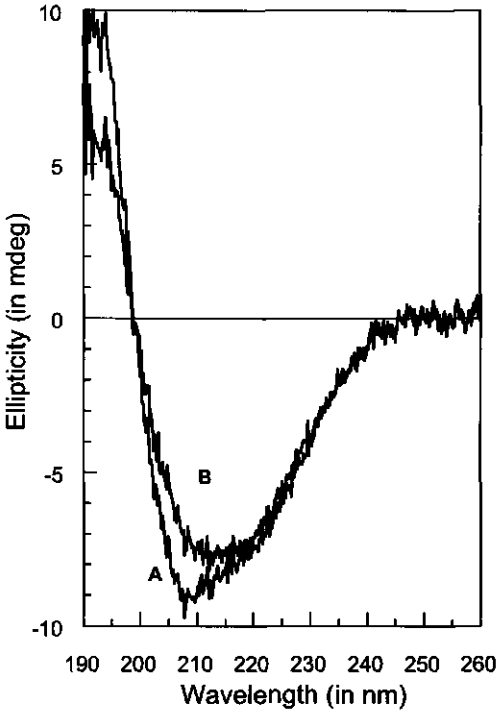

Figure 5.7: Far-UV CD spectra of IgG adsorbed on Teflon particles (1.68 mg m^{-2}) in a 5 mM phosphate buffer at pH 6.0, for two temperature values: A = 20 °C and B = 85 °C.

The presence of a hydrophobic surface has a strong effect on the adsorption-induced conformational changes. Now the changes may result from differences in hydrophobicity between the different segments of the IgG molecule. Model calculations following the procedure described by Connolly [49] have shown that the surface of the F(ab) fragment is in average slightly more hydrophobic than that of the Fc fragment [50]. Furthermore, it is not unlikely that the hydrophobic patches, available for the solvent molecules, are primarily found in the cavity formed by the variable domains of an IgG molecule. The interactions between the variable domains of the heavy and light chains are dominated by hydrophobic forces, and the antigen binding sites often obtain their specificity by creating this hydrophobic cavity. As a result of interacting with a hydrophobic surface the IgG molecule and, in particular, the more hydrophobic variable domains of the antigen binding area are likely to unfold, which in turn may influence the antigen binding specificity.

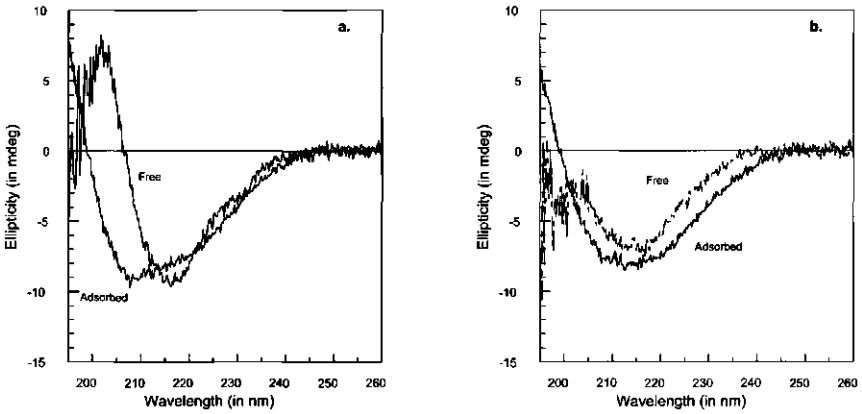

Figure 5.8: Far-UV CD spectra of free (0.2 mg m^{-1}) and adsorbed (1.68 mg m^{-2}) IgG in a 5 mM phosphate buffer, pH 6.0. a: at 20 °C, b: at 75 °C.

Analysis of the CD spectra in the wavelength range 195-240 nm gave good results with respect to the RMS error, i.e., an average of 7.0 was obtained. However, comparison of the fitted curve with the experimental data clearly showed that the α-helix content was overestimated. However, when the wavelength of the light beam becomes to small, the detected signal will be strongly affected due to an increased light scattering. Thus, we may expect a strong effect on the intensity of the ellipticity in the wavelength range below 200 nm. To check whether this apparent overestimation of the α-helix content could be reduced, a second fit was performed in the wavelength range 199-240 nm. The results of this fit were very good as the fitted curve matched the experimental data exactly. The secondary structure element contents for the different temperatures, obtained from the latter fit are summarised in Table 5.2.

Table 5.2. Content (in %) of structure elements in IgG adsorbed on Teflon particles (1.68 mg m^{-2}) at different temperatures (only wavelength > 199 used for fit). Medium: 5 mM phosphate buffer, pH 6.0.

Temperature (in °C)	α-helix	β-sheet	β-turn	random coil	RMS error
20	17	32	12	39	5.5
30	17	33	12	38	6.1
40	15	36	11	38	5.9
50	12	38	10	40	5.5
60	11	40	9	40	5.4
65	11	40	9	40	4.7
70	11	41	8	40	5.6
75	11	41	8	40	5.0
80	10	42	8	40	4.7
85	9	43	8	40	5.8

As compared to the native IgG, the fractions of α-helix and random coil conformation are strongly increased due to the adsorption, whereas the β-sheet content is strongly diminished. With increasing temperature the α-helix and β-turn contents gradually decrease whereas the fraction of β-sheet structures increases. The strong α-helix induction observed upon adsorption on a hydrophobic surface has more often been observed for the adsorption of proteins on hydrophobic surfaces [29,51]. Because no favourable hydrogen bonds can be formed between the IgG and a hydrophobic surface, the adsorbed molecules may tend to form intramolecular hydrogen bonds, thereby promoting the formation of α-helixes.

These results clearly show that IgG molecules undergo structural rearrangements upon contact with a *hydrophobic* surface. This is in contrast with the results described by Buijs et al. [23] for the adsorption of the same IgG onto a *hydrophilic* surface.

5.4 Conclusions

It is demonstrated that combining Differential Scanning Calorimetry and Circular Dichroism Spectroscopy provides useful information about heat induced denaturation of immunoglobulin G. At elevated temperatures IgG unfolds, which, in turn, causes formation of aggregates. The aggregation step is likely to cause the irreversibility of the structural transition. The DSC thermogram shows one endothermic transition at a temperature at which the major change in the secondary structure indicated in the CD spectrum is observed as well.

The secondary structure of the native IgG, calculated from the CD spectra, compares well with that measured by other techniques. Due to the unfolding of the IgG the fractions of β-sheet and β-turn decrease, whereas those of random coil and, to a lesser extent, α-helix conformations increase. It was observed that even after heat induced denaturation a significant part of the secondary structure remains.

IgG also changes its structure due to the adsorption on a hydrophobic surface. The fractions of α-helix and random coil conformation are strongly increased after adsorption, whereas the β-sheet content is strongly reduced. Heating adsorbed IgG results in a gradual decrease of the α-helix and β-turn contents, whereas the fraction of β-sheet structure increases. After heating up to 75 °C the structure of dissolved and adsorbed IgG, respectively, still deviate from each other although the difference is much less than at lower temperatures.

In view of these conclusions it can be stated that, in addition to, or instead of, unfavourable molecular orientations, changes in the three-dimensional (secondary and possibly tertiary) structure may explain the lower antigen binding affinity of IgG molecules adsorbed on hydrophobic surfaces.

Acknowledgement

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5.5 References

1. Sevier, E.D. (1985) *J. Med. Technol. 2*, 287.
2. Frances, R., Batzer, M.D. (1980) *Fertil. Steril. 34*, 1.
3. van Erp, R., Linders, Y.E.M., van Sommeren, A.P.G., Gribnau, T.C.J. (1992) *J. Immunol. Methods 152*, 191.
4. Lichtenbelt, J.W.T., Heuvelsland, W.J.M., Oldenzeel, M.E., Zsom, R.L.J. (1993) *Colloids Surfaces B: Biointerfaces 1*, 75.
5. Haynes, C.A., Norde, W. (1994) *Colloids Surfaces B: Biointerfaces 2*, 417.
6. Hidalgo-Alvarez, R., Galisteo-González, F. (1995) *HCR Comprehensive Review 2*, 249.
7. Norde, W. (1996) *Macromol. Symp. 103*, 5.
8. Lee, S.H., Ruckenstein, E. (1988) *J. Colloid Interface Sci. 125*, 365.
9. Wesslén, B., Kober, M., Freij-Larsson, C., Ljungh, Å., Paulsson, M. (1994) *Biomaterials 15*, 278.
10. Elgersma, A.V., Zsom, R.L.J., Lyklema, J., Norde, W. (1992) *J. Colloid Interface Sci. 152*, 410.
11. Freij-Larsson, C., Nylander, T., Jannasch, P., Wesslén, B. (1996) *Biomaterials 17*, 2199.
12. Lee, J., Martic, P.A., Tan, J.S. (1989) *J. Colloid Interface Sci. 131*, 252.
13. Peula, J.M., Hidalgo-Alvarez, R., De Las Nieves, F.J. (1995) *J. Biomater. Sci. Polymer Edn. 7*, 231.
14. Puig, J., Fernández-Barbero, A., Bastos-González, D., Serra-Domnènech, J., Hidalgo-Alvarez, R. (1994) in *Proceedings of the International Symposium on Surface Properties of Biomaterials* (West, R., Batts, G. eds.), pp. 39, Butterworth-Heinemann, London.
15. Buijs, J., Lichtenbelt, J.W.T., Norde, W., Lyklema, J. (1995) *Colloids Surfaces B: Biointerfaces 5*, 11.
16. Edmundson, A.B., Ely, K.R. (1986) in *Handbook of experimental Immunology in four volumes* (Weir, D.M. ed.), Blackwell Scientific Publications, Oxford.
17. Chothia, C., Novotny, J., Bruccoleri, R., Karplus, M. (1985) *J. Mol. Biol. 186*, 651.
18. Padlan, E.A. (1997) *Mol. Immunol. 31*, 169.
19. Deisenhofer, J. (1981) *Biochemistry 20*, 2361.
20. Marquart, M., Deisenhofer, J., Huber, R., Palm, W. (1980) *J. Mol. Biol. 141*, 369.
21. Singh, B.R., Fuller, M.P. (1991) *Appl. Spectrosc. 45*, 1017.
22. Jeon, J.S., Sperline, R.P., Raghavan, S. (1992) *Appl. Spectrosc. 46*, 1644.
23. Buijs, J., Norde, W., Lichtenbelt, J.W.T. (1996) *Langmuir 12*, 1605.

24. Kirsch, J.L., Koenig, J.L. (1989) *Appl. Spectrosc. 43*, 445.
25. Fasman, G.D. (1996) *Circular Dichroism, the conformational analysis of biomolecules*, Plenum Press, New York.
26. Kats, M., Richberg, P.C., Hughes, D.E. (1995) *Anal. Chem. 67*, 2943.
27. Tetin, S.Y., Linthicum, D.S. (1996) *Biochemistry 35*, 1258.
28. Rousseaux, J., Aubert, J.P., Loucheux-Lefebvre, M. (1982) *Biochim. Biophys. Acta 701*, 93.
29. Maste, C.L.M., Pap, E.H.W., van Hoek, A., Norde, W., Visser, A.J.W.G. (1996) *J. Colloid Interface Sci. 180*, 632.
30. Norde, W. (1992) *Clinical Materials 11*, 85.
31. Takeda, K., Hamada, S., Wada, A. (1993) *J. Protein Chem. 12*, 223.
32. Haynes, C.A., Norde, W. (1995) *J. Colloid Interface Sci. 169*, 313.
33. McCarthy, D.A., Drake, A.F. (1989) *Mol. Immunol. 26*, 875.
34. Greenfield, N., Fasman, G.D. (1969) *Biochemistry 8*, 4108.
35. Chang, C.T., Wu, C.-S.C., Yang, J.T. (1978) *Anal. Biochem. 91*, 13.
36. de Jongh, H.H.J., Goormaghtigh, E., Killian, J.A. (1994) *Biochemistry 33*, 14521.
37. Brahms, S., Brahms, J. (1980) *J. Mol. Biol. 138*, 149.
38. Buchner, J., Renner, M., Lilie, H., Hinz, H.-J., Jaenicke, R., Kiefhaber, T., Rudolph, R. (1991) *Biochemistry 30*, 6922.
39. Martsev, S.P., Kravchuk, Z.I., Vlasov, A.P., Lyakhnovich, G.V. (1995) *FEBS Letters 361*, 173.
40. Tischenko, V.M., Zav'yalow, V.P., Medgyesi, G.A., Potekhin, S.A., Privalov, P.L. (1982) *Eur. J. Biochem. 126*, 517.
41. Vlasov, A.P., Kravchuk, Z.I., Martsev, S.P. (1996) *Biochemistry (Moscow) 61*, 155.
42. Privalov, P.L. (1980) *Pure & Appl. Chem. 52*, 479.
43. Barone, G., Giancola, C., Verdoliva, A. (1992) *Thermochim. Acta 199*, 197.
44. Bremer, L.G.B., Walstra, P., van Vliet, T. (1995) *Colloids Surfaces A: Physicochem. Eng. Aspects 99*, 121.
45. Byler, D.M., Susi, H. (1986) *Biopolymers 25*, 469.
46. Dijt, J.C., Cohen Stuart, M.A., Fleer, G.J. (1994) *Adv. Colloid Interface Sci. 50*, 79.
47. Buijs, J., van den Berg, P.A.W., Lichtenbelt, J.W.T., Norde, W., Lyklema, J. (1996) *J. Colloid Interface Sci. 178*, 594.
48. Buijs, J., White, D.D., Norde, W. (1997) *Colloids Surfaces B: Biointerfaces 8*, 239.
49. Connolly, M.L. (1983) *J. Appl. Cryst. 16*, 548.
50. Buijs, J. PhD thesis, Wageningen Agricultural University, Wageningen, the

Netherlands, 1995.

51. Zoungrana, T., Findenegg, G.H., Norde, W. (1997) *J. Colloid Interface Sci. 190*, 437.
52. Chapter 4, this thesis.

Chapter 6

INFLUENCE OF PRE-ADSORBED (BLOCK COPOLYMER) MOLECULES ON IGG CONFORMATION

Abstract

The effect of pre-adsorbed molecules on the secondary structure of subsequently adsorbing monoclonal immunoglobulin G (IgG) molecules is studied by circular dichroism spectroscopy. The Teflon latex used as the sorbent surface is precoated with triblock copolymers of the type poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO). The water-soluble PEO buoy parts protrude into the solution and form a steric barrier that reduces the surface area available for adsorption and structural rearrangements of the IgG molecules. Structural rearrangements were less extensive with increasing surface coverage of the polymer. It was found that pre-adsorbed IgG molecules have comparable effects on the secondary structure of subsequently adsorbing IgG; a more native-like structure is retained at higher pre-adsorbed amounts. Hence, appropriate partial pre-coating of a surface is an effective way to control the secondary structure of adsorbed IgG.

6.1 Introduction

In most proteins the biological activity requires structural integrity of the molecules, irrespective of this molecule being an enzyme, an antibody, or a receptor. The conformation of proteins, which is determined by an intricate balance of interactions between all components in the system, is only marginally stable and sensitive to changes in environmental conditions such as pH, temperature and ionic strength [1-3]. In line with this, physical adsorption of proteins onto solid surfaces, as employed in industrial applications like immunoassays, biosensors or colloidal drug carriers, can significantly alter the conformation [4, 5]. As a result, the biological functioning of adsorbed proteins is often impaired. To preserve biological activity one therefore frequently aims for procedures to reduce, or eliminate adsorption-induced conformational changes.

In general, adsorption-induced conformational changes involve some degree of spreading of the protein at the sorbent surface. This spreading is hindered by the presence of other protein molecules on the surface [6]. For instance, for Savinase on Teflon and silica [7], catalase on silica [35], albumin and fibrinogen on hydrophobic self-assembled monolayers and hemoglobin on various surfaces [8] it is observed that adsorption-induced conformational changes are less pronounced at higher surface coverages. For this reason, reducing the fraction of the surface area available for the adsorbing protein molecules may be a meaningful approach to suppress the degree of conformational changes and increase the biological activity per molecule of the adsorbed proteins. Such a reduction of available surface area can be achieved by pre-coating the sorbent surface with molecules that form a barrier inhibiting unfolding of the protein molecule. The pre-adsorbed molecules need not necessarily be of the same type as the protein molecules in which one is interested: a similar effect may also be obtained by pre-coating a surface with other possibly more protein-inert molecules.

Pre-adsorption of triblock copolymers composed of poly(ethylene oxide), PEO, and poly(propylene oxide), PPO, and PEO again is commonly used [9-12] to prevent or reduce subsequent protein adsorption. The copolymer adsorbs on a hydrophobic surface through the water-insoluble PPO anchor part leaving the water-soluble PEO buoy parts to protrude into the solution [13-15]. These buoy parts form a steric barrier that, on a fully covered surface, prevents protein adsorption. If, however, the amount of pre-adsorbed polymer is low, a rather open, low density polymeric layer is formed that contains uncovered areas in which proteins are still able to adsorb onto the surface [16-18]. The buoy parts in

these layers may prevent unfolding of the adsorbed protein. In addition, the pre-adsorbed polymers may alter the properties of the sorbent surface (e.g. its hydrophobicity) which are known to affect the conformational rearrangements in adsorbed proteins. Furthermore, pre-adsorbed triblock copolymers can direct asymmetrically shaped proteins, i.e. IgG molecules, to adsorb in a preferential orientation [18].

In a previous study we performed a structural analysis of a monoclonal IgG adsorbed on different silica surfaces (hydrophilic, hydrophobic, hydrophobic with pre-adsorbed triblock copolymers) using ATR-FTIR spectroscopy [17]. Unfortunately with this technique it was not possible to obtain reliable data for IgG adsorbed on bare hydrophobic silica because of interference of hydrophobic dehydration water. Therefore, such a spectroscopic comparison between IgG adsorbed on a bare hydrophobic surface and on a hydrophobic surface precoated with triblock copolymers was impossible and, hence, the effect of the pre-adsorbed polymers on the conformation of subsequently adsorbed IgG could not unambiguously be determined. However, the protein structure in solution as well as adsorbed on a hydrophobic surface [4, 19] can be studied by Circular Dichroism (CD) spectroscopy.

The main purpose of the present work is to determine by CD the effect of pre-adsorbed polymer molecules on the secondary structure of adsorbed monoclonal IgG molecules. We used PEO-PPO-PEO triblock copolymers to pre-coat the sorbent surface. The effect of these layers on the secondary structure of adsorbed IgG is studied by taking the coverage of the sorbent by the pre-adsorbed layer as the experimental variable. In addition, the effects of adsorption conditions, such as incubation time and pH are studied. Hydrophobic perfluoro-alkoxy fluoro carbon Teflon particles are selected as the sorbent surface.

6.2 Experimental

6.2.1 Materials

Protein

Mouse monoclonal Immuno gamma Globulin (IgG) from isotype 1 directed against the human pregnancy hormone (hCG, Human Chorionic Gonadotropin) was a gift from Organon Teknika (Boxtel, The Netherlands).

The isoelectric points of the whole IgG and its fragments $F(ab')_2$ and F(c) are 6.0,

5.9 and 6.1 respectively, as determined by isoelectrical focusing.
Concentrated IgG solutions were stored at -20°C and thawed once shortly before use. Prior to every experiment protein solutions were filtered through an 0.2 μm Acrodisc filter to remove aggregates. The extinctions at 280 nm were converted into protein concentrations using an extinction coefficient of 1.45 $cm^2\ mg^{-1}$.

Chemicals
Triblock copolymer of the type PEO-PPO-PEO (Synperonic P75), hereafter abbreviated as P75, was a gift from ICI (Rotterdam, The Netherlands). The two PEO (poly(ethylene oxide)) parts are water-soluble but the PPO (poly(propylene oxide)) part is water-insoluble. The average molecular weights of the whole polymer, its PEO, and PPO parts are 4150, 1075 Da (24 monomers) and 2000 Da (34 monomers) respectively.
Chemicals for buffer preparation, Na_2HPO_4, NaH_2PO_4, were of analytical grade and used without further purification. Water was purified by reverse osmosis and passed subsequently through a superQ system (Millipore).

Adsorbent
The Teflon latex used as the sorbent was a gift from Du Pont de Nemours (Le Grand-Saconnex, Switzerland). The particles are monodisperse, hydrophobic (contact angle of a drop of water on a tablet of dried material was 96°) and have a diameter of 215 nm and a specific surface area of 13 $m^2\ g^{-1}$ [19]. The latex particles are negatively charged due to sulfonate groups which stem from the polymerisation initiator, potassium persulphate. As sulphate groups are strong, the particles carry a constant (negative) surface charge density over the pH range (pH 5-8) used in our experiments. The Teflon particles do not contain UV-absorbing groups and their refractive index (1.35) is close to that of water (1.33). Hence, these particles absorb and scatter negligibly in the far UV and are therefore well suited to be used as an adsorbent when measuring circular dichroism of adsorbed proteins.

6.2.2 Method
Teflon particles are precoated with polymer by incubation for 15 minutes in polymer solutions of various concentrations. From reflectometry experiments we know that under these experimental conditions all polymer molecules are completely adsorbed.

By adjusting the number of Teflon particles per experiment it was possible to

control the protein surface concentration at fixed total protein concentration.
The coverage of the (precoated) Teflon surface by the protein is adjusted while the total protein concentration is maintained constant. For IgG an incubation time of 30 minutes is used, unless stated otherwise.
A background spectrum of the (precoated) particles is taken before each measurement.

Circular Dichroism
CD spectra were recorded on a JASCO spectropolarimeter, model J-715 (JASCO International Co., Tokyo, Japan). Solutions of protein as well as dispersions of the protein-covered particles were scanned between 190 nm and 260 nm. The CD measurements and the fitting of the spectra were performed as described in a previous paper [4].

6.3 Results

6.3.1 IgG conformation as a function of its surface coverage. Absence of pre-adsorbed polymer
Representative far UV CD spectra of IgG in solution and adsorbed at hydrophobic Teflon particles (1.68 mg m^{-2}) are shown in figure 6.1. The spectrum of IgG in solution has extremes at 217 nm (negative) and at 203 nm (positive) and intersects the zero intensity line at a wavelength of 206 nm. It reflects the so-called immunoglobulin fold, indicating a high content of beta sheet [20-22]. As expected [3, 23], the spectra of adsorbed IgG strongly differ from that of IgG in solution indicating major adsorption-induced changes in the secondary structure of IgG. The contents of secondary structure elements calculated from these spectra are summarised in table 6.1.

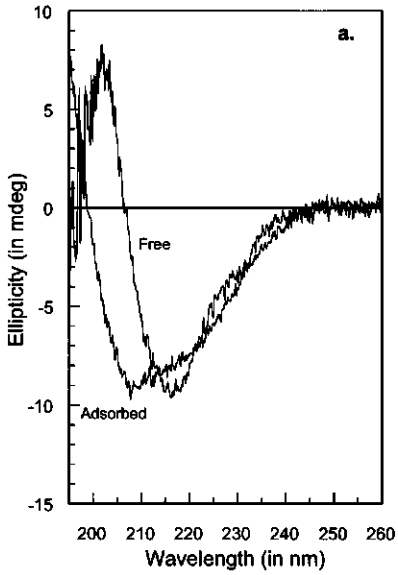


Figure 6.1: Far-UV CD spectra of free (0.2 mg ml^{-1}) and adsorbed (1.68 mg m^{-2}) IgG in a 5 mM phosphate buffer, pH 6.0 at 20 °C.

Table 6.1: Content (%) of structure elements in dissolved and adsorbed IgG.

structure element	% in dissolved native IgG	% in adsorbed IgG
α-helix	0	23
β-sheet	76	34
β-turn	14	9
random coil	10	34

Dissolved native IgG consists for circa 76% of β-sheet and contains only minor fractions of β -turn and coil structures and essentially no α-helix. Upon adsorption the β-sheet content decreases considerably whereas the fractions of α-helix and random coil increase. Upon adsorption, the intra-molecular interactions, that form the basis of the secondary structure of proteins, may be exchanged by interactions between the molecule and the surface. If such protein-surface interactions are favourable this will result in a decrease in secondary structure (and a favourable

increase in conformational entropy of the protein). On the other hand, if favorable interactions between peptide units and the surface are not possible extra intra-molecular interactions may be formed, and in this way promote the formation of ordered structure [24]. On the hydrophobic Teflon surface, the lack of surface groups that can form favourable hydrogen bonds with adsorbing IgG molecules results in the formation of intra-molecular α-helices. α-helix induction upon adsorption on a hydrophobic surface (at low surface coverage) is also observed for other protein molecules [19, 25].

To determine the effect of pre-adsorbed IgG molecules on the secondary structure of subsequently adsorbing IgG molecules we varied the surface coverage. At lower surface pre-coverage, the fraction of surface area remaining available for later arriving protein molecules to adsorb and adjust their structure is larger. The fractions of structure elements as a function of the adsorbed amount of IgG are graphically presented in fig. 6.2. From reflectometry [18] we know that under these experimental conditions all IgG molecules are adsorbed.

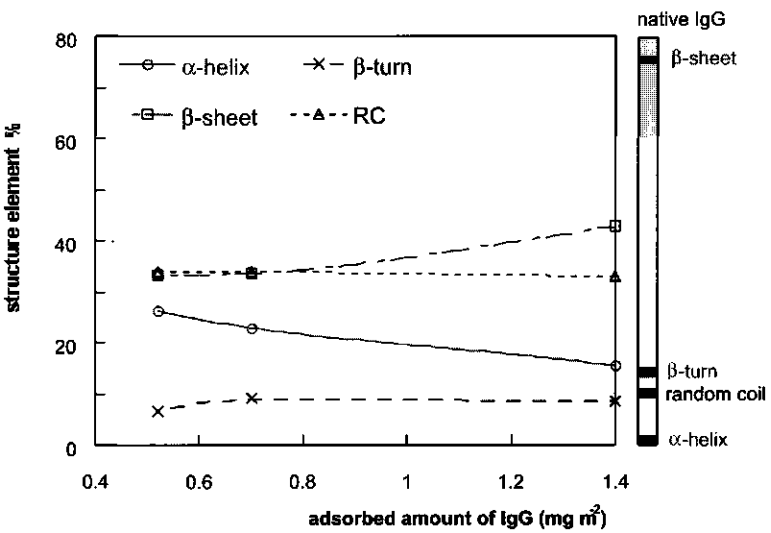


Figure 6.2: Fractions of structure elements plotted as a function of the adsorbed amount of IgG. For comparison the secondary structure composition of native IgG is shown on the right.

The β -turn and random coil contents are not very sensitive to the adsorbed

amount. However, the fraction of α-helix decreases with increasing adsorbed amount whereas the fraction of β-sheet increases. Clearly, structural changes are smaller when the degree of surface coverage is larger; a more native-like structure is retained at higher adsorbed amounts. In other words, pre-adsorbed IgG molecules suppress structural rearrangements in subsequently adsorbing molecules by reducing the available area for spreading at the surface. This is found for many different proteins adsorbing on various solid surfaces [6,7,26,35]. For monolayers of IgG molecules at the air-water interface a similar effect is observed by Tronin et al. [27]. At low surface pressure, i.e. at low surface coverage, the IgG film is expanded; the effective molecular cross section grows as a result of unfolding of the molecules. At higher surface pressure the film is more compressed. In other words, unfolding of the IgG molecules is prevented by increasing the surface pressure or, for that matter, by increasing the surface coverage.

6.3.2 IgG conformation on a triblock copolymer precoated surface

We demonstrated that pre-adsorbed IgG molecules can suppress the degree of structural changes in subsequently adsorbing IgG molecules. Now it will be investigated whether a similar effect can also be obtained by precoating the surface with a triblock copolymer.

It is well known that interactions of some ionic surfactants with globular proteins induce major changes in the protein conformation [28, 29], whereas in general, nonionic surfactants do not interact, or interact only mildly with proteins [30] so that no severe changes in the secondary structure are induced. To check whether specific interactions of the (nonionic) polymer influence the conformation of IgG, spectra of IgG are measured in solutions containing varying concentrations of P75 up to 1 mg ml^{-1}.
The obtained spectra (not shown) were identical to that of dissolved IgG for every polymer concentration. This demonstrates that in solution the presence of polymer does not induce any changes in the conformation of native IgG. Absence of interaction in solution between a PEO-PPO-PEO triblock copolymer and albumin, fibrinogen, and immunoglobulin, was also reported by Tan and Martic [31].

With increasing adsorbed amount of P75, the rate of adsorption and the maximum adsorbed amount of subsequently adsorbing IgG decreases strongly [18]. Moreover, an IgG-repelling surface was created at polymer coverages of 0.7 mg m^{-2} [18]. For P75, saturation adsorption at a hydrophobic surface

corresponds to an adsorbed amount of 0.8 mg m^{-2}.

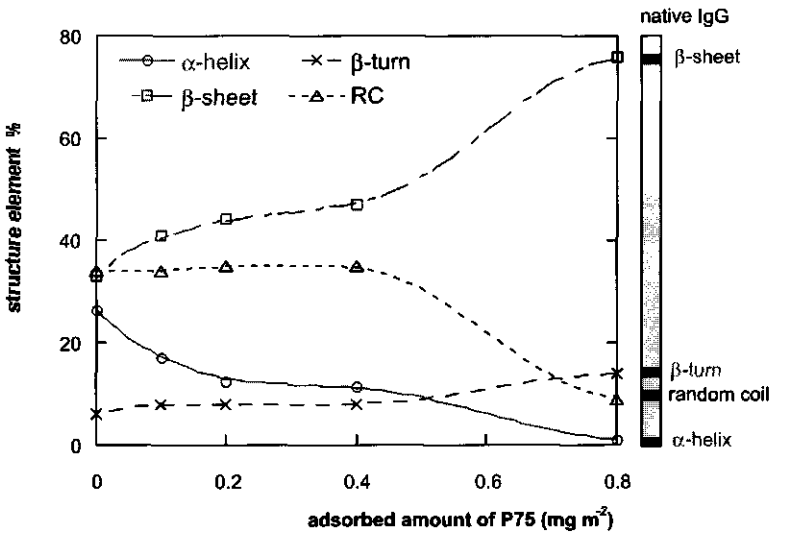


Figure 6.3: Fractions of structure elements of adsorbed IgG as a function of the pre-adsorbed amount of P75. For comparison the secondary structure composition of native IgG is shown on the right.

The Teflon particles are precoated with P75 up to surface coverages of 0.8 mg m^{-2}. CD spectra of IgG adsorbed at polymer precoated particles are measured and the corresponding secondary structure compositions are graphically presented in fig 6.3.

As found with pre-adsorbed IgG, the β-turn and random coil content are the least sensitive for the pre-adsorbed amount of P75. In adsorbed IgG the fraction of RC amounts to approximately 35% and it is invariant with the adsorbed amount of IgG or P75, whereas in solution it only amounts to about 10%. Thus, the fraction of random coil structure serves well to probe whether IgG is adsorbed or not.

At intermediate amounts of P75 the fraction of α-helix decreases whereas the fraction of β-sheet increases with increasing P75 surface coverage. Thus, pre-adsorbed triblock copolymer P75 suppresses the degree of structural changes in subsequently adsorbing IgG and a more native-like structure is retained. From the fraction of random coil structure, 35%, we conclude that at these surface coverages of P75 all IgG molecules are in the adsorbed state.

In a previous paper we reported on the effect of pre-adsorbed polymers on the structure of adsorbed proteins using ATR-FTIR spectroscopy [17]. Information on the exact adsorbed amount of polymer was not available, but it was lower than the maximum in order not to fully block the surface for subsequent protein adsorption. The results of that study are in good agreement with those obtained in the present study, i.e., the presence of the polymer on the surface allowed neither IgG nor BSA to rearrange its structure extensively.

It is observed that at high surface coverage of P75, approximately 0.6 mg m^{-2} and 0.8 mg m^{-2}, the conformation of the IgG is similar to the conformation of native dissolved IgG. Furthermore, the fraction of random coil structure, 10%, indicates that the IgG molecules are, as expected, in solution rather than adsorbed.

Carignano et al. [32] performed a theoretical study of model systems resembling our experimental system of pre-adsorbed PEO chains and a protein that may change its conformation upon adsorption. The fractions of native and denatured proteins were calculated as a function of polymer grafting density. As a model for PEO chains on a hydrophobic surface they used a flexible chain consisting of segments that are attracted by the surface. The model protein molecule is assumed to be spherical in solution, upon adsorption it can either retain its spherical shape or denature into a pancake-like configuration. The contact energy for pancakes with a bare surface was assumed to be twice as high as for spheres. As a result, on a bare surface the amount of adsorbed pancakes was twice that of adsorbed spheres.
An increase in the surface coverage by PEO chains resulted in a strong decrease of the amount of adsorbed pancakes while the amount of adsorbed spheres increased. The density of spheres on the surface decreased once there were no more pancakes on the surface. This behaviour can be explained by the strong repulsion that a pancake configuration experiences on a surface precoated with polymers. In the pancake configuration most of the volume of the protein is close to the surface where, due to the polymer-surface attraction, the polymer segments are located as well.
These calculations provide agreement with our experimental observations. Unfortunately, CD experiments are not suitable to discriminate between a protein layer in which each molecule is unfolded to some extent and a layer in which a part of the protein population is completely unfolded and the rest has a native conformation, so this part of the assumptions of Carignano and Szleifer could not

be tested experimentally.

Although IgG and P75 are very different molecules, increasing pre-adsorbed amounts of these molecules have comparable effects on the secondary structure of subsequent adsorbing IgG. This trend suggests a similar underlying mechanism, viz., a reduction in the available area for spreading at the sorbent surface.

6.3.3 Biological activity of IgG adsorbed on precoated surfaces

Elsewhere we observed [18] an increased biological activity of IgG molecules both at higher adsorbed amounts of IgG and on surfaces precoated with polymer. This increase is likely the result of both retaining a more native-like structure and a more favourable orientation. In [18] we present a detailed study of the IgG adsorption process on (polymer) precoated surfaces and its effect on the immunological activity.

The positive effect of surface coverage on the biological activity was also observed by Tronin et al. [27]. They found that at an air-water interface at high surface pressure IgG was still active, whereas a complete loss of activity was registered at low surface pressure.

6.3.4 Effect of adsorption conditions on IgG conformation

Incubation time

The time dependence of the structural changes in adsorbed IgG are studied for various adsorption conditions: different pH values, different adsorbed amounts of IgG and relatively low adsorbed amounts of triblock copolymer (to ensure that all IgG molecules are adsorbed at the surface). To enable determination of the secondary structure after short periods of incubation of the protein with the sorbent surface the spectra were an average of only 2 scans. At the start of the IgG adsorption a spectrum was taken every two minutes up to 15 minutes of incubation time. Thereafter, spectra were taken after 20, 30 and 60 minutes and 2, 3 and 24 hours.

The spectra obtained for each adsorption condition are almost invariant with incubation time. This indicates that essentially all changes in the protein structure occur before the first spectrum is taken and that no additional reconformation occurs after that, up to a day. In our measurements approximately 2 minutes were necessary to fill the cuvette with protein solution, and to take the first spectrum, implying that sufficient time may have elapsed for the adsorbed protein to change its conformation. This also is in agreement with our ATR-FTIR results [17].

pH

The spectra of dissolved IgG are identical over the pH range 5-8. However, the spectra of the adsorbed IgG do depend on pH. The variation in secondary structure composition is calculated from these spectra and summarised in table 6.2.

Table 6.2: Content (%) of structure elements in adsorbed IgG at various pH of adsorption.

structure element	% in adsorbed IgG adsorbed at				% in dissolved native IgG
	pH 5	pH 6	pH 7	pH 8	
alfa helix	28	22	25	28	0
beta sheet	28	34	25	26	76
beta turn	7	9	7	6	14
random	36	35	42	40	10

Adsorption-induced conformational changes are at a minimum at the isoelectric point (i.e.p.) of the IgG, pH 6. Progressed structural rearrangements at pH values away from the i.e.p. may be explained by the excess of positive or negative charges on the protein which may cause intramolecular charge-charge repulsion and promote a more expanded and flexible structure [36]. In solution, these intramolecular changes may not affect the secondary structure [34], but the interactions between the strands are weakened, thus decreasing the protein stability against other destabilising agents such as temperature or the presence of a sorbent. Thus, at pH values away from the i.e.p. the adsorbed IgG molecules are somewhat more spread on the surface. This more unfolded structure can partially explain the decrease in adsorption saturation on the acid and alkaline sides of the isoelectric region, as measured with reflectometry [37]. Additionally, adsorption saturation depends on the orientation (distribution) of the molecules at the surface, and on the surface coverage.

6.4 Conclusion

The fraction of random coil structure of an IgG molecule can be used to determine whether it is in an adsorbed state or in solution.

Pre-adsorbed molecules can suppress the degree of structural changes in subsequently adsorbing IgG molecules by reducing the available surface area. This effect was observed on surfaces precoated with IgG and triblock copolymer molecules. Hence, partial pre-coating of a surface is an effective way of controlling the conformation and the biological activity of adsorbed IgG. In a following paper we will extend our study on the effect of pre-adsorbed polymer on the adsorption and biological activity of IgG in an ELISA application.

Adsorption-induced structural changes in IgG occur within seconds and no additional reconformation occurs over a timescale of hours. This shows that long term reorientation of the IgG molecules on the surface [18] does not involve further structural changes.

The pH at which the adsorption process takes place has an effect on the secondary structure of adsorbed IgG. The structural integrity is best retained around the i.e.p. of the IgG. Hence, solution conditions may be an interesting variable in the production of immunoassays.

6.5 References

1. Welfle, K., Misselwitz, R., Hausdorf, G., Höhne, W., and Welfle, H. (1999) *Biochim. Biophys. Acta 1431*, 120.
2. Vlasov, A. P., Kravchuk, Z. I., and Martsev, S. P. (1996) *Biokhimiyia 61*, 212.
3. Buijs, J., Norde, W., and Lichtenbelt, J.W.T. (1996) *Langmuir 12*, 1605.
4. Vermeer, A.W.P., Bremer, M.G.E.G., and Norde, W. (1998) *Biochim. Biophys. Acta 1425*, 1.
5. Geddes, N.J., Furlong, D.N., Zientek, P., Than, K.A., and Edgar, J.A. (1993) *J. Colloid Interf. Sci. 157*, 491.
6. Wertz, C. F., and Santore, M.M. (1999) *Langmuir 15*, 8884.
7. Maste, M.C.L., Norde, W., and Visser, A.J.W.G. (1997) *J. Colloid Interf. Sci. 196*, 224.
8. Kondo, A., and Mihara, J. (1996) *J. Colloid Interf. Sci. 177*, 214.
9. Lee, J. H., Kopecek, J., and Andrade, J. (1989) *J. Biomed. Mater. Res. 23*, 351.
10. Amiji, M., and Park, K. (1992) *Biomaterials 13*, 682.
11. Schroën, C.G.P.H., Cohen Stuart, M.A., van der Voort Maarschalk, K., van der Padt, A., and van 't Riet, K. (1995) *Langmuir 11*, 3068.
12. Freij-Larsson, C., Nylander, T., and Wesslen, B. (1996) *Biomaterials 17*, 2199.
13. Lee, J., Martic, P.A., and Tan, J.S. (1989) *J. Colloid Interf. Sci. 131*, 252.
14. Tiberg, F., Malmsten, M., Linse, P., and Lindman, B. (1991) *Langmuir 7*, 2723.
15. O'Connor, S.M., Gehrke, S.H., and Retzinger, G.S. (1999) *Langmuir 15*, 2580.
16. McPherson, T., Kidane, A., Sleifer, I., and Park, K. (1998) *Langmuir 14*, 176.
17. Giacomelli, C.E., Bremer, M.G.E.G., and Norde, W. (1999) *J. Colloid Interf. Sci. 220*, 13.
18. Chapter 3, this thesis.
19. Maste, M.C.L., Pap, E.H.W., Hoek, A. v., Norde, W., and Visser, A.J.W.G. (1996) *J. Colloid Interf. Sci. 180*, 632.
20. Amzel, L.M., and Poljak, R.J. (1979) *Annu. Rev. Biochem. 48*, 961.
21. Davies, D.R., and Metzger, H. (1983) *Annu. Rev. Immunol. 1*, 87.
22. Padlan, E.A. (1994) *Mol. Immunol. 31*, 169.
23. Vermeer, A.P.W., and Norde, W. (2000) *J. Colloid Interf. Sci. 225*, 394.
24. Norde, W. (1998) *Biopolymers at interfaces, Driving forces for protein adsorption at solid surfaces*, Vol. 75, Marcel Dekker, Inc., New York.
25. Zoungrana, T., Findenegg, G.H., and Norde, W. (1997) *J. Colloid Interf. Sci. 190*, 437.
26. Norde, W., and Favier, J. P. (1992) *Colloids Surf. 64*, 87.

27. Tronin, A., Dubrovsky, T., Dubrovskaya, S., Radicchi, G., and Nicolini, C. (1996) *Langmuir 12*, 3272.
28. Rao, K. S., and Prakash, V. (1993) *J. Biol.Chem. 268*, 14769.
29. Subramanian, M., Seshadri, B. S., and Venkatappa, M. P. (1986) *J. Biosci. 10.*
30. Ananthapadmanabhan, K. P. (1993) *Protein-surfactant interactions*, CRC Press, Boca Raton, Fl.
31. Tan, J.S., and Martic, P.A. (1990) *J. Colloid Interf. Sci. 136*, 415.
32. Carignano, M.A., and Szleifer. I. (2000) *Colloids Surfaces B 18*, 169.
34. Chehin, R., Iloro, I., Marcos, M.J., Villar, E., Shnyrov, V.L., and Arrondo, J.L.R. (1999) *Biochemistry 38*, 1525.
35. Kondo, A., Murakami, F., Kawagoe, M., and Higashitani, K., (1993) *Appl. Microbiol. Biotechnol.* 39, 726.
36. Kleijn, M., and Norde, W. (1995) *Heterogeneous Chem. Rev. 2*, 157.
37. Chapter 2, this thesis.

7.1 Introduction

Since their introduction in the early nineteen seventies [1], solid phase immunoassays (SPI) form the basis of many commercially available immunodiagnostic tests. Even though a large number of successful technical variations have been introduced, the fundamental principle has not changed: SPI configurations consist of an immobilized capture reagent (an antibody, antigen or a fragment of one of these), the substance to be measured and a detection system that at least includes a second specifically binding agent carrying a detectable label. In an enzyme-linked immunosorbent assay (ELISA)[1], the label is an enzyme that causes a matching substrate to change its colour or otherwise reflects its presence quantitatively. The major advantages of such assays are their high stability and sensitivity, the potential for obtaining rapid quantitative results and the possibility of using relatively simple equipment [2].

To achieve high sensitivity in ELISA assays (1) maximized biological activity of the immobilized immunoproteins and (2) minimized non-specific binding of reactants to each other and to residual free solid surface are prerequisites. Yet, capture proteins are generally immobilized through physical adsorption [3], a process which is known to result in a partial loss of immunoreactivity [4]. This loss of reactivity may be caused by a spatially reduced accessibility of the antigen binding sites or by conformational changes in the antibodies, especially when hydrophobic surfaces are used. The antigen binding sites become inaccessible for antigens when they are in the vicinity of the sorbent surface. To avoid these problems a variety of immobilisation techniques have been proposed, e.g. covalent coupling to the solid surface [4], streptavidine bridging [2], immobilisation using a previously adsorbed antiglobulin [2] or protein A [5]. Although some of these techniques may indeed lead to increased average biological activity of IgGs, they may also introduce new difficulties: more costly manufacturing procedures, the need for expensive chemicals and dedicated solid surfaces to name some. The main reason for industry to persist in using physical adsorption is its easy processing and its acceptable product performance.

With these considerations in mind the present paper will focus on achieving improved immuno-performance of adsorbed proteins. Previously [6], we showed that, in an experimental model system that the anisotropic shape of the IgG molecules can be exploited advantageously to let them adsorb in a preferential orientation, leading to a considerably higher specific activity of adsorbed IgG. This was realised by creating a 'molecular sieve' on the sorbent surfaces by pre-

adsorbed molecules. The underlying principles and assumptions are described in the theoretical section.

To examine whether these favourable model results apply to industrially manufactured diagnostic methods we implement a triblock copolymer pre-adsorption procedure in an ELISA assay designed for the early diagnosis of pregnancy. Using radioactively labelled IgG and hCG molecules allowed us to monitor the adsorption of IgG and the subsequent specific binding of hCG.

7.2 Theoretical aspects of 'molecular sieving' and its effect on subsequent IgG adsorption

Pre-adsorbed molecules are not necessarily of the same type as the specific IgG molecules one needs for the intended immunoassay. One might think of, for example, other proteins or even synthetic polymers. In our studies we applied Synperonic a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymer, abbreviated as P75. Synperonic triblock copolymers are often used to block non-specific protein binding [7-10].

The adsorption of P75, adsorbing from water on a hydrophobic surface results in a conformation in which the more hydrophobic PPO part adsorbs on the surface with the more hydrophilic PEO parts protruding into the solution [11-13]. When the surface concentration of P75 is not too high, a rather open, monolayer may be formed that contains uncovered hydrophobic areas (fig. 7.1). These areas vary in size depending on (1) the molecular properties of the polymer used, and (2) and the surface concentration of the polymer.

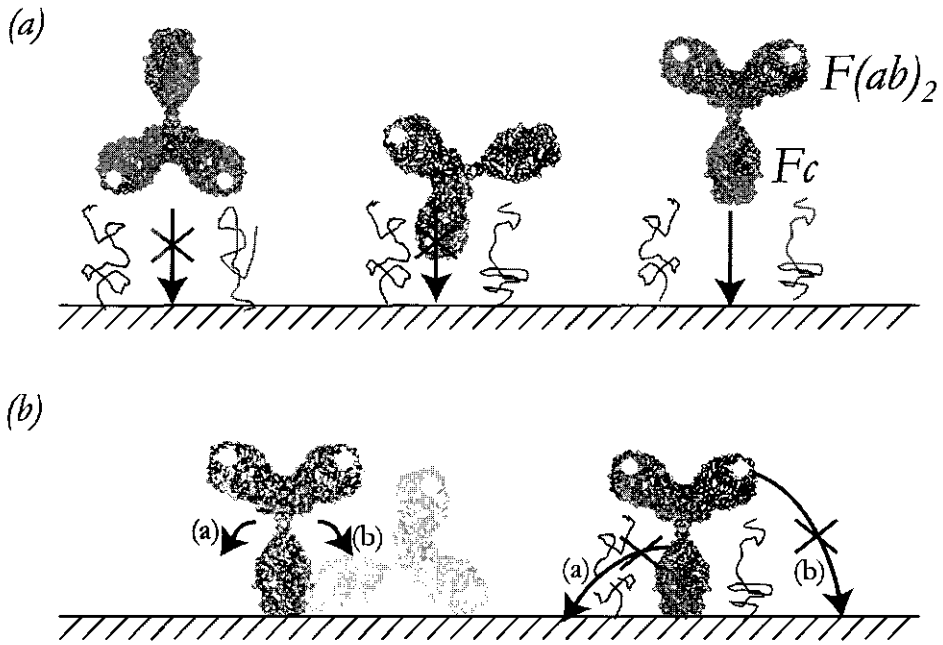


Figure 7.1: (A) Oriented adsorption of IgG on a precoated surface, leading to a biologically more active layer. (B) Prevention of (a) spreading and (b) tilting of adsorbed IgG by pre-adsorbed molecules. The antigen binding sites are represented by the white dots at the top of the F(ab) parts.

Figure 7.1 gives an impression of the role of the polymer layer on the adsorption of IgG molecules. The IgG molecules arrive at the periphery of the polymer layer at the sorbent surface in random orientations (fig. 7.1a).
The pre-adsorbed polymer layer may influence the adsorbing IgG molecules in two ways

1. Just before adsorption, the parts of the polymer that protrude into the solution form a barrier preventing IgG molecules from adsorbing at those sites. The sizes of the open areas in the polymer layer may be tailored such that only those IgG molecules that are oriented with their smaller Fc parts towards the surface can enter the polymer layer and adsorb onto the surface, thereby forcing the larger antigen binding part ($F(ab)_2$) directed outward so that the antigen binding sites remain accessible to antigens (fig. 7.1a). In other words, the pre-adsorbed polymer layer creates a 'molecular sieve' which induces size-excluded attachment of IgG at the sorbent surface. In this way, orientation of the IgG molecules is promoted resulting in an increased specific biological activity of

the adsorbed IgG molecules.

2. Following adsorption, pre-adsorbed molecules may suppress changes in the orientation and conformation of subsequently adsorbing immunoglobulins by reducing the available surface area (fig. 7.1b). Generally, IgG molecules adsorbing at a surface tend to adjust their orientation and conformation in such a way as to optimise their interaction with the surface. Given their non-globular geometry, this usually leads to a rather side-on orientation and a spread molecule and, hence, a relatively low maximum adsorption. In such an orientation the antigen binding sites, which are located at the far ends of the antigen binding domains, are poorly accessible for binding antigens. However, orientational and conformational changes can only take place when there is sufficient space and time. The PEO-parts that protrude into the solution may form a barrier that prevents this undesired tilting and spreading of the IgG molecules.

In this study we will test the validity of these processes in a microtiter plate study.

7.3 Experimental

7.3.1 Materials

Adsorbent

Flat-bottomed 96-well polystyrene microtiter plates were obtained from Organon Teknika BV (Boxtel, The Netherlands).

Chemicals

Triblock copolymer of the type PEO-PPO-PEO (Synperonic P75), hereafter denoted as P75, was a gift from ICI (Rotterdam, The Netherlands). The two PEO (poly(ethylene oxide)) parts are relatively hydrophilic and the PPO (poly(propylene oxide)) part is relatively hydrophobic. The average molecular weights of the whole polymer, its PEO, and PPO parts are 4150, 1075 (24 segments) and 2000 (34 segments) respectively.

Chemicals for buffer preparation, Na_2HPO_4 and NaH_2PO_4 (Merck) were of analytical grade and used without further purification. Milli-Q water quality was used throughout.

Proteins

Mouse monoclonal immunoglobulins G (IgG 7B and IgG 3A) from isotype 1

directed against the human pregnancy hormone (hCG, Human Chorionic Gonadotropin), highly purified hCG and normal goat serum were a gift from Organon Teknika. BSA was a commercial product (Sigma). The adsorption and immunoreaction characteristics of IgG and hCG were studied after ^{125}I-iodination of IgG 7B using Iodobeads (Pierce) and ^{35}S- sulfonation of hCG and IgG 3A using Sulphur Labelling Reagent (Amersham, Little Chalfont, England). ^{125}I-IgG 7B is used as the capture antibody and ^{35}S-IgG 3A is used as the secondary antibody. Protein concentrations were determined using a Micro BCA Protein Assay Reagent (Pierce, Oud-Beijerland, The Netherlands). P75, IgG and BSA solutions were prepared with 5 mM phosphate buffer.

7.3.2 Method

Method validation

In order to study the effect of coating procedures on adsorption and biological activity in microplate wells, we first need to develop reliable and reproducible procedures. Therefore, it is necessary to first determine (1) whether the adsorption behaviour of IgG is affected by the presence of a radio-active label in the molecule and (2) whether the sulphur and iodide signals as detected by the liquid scintillation counter overlap. To that end, the following experiments were carried out.

1. IgG from mixtures of varying labelled/unlabelled ratios is adsorbed on microtiter wells. Figure 7.2 shows that the signal and, consequently, the amount of adsorbed IgG varies linearly with the labelled fraction. This allows us to consider the adsorption behaviour of labelled and original IgG to be identical.

2. Figure 7.3 presents pulse height spectra of ^{125}I-IgG and ^{35}S-hCG, calibrated in energy units, as measured with the liquid scintillation counter. Below 50 keV the spectra overlap, and the overall signal is the sum of the iodide and the sulphur signals. Consequently, the sulphur signal and accordingly the number of hCG molecules cannot generally be determined independently. However, under certain specified conditions the shapes of the pulse height-energy spectra of radioactive atoms are independent of their concentrations. In particular, the ratio of the sulphur signal in the region of 50-160 keV to the signal in the complete region (5-160 keV) is invariant. Therefore, once this ratio is determined, the total sulphur-based signal and, hence, the number of hCG molecules can be determined accurately and independently by measuring in the 50 - 160 keV region only.

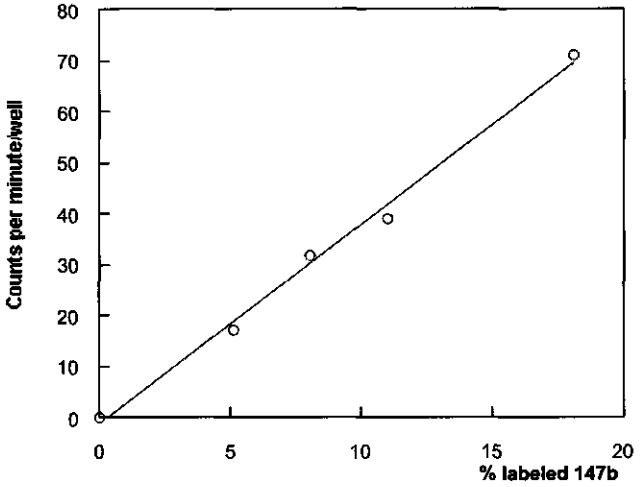


Figure 7.2: Effect of labelling IgG on its adsorption behaviour, cpm as a function of the composition of the IgG mixture.

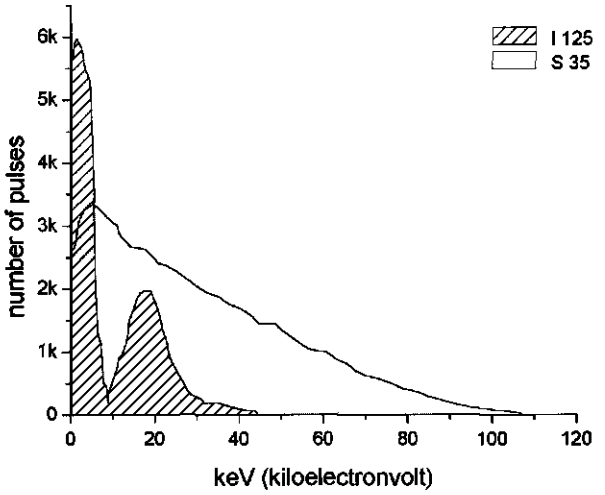


Figure 7.3: Pulse height spectra of ^{125}I-IgG and ^{35}S-hCG.

Estimation of P75 surface concentration

From our previous reflectometry experiments using model systems [6] it is known that the conformation of the adsorbed P75 is constant with time and surface concentration within the timescale of our experiments. In other words, the amount of P75 adsorbed on the walls of a microtiter well is the only accessible parameter

when relating its pre-adsorption to the functional behaviour of subsequently adsorbed IgGs. However, a direct method of measuring this adsorbed amount is not available so far.

Both from our previous reflectometry experiments using model systems and from the literature [11] it is known that the initial adsorption rate of IgG on pre-adsorbed P75 layers depends on the P75 surface concentration. At a given P75 surface concentration the reduction of the initial adsorption rate of IgG relative to that on a bare surface, is expected to be similar between microtiter wells and model surfaces. Reflectometry on model surfaces allows continuous measurement of the adsorbed amount of P75 as well as the subsequent initial IgG adsorption rate. The relative initial IgG adsorption rate as a function of the P75 surface concentration, as measured with reflectometry, is plotted in figure 7.4. Thus, by measuring the relative initial rate of adsorption of IgG in ELISA an estimation of the P75 surface concentration may be made on the basis of this figure.

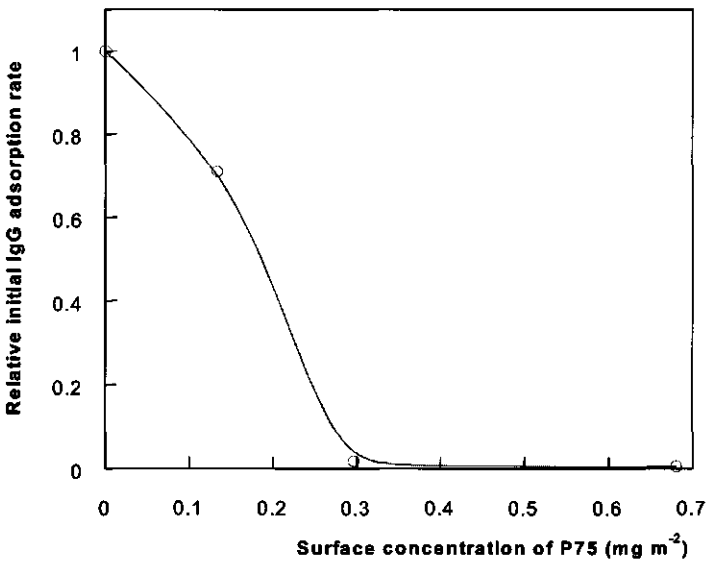


Figure 7.4: Relative initial adsorption rate of IgG as a function of the P75 surface concentration measured by reflectometry.

In ELISA the initial IgG adsorption rate can be determined by incubating a series of microtiter wells, equally covered with P75, with IgG solution. The adsorption time is varied per set of three wells and the resulting adsorbed amount is measured. From this data a plot of the amount of IgG adsorbed as a function of time can be

constructed. The initial adsorption rate is determined by taking the tangent to the $\Gamma(t)$ curve at $t=0$.

ELISA protocol

All incubations were performed under constant agitation at room temperature on a plateshaker (Sarstedt TPM2, 900 rpm), unless stated otherwise. In between the different steps of the protocol, plates were routinely rinsed four times with phosphate buffer.

Microtiter wells were incubated overnight with 200 µl P75 solution (0-340 µg ml^{-1}). The wells were subsequently incubated with 150 µl IgG solution (15 µg ml^{-1}) containing a defined mixture of labelled and unlabelled IgG. Excess free surface area on the microtiter well was blocked by a 30 minutes incubation with 150 µl BSA solution (1000 µg ml^{-1}). Subsequently 100 µl hCG solution (10300 µg ml^{-1}) was incubated for 30 minutes and afterwards the final washing procedure was performed. Each well was put in a scintillator flask containing 4 ml scintillation liquid and vigorously shaken for 45 seconds using a Vortex. Each well was counted separately in a gamma counter (Autogamma 5000, Packard Instruments, Downers Grove, Il.) for 30 minutes and in a liquid scintillation counter (LSC; TrioCarb 1900CA, Packard Instruments, Downers Grove, Il.) for 45 minutes in the range of 50 - 160 keV, using the established fact of absence of overlap between the iodide and the sulphur signals. In each counting session, untreated wells were counted to obtain an average background signal that was subtracted from all measurements.

7.4 Results

To establish specific activities of adsorbed IgG unambiguously, it is necessary to determine whether the extremely high hCG concentration used in this study leads to unacceptable non-specific adsorption. Considerable non-specific adsorption of hCG and secondary antibody will result in a substantial overestimation of the activity of adsorbed IgG and undesirably high background signals in ELISA, respectively.

7.4.1 Non-specific adsorption

Non-specific binding to the microtiter surface and other coated reactants of hCG and secondary antibody (in these experiments radio-actively labelled IgG 3A) is studied under various conditions. To discriminate between specific hCG binding and non-specific hCG or secondary antibody binding, bare as well as non-

specifically and specifically coated microplates were used in parallel. Non-specific binding was calculated from binding of hCG and IgG 3A to bare microtiter wells (polystyrene) and to microtiter wells precoated with P75 or BSA, respectively. Specific binding of hCG was measured using a IgG 7B-coated microplate. For both sets of plates, three different incubation media were used, i.e. phosphate buffer, phosphate buffer containing BSA (1000 μg ml^{-1}), and normal goat serum (figure 7.5 and figure 7.7).

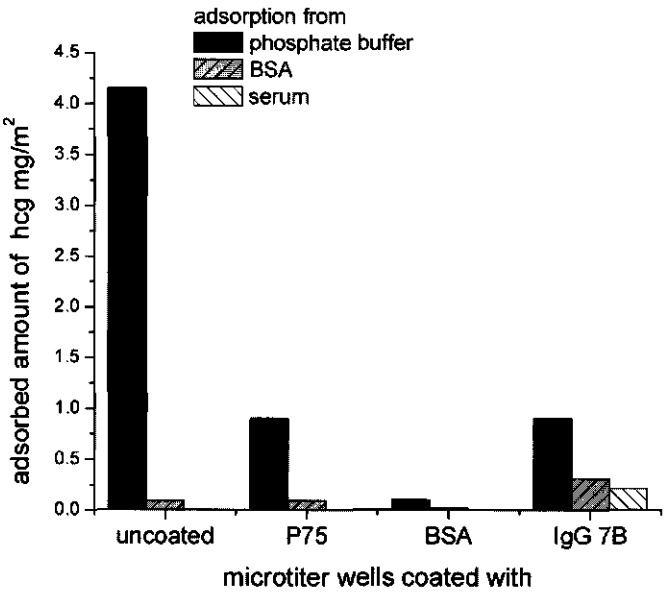


Figure 7.5: Non-specific and specific adsorption of hCG from different media on: bare wells, wells precoated with P75, BSA and IgG 7B.

Figure 7.5 shows the contributions of the various binding possibilities for hCG. In protein-free medium hCG binds in large amounts to the bare polystyrene surface. This amount of (non-specifically) bound hCG is reduced considerably when wells are precoated with P75 or -even better- BSA. Apparently, BSA is a reasonably good blocking agent. Regardless of the pretreatment of the plate, additional use of BSA in the incubation medium reduces non-specific binding even further. In this medium, the amount of specifically bound hCG exceeds the 'noise' of non-specifically bound hCG. Using full serum as the incubation medium, with its large variety of proteins, reduces non-specific binding on each of the investigated plates down to a level that can hardly be discriminated from the background. The ratio of

specifically to non-specifically bound hCG (figure 7.6) i.e., the signal to noise ratio, convincingly shows the improvement that can be achieved by proper selection of the incubation conditions.

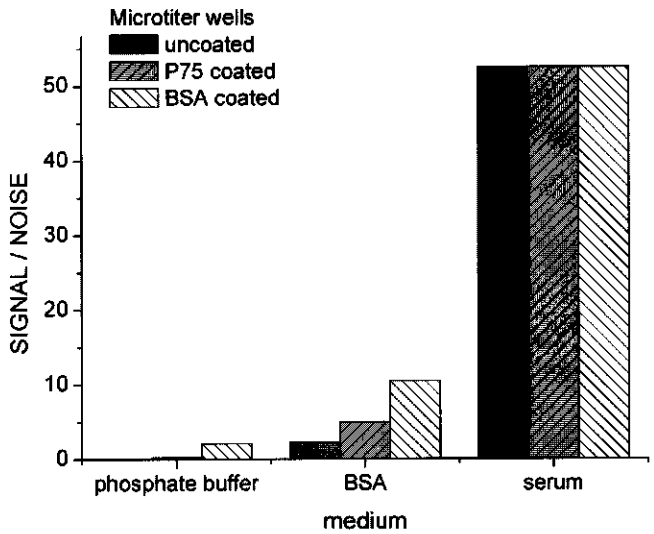


Figure 7.6: Signal to noise ratio for hCG binding from different media.

Considering the binding of secondary antibody IgG 3A, similar experiments were performed. As could be expected, in phosphate buffer this protein binds in large amounts to bare polystyrene. More surprisingly, it binds in even larger amounts to wells precoated with IgG 7B (figure 7.7). This indicates that our coated IgG 7B and our secondary antibodies 3A strongly interact. Similar to our hCG data, non-specific binding is reduced considerably in a BSA-rich medium, and even further in goat serum. Yet, the non-specific binding of IgG 3A to the coated IgG could not entirely be prevented, not even in normal goat serum. This will result in a higher background signal. Clearly, lower background signals could be achieved when the amount of IgG in the coat can be reduced.

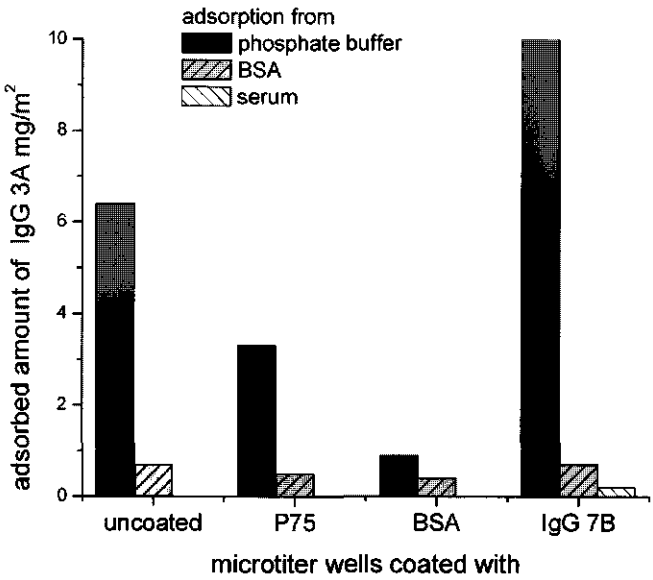


Figure 7.7: Non-specific adsorption of IgG 3A from different media onto: bare wells, wells precoated with P75, BSA and IgG 7B.

These experiments provide strong evidence that the selection of the incubation medium and of the plate pretreatment are of utmost importance to reduce non-specific interactions. Therefore, in further experiments hCG binding was determined in a medium of normal goat serum, using BSA as the blocking agent after IgG 7B coating.

Having in this way optimised our assay and analysis protocols, the number of biologically active antigen binding sites per well can be determined with confidence since the affinity constant for the binding of hCG to the physically adsorbed IgG 7B is extremely large (about $2\ 10^9$ l mol^{-1}) [14], and the hCG concentration used is in large excess of the available antigen binding sites offered by the adsorbed IgG. Finally, kinetic studies (not shown) indicated that hCG binding does indeed reach equilibrium within the applied incubation time.

7.4.2 IgG adsorption

The adsorption of IgG 7B is studied on both bare and P75 precoated microtiter wells. The incubation method and the IgG concentration are varied.

Experiments in which the bare microtiter plate is continuously agitated during adsorption not only show a higher initial adsorption rate; they also display a higher maximum adsorbed amount (fig. 7.8). The maximum amount of IgG adsorbed clearly depends on the rate of supply; for instance, after 24 hours of static incubation the adsorbed amount is lower than after 1 hour of permanent homogenisation.

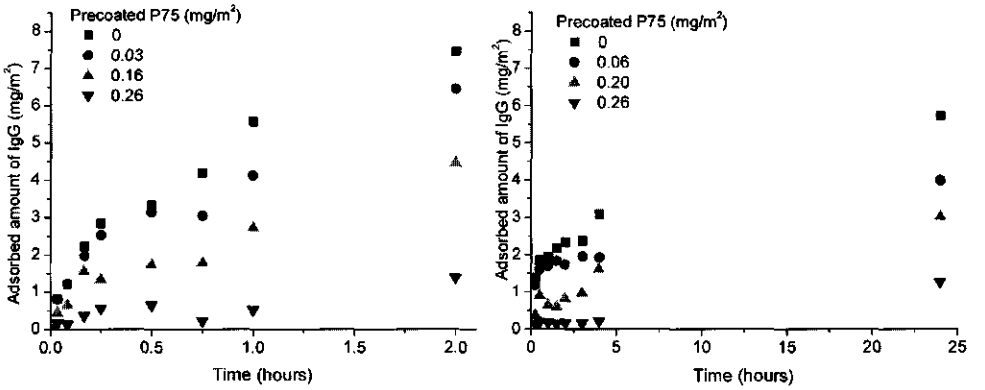


Figure 7.8: IgG adsorption on microtiter wells precoated with P75. IgG incubation (left) under constant agitation and (right) statically.

This striking effect of agitation shows that convection prevails over diffusion-controlled adsorption [15]. The difference in plateau value means that in the adsorbed state the (non-globular) IgG molecules occupy a larger surface area with increasing adsorption time. Most probably this is caused by relaxation (reorientation and reconformation) of the molecules. Indeed, from CD measurements, we concluded that structural changes in IgG on a hydrophobic surface progress further when the amount of IgG adsorbed is lower [16].
Reorientation and reconformation can only take place when there is sufficient space and time available [17-19]. From the observed trends it can be inferred that the rate of these relaxation processes must be comparable to the rate of deposition at the sorbent surface. Beyond some critically high supply rate, adsorbed molecules no longer have time for relaxation before adjacent surface is covered by additional molecules. Additional support for this mechanism can be gained from varying the protein concentration in the incubation medium, because increasing concentration implies higher fluxes. Figure 7.9 shows the binding curves of a large series of IgG concentrations.

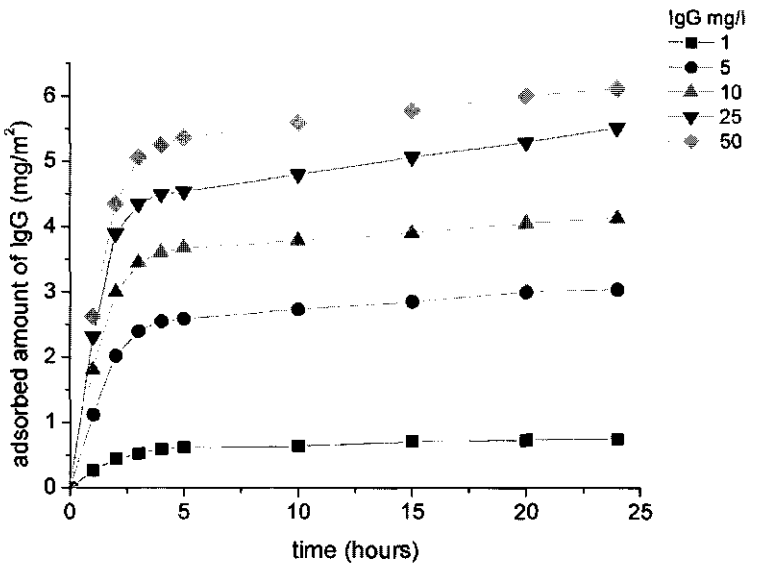


Figure 7.9: IgG adsorption for several IgG concentrations.

Here too, adsorption from a solution of lower IgG concentration results in a lower maximum adsorption. Evidence for the existence of the above-mentioned critically high supply rate is not yet contained in fig. 7.9, to that end additional experiments at even higher IgG concentrations may have to be performed.

From these experiments we can conclude that in ELISA, as in the experimental model system [6], the orientation and conformation of adsorbed IgG can be influenced by previously adsorbed molecules, in this case IgG molecules.

The effect of other pre-adsorbed molecules, in our case triblock copolymer P75, on subsequently adsorbing IgG molecules will be discussed below. From reflectometry experiments with model systems [6] it is known that the significant characteristic is the surface concentration of P75. The estimated P75 surface concentrations in our experiments are in the range of zero to 0.27 mg m^{-2}, details are given in the captions of fig. 7.8. Surface concentrations compare to 0 to 34 % of saturation adsorption of P75.

Similar to the protein adsorption findings on a bare plate, agitation has its effect on the initial binding rate and the adsorbed mass. At increasing amounts of pre-adsorbed 75, the initial adsorption rate of IgG decreases (figure 7.8), as does the maximum amount of protein that is bound. At high surface concentrations by P75 no differences in the maximum adsorbed amount are found any more: apparently the IgG can no longer relax on a sufficiently dense precoated surface.
In general, the binding probability of an IgG molecule on a freely accessible bare part of a hydrophobic surface approaches unity [6]. Therefore, the observed reduction in initial adsorption rate (fig. 7.8) indicates that the binding probability reduces because a smaller fraction of the incoming IgG molecules can interact with the sorbent surface due to the repulsion by the polymer.

7.4.3 The specific activity and binding capacity of adsorbed IgG

The dependence of the initial adsorption rates and the plateau values on the polymer surface concentration indicates that the orientation and/or the conformation of the later adsorbing IgG molecules may be influenced. Whether a biologically more active IgG layer is created by the presence of the pre-adsorbed polymer can be investigated by measuring the specific activity of IgG in the adsorbed layer.

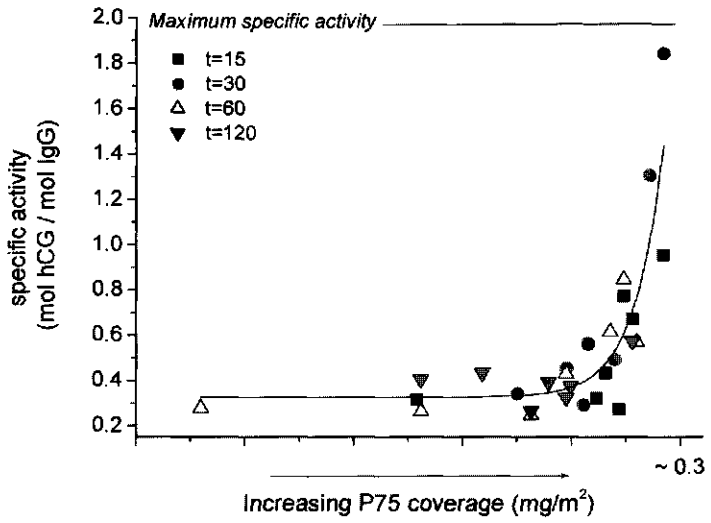


Figure 7.10: Specific activity of IgG as a function of P75 surface concentration, measured after 15, 30, 60 and 120 minutes of IgG incubation.

We observed that the amount of IgG adsorbed depends on the estimated P75 surface concentration. Based on these reliable estimates figures 7.10 and 7.11 represent the trends in the effect that surface concentration of P75 has on the specific activity of the adsorbed IgG molecules for its antigen hCG. IgG adsorption times were varied between 15, 30, 60 and 120 minutes.

Once adsorbed on bare microtiter wells, or at very low P75 surface concentration, the specific activity of IgG is approximately 0.3 mole/mole at all investigated incubation times (fig. 7.10). In solution, each molecule of IgG possesses two antigen binding sites. In other words, after adsorption on practically empty surfaces only 15% of all antigen binding sites are capable of binding antigens. Loss of internal structure of the IgG and/or a reduced geometric accessibility of binding sites due to an adverse orientation on the surface may explain part of this low activity [16,20]. Similar losses in functionality have been reported by many others, both for IgG [3] and for other proteins [21]. The specific activity of IgG on bare microtiter wells is -on the time scales of our experiments- independent of the coating time.

Fortunately, at high P75 surface concentration the specific activity of adsorbed IgG molecules strongly increases with this concentration regardless of the incubation time of the IgG. At these high surface concentrations specific activities as high as 1.8 mole/mole can be achieved, i.e. over 90% of all antigen binding sites are active. When IgG is forced to adsorb in between pre-adsorbed polymer molecules, the specific activity increases by well over a factor of five. These data demonstrate that, in full agreement with our earlier model studies on this subject [6], a more favourably oriented IgG layer is created and that the sieving of the IgG by the polymer, as described in the introduction, not only takes place in our model experiments but also occurs in ELISA experiments! Within the given experimental accuracy no differences in the specific activity can be distinguished between differing IgG incubation times. This trend is in line with our model studies.

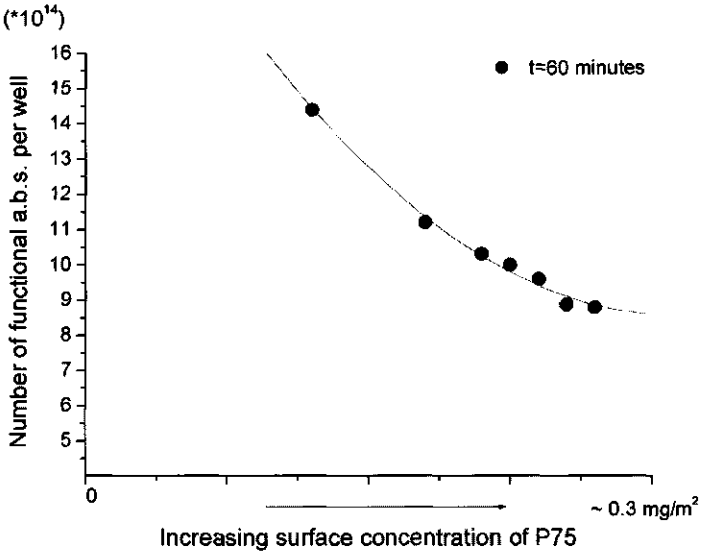


Figure 7.11: Total number of functional antigen binding sites per well as a function of P75 surface coverage.

In practical immunoassay applications, the capture rate of the analyte is determined by the binding capacity of the solid phase. This binding capacity is determined by both the amount of adsorbed IgG and its average biological activity. Even when all IgG molecules are oriented in a perfect way the number of antigen binding sites may be low because of the small amount of adsorbed IgG. A certain minimum number of antigen binding sites are needed to make a test successful. The number of functional antigen binding sites- the binding capacity- per well as a function of the actual IgG surface concentration is plotted in fig. 7.11. The curves for the different incubation times show the same trends and therefore we give, by way of example, only the curve for 60 minutes. Given the applied pre-treatment of the microplate it is possible to practically maintain the number of functional antigen binding sites rather high despite a drastic loss in adsorbed mass. Even at high P75 precoating and, therefore, low IgG adsorption the number of active antigen binding sites per well definitely exceeds the amount of antigen normally present in a test sample, and hence we still fulfill the practical demand for rapid and effective assay performance. This finding opens unique options for reduction of non-specific binding due to cross reactivity and for better process control.

7.5 Conclusions

In a microplate format, as in model systems, adsorption of IgG is strongly

influenced by the presence of previously coated molecules. Pre-adsorbed molecules form a 'molecular sieve' on the sorbent surface, which induces oriented adsorption of IgG yielding a higher specific biological activity of the IgG molecules. The underlying mechanism may be generic in origin and effective to orient a variety of other non-spherical molecules or particles on a solid surface.

Advantages of this new degree of freedom in adsorption-based assay development can be the prospect of using highly specific antibodies which normally lose their bio-activity upon adsorption, the possibility of tailoring non-specific interactions, the application in multi-analyte assays and using smaller amounts of expensive biomaterials.

Our studies indicate that control of the amount of precoated material may be critical, and further work in this area holds promises.

7.6 References

1. Engvall, E. and Perlmann, P. (1971) *Immunochemistry 8*, 871.
2. Butler, J.E. Enzyme-Linked Immunosorbent Assay.
3. Butler, J.E., Ni, L., Nessler, R., Joshi, K.S., Suter, M., Rosenberg, B., Chang, J., Brown, W.R. and Cantarero, L.A. (1992) *J. Immunol. Methods 150*, 77.
4. Molina-Bolivar, J.A., Galisteo-Gonzalez, F. and Hidalgo-Alvarez, R. (1998) *J. Biomater. Sci. Polymer Edn. Vol 9*, No.10, 1089.
5. 5 Murata, M., Arakawa, M., Yoshida, T., and Hato, M. (1998) *Colloids Surf. B 12*, 35.
6. Chapter 3, this thesis.
7. Lee, J.H., Kopecek, J., and Andrade, J. (1989) *J. Biomed. Mater. Res. 23*, 351.
8. Amiji, M., and Park, K. (1992) *Biomaterials 13*, 682.
9. Schroën, C.G.P.H., Cohen Stuart, M.A., van der Voort Maarschalk, K., van der Padt, A., and van 't Riet, K. (1995) *Langmuir 11*, 3068.
10. Freij-Larsson, C., Nylander, T., and Wesslen, B. (1996) *Biomaterials 17*, 2199.
11. Lee, J., Martic, P.A., and Tan, J.S. (1989) *J. Colloid Interf. Sci. 131*, 252.
12. Tiberg, F., Malmsten, M., Linse, P., and Lindman, B. (1991) *Langmuir 7*, 2723.
13. O'Connor, S.M., Gehrke, S.H., and Retzinger, G.S. (1999) *Langmuir 15*, 2580.
14. Van Erp, R. Ph.D. Thesis, Utrecht University 1991.
15. Beumer, T., Stoffelen, E., Smits, J., and Carpay, W. (1992) *J. Immunol. Methods 154*, 77.
16. Chapter 6, this thesis.
17. Maste, M.C.L., Norde, W., and Visser, A.J.W.G. (1997) *J. Colloid Interf. Sci. 196*, 224.
18. Wertz, C.F., and Santore, M.M. (1999) *Langmuir 15*, 8884.
19. Kondo, A., Murakami, F., Kawagoe, M., and Higashitani, K. (1993) *Appl. Microbiol. Biotechnol. 39*, 726.
20. Vermeer, A.W.P., Bremer, M.G.E.G., and Norde, W. (1998) *Biochim. Biophys. Acta 1425*, 1.
21. Zoungrana, T., Findenegg, G. H., and Norde, W. (1997) *J. Colloid Interf. Sci. 190*, 437.

SUMMARY

Preservation of biological functioning of proteins during immobilisation is of special interest in various biomedical and biotechnical applications. In industry physical adsorption of immunoglobulins (IgGs) onto solid surfaces is still the predominant immobilisation procedure because it is relatively easy to perform. Physical adsorption, however, often results in an undesired loss of biological activity. This loss of activity may be caused by changes in the specific folding (the conformation) of the IgG or by a reduced accessibility of the antigen binding sites by blocking, for instance when IgG molecules are adsorbed with their antigen binding sites oriented towards the sorbent surface.

To control the orientation and conformation of adsorbed IgG molecules we studied the interactions involved in physical adsorption of IgG molecules on solid surfaces. Our goal was to optimize the biological activity of adsorbing IgG molecules. For this, we introduced a new method to achieve oriented physical adsorption of IgG. This concept is based on the anisodimensionality of IgG molecules and resembles 'molecular sieving' on the sorbent surface. The 'sieve' is created by preadsorbed molecules that form a steric barrier preventing adsorption at some sites, but leaving patches of uncovered surface area. The open areas in the preadsorbed layer can be tuned in such a way that only the smaller part of anisodimensional molecules can enter and adsorb. In the case of IgG this means that only the Fc part can adsorb to the surface and thereby forcing the larger antigen binding parts ($F(ab)_2$) directed towards the solution and, hence, accessible to bind antigens. A 'sieve' formed either with preadsorbed IgG molecules or with triblock copolymers of poly(ethylene oxide), PEO, and poly(propylene oxide), PPO, of the type PEO-PPO-PEO was proven to yield a higher specific biological activity of subsequently adsorbing IgG.

In brief, the effect of a 'molecular sieve' on IgG adsorption is essentially threefold. Firstly, *it induces oriented* IgG adsorption. *Secondly, it prevents extended* undesirable structural changes in adsorbed IgG and thirdly, it prevents undesirable reorientation of the adsorbed IgG.

In chapter 1 it is explained that in many applications there is a need to control the biological activity of adsorbed IgG. The physical properties and characteristics of

IgG molecules and the interactions involved in physical adsorption of proteins are described and, more importantly, our variant of 'molecular sieving' is introduced. Finally, an outline of this thesis is given.
The influence of electrostatic interactions on the adsorption of IgG is examined both theoretically and experimentally in chapter 2. The long range interaction between IgG and the sorbent surface is treated in terms of the DLVO theory. We attempted to make use of the dipolar character of the IgG molecules to control their orientation upon adsorption. It is concluded that electrostatic interactions have a strong influence on the adsorption behaviour of IgG molecules on hydrophilic charged surfaces. Due to extensive desorption of IgG from both positively and negatively charged surfaces, electric field-induced orientation of IgG could not be established unambiguously.

Chapter 3 is mainly dedicated to the orientational aspects of IgG adsorption. In this chapter the phenomenon of 'molecular sieving' is demonstrated first theoretically using a Random Sequential Adsorption (RSA) model and second experimentally by a set of reflectometry experiments on surfaces partially covered with preadsorbed layers of either IgG or triblock copolymers of PEO-PPO-PEO. The rate of IgG adsorption and the maximum adsorbed amount decreases with increasing adsorbed amount of triblock copolymer. On the precoated layers, IgG is indeed adsorbed in a preferential orientation which yielded a higher specific biological activity of the IgG molecules. Furthermore, we observed that the preadsorbed layers prevent undesirable reorientation of adsorbed IgG.
The mass flux towards the surface also has a profound effect on the adsorbed amount and, consequently, on the orientation of IgG.

In chapters 4, 5 and 6 conformational changes in IgG are studied. In chapter 4 a structural analysis of a monoclonal IgG adsorbed on different silica surfaces (hydrophilic, hydrophobic, hydrophobic with preadsorbed triblock copolymers) using ATR-FTIR spectroscopy is given. The secondary structure of adsorbed IgG on a hydrophilic silica surface resembles that of native IgG in solution. The presence of preadsorbed triblock copolymers on the hydrophobic silica surface cause a decrease in the adsorbed amount of IgG and, more importantly prevent substantial structural rearrangements in the adsorbed IgG molecules.

In chapters 5 and 6, Circular Dichroism (CD) is used as a spectroscopic technique for studying protein structure in the adsorbed state. Chapter 5 gives information on the structural changes of IgG molecules induced by adsorption on a hydrophobic

surface and compares these changes with those induced by heat treatment. Neither heat-induced nor adsorption-induced structural changes lead to complete unfolding into an extended polypeptide chain, but leave a significant part of the IgG molecule in a globular or corpuscular form. The structural changes induced by heating and by adsorption are different.

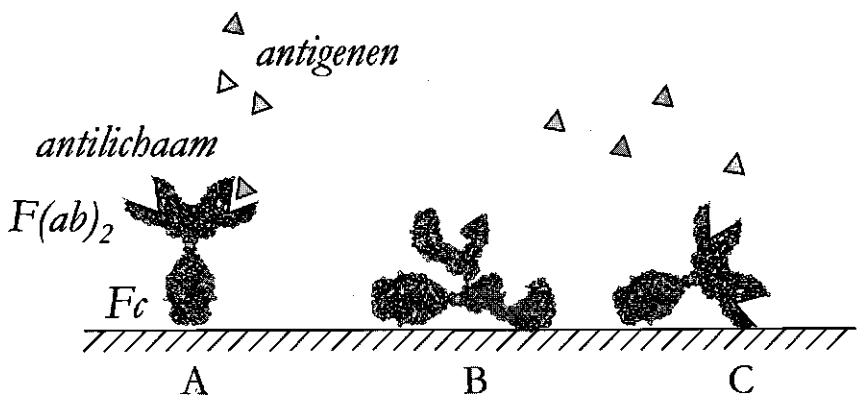
The effect of preadsorbed IgG and triblock copolymer molecules on the secondary structure of subsequently adsorbing IgG molecules is studied in chapter 6. Structural rearrangements were less extensive with increasing surface coverage of the polymer. It was found that preadsorbed IgG molecules have comparable effects on the secondary structure of subsequent adsorbing IgG; a more native-like structure is retained for the higher adsorbed amounts. Hence, partial pre-coating of a surface is an effective way to control the secondary structure of later adsorbed IgG molecules.

To examine whether the model results apply to industrially manufactured diagnostic methods we implemented in chapter 7 the triblock copolymer pre-adsorption procedure in a microplate assay. Radio-actively labelled IgG and hCG molecules allowed us to monitor the adsorption of IgG and the subsequent specific binding of hCG. The data obtained are in agreement with our earlier model studies and demonstrate that sieving of the IgG by the polymer does take place, resulting in the creation of a more favourably oriented IgG layer. Our studies indicate that the amount of precoated material is critical in the formation of an operational 'sieve'.

SAMENVATTING

Antilichamen, ook wel immunoglobulinen genaamd, zijn eiwitten die een belangrijke rol spelen bij de bescherming van mens en dier tegen ziekteverwekkers zoals virussen en bacteriën. Antilichamen worden gemaakt door speciale bloedcellen wanneer een lichaamsvreemde stof, antigeen genaamd, binnen dringt. Antilichamen maken binnengedrongen antigeenmoleculen onschadelijk door zich eraan te binden. Elk antilichaam heeft een moleculaire structuur die precies 'past' op de structuur van een speciaal antigeenmolecuul, zoals een sleutel op een slot. Hierdoor kunnen antilichamen het betreffende antigeen temidden van miljoenen andere stoffen herkennen en binden.

Antilichamen bestaan uit vier ketens. Deze ketens zijn zodanig gevouwen dat een groot Y-vormig molecuul ontstaat (figuur 1). Zo'n molecuul bestaat uit 3 delen: twee identieke delen (F(ab)) die elk een bindingsplaats voor het antigeen bevatten en een deel (Fc) dat zorgt voor de hechting van het antilichaam aan lichaamscellen. De twee F(ab) delen zijn via een scharnierachtige koppeling met elkaar verbonden en vormen zo een groter geheel ($F(ab)_2$).



Figuur 1: Het effect van hechting op de antigeenbindingsactiviteit van antilichamen. Optimale oriëntatie en structuur, en dus maximale activiteit (A). Verlies van activiteit door structuurveranderingen (B) of een ongunstige oriëntatie op het oppervlak (C).

In medische testen wordt veel gebruik gemaakt van de eigenschap van antilichamen om heel specifiek bepaalde biologische moleculen te binden. Zo worden antilichamen bijvoorbeeld gebruikt om ziekteverwekkers of hormonen in bloed of urine aan te tonen. In deze testen worden antilichamen vaak gehecht aan vaste oppervlakken. Dit vergemakkelijkt het aantonen en scheiden van antilichamen die antigenen hebben gebonden. Echter, door de hechting op een oppervlak verliezen de antilichamen ten dele hun vermogen om antigenen te binden (figuur 1). Voor dit verlies aan bindingsactiviteit kunnen twee belangrijke oorzaken gegeven worden. Ten eerste, de specifieke vouwing van de ketens kan veranderen waardoor de structuur van het antilichaam niet meer 'past' op het antigeen en het antilichaam het antigeen niet meer herkent (figuur 1B). Ten tweede, intacte bindingsplaatsen kunnen geblokkeerd en dus onbereikbaar zijn voor antigenen, bijvoorbeeld wanneer ze zich in de buurt van het oppervlak bevinden (figuur 1C). Voor een optimale bindingsactiviteit op een oppervlak moet dus zowel de oriëntatie als de structuur van antilichamen gunstig zijn. Er is veel onderzoek uitgevoerd om hechtingsmethoden te ontwikkelen waarbij aan bovenstaande voorwaarden wordt voldaan. Hoewel deze nieuwe methoden soms hebben geleid tot een redelijk behoud van antigeenbindingscapaciteit, kleefden er ook vaak duidelijke nadelen aan zoals een moeilijke productieprocedure of de noodzaak kostbare chemicaliën of speciale oppervlakken te gebruiken.

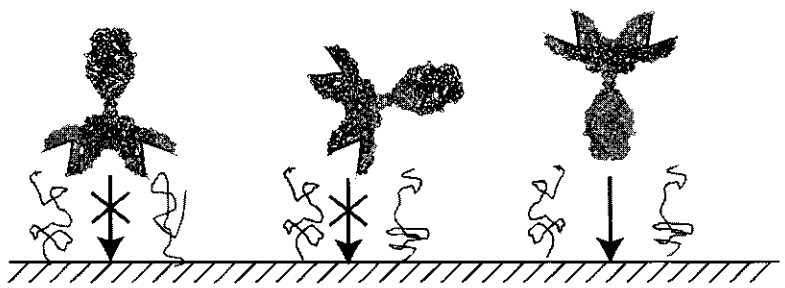
Inzicht in de wisselwerkingen tussen antilichamen en vaste oppervlakken is van groot belang voor de ontwikkeling van nieuwe hechtingsmethoden. Het in dit proefschrift beschreven onderzoek is uitgevoerd om inzicht in deze wisselwerkingen te verkrijgen. Dit inzicht wordt gebruikt om op een eenvoudige manier de oriëntatie en structuur van antilichamen op een oppervlak te sturen en hiermee de antigeenbindingsactiviteit van de antilichamen te optimaliseren.

Eerst is in modelsystemen met verschillende technieken de effecten van lading (pH en zoutsterkte), het type oppervlak en de verblijftijd op het oppervlak op de structuur en oriëntatie van de antilichamen bestudeerd. Op basis van deze gegevens is besloten om voor de gewenste oriëntatie op een oppervlak gebruik te maken van de specifieke Y-vorm van de antilichamen. Vervolgens is een nieuwe hechtingsmethode ontwikkeld.

'Moleculair zeven'

Principe

De nieuwe hechtingsmethode (figuur 2) is gebaseerd op de werking van een zeef: kleine deeltjes kunnen wel door de zeefgaten maar grote deeltjes niet. Iets vergelijkbaars geldt ook voor hechtende eiwitten op een oppervlak: een eiwit kan alleen op een oppervlak hechten als er voldoende vrije ruimte is. Omdat het antigeen bindende deel ($F(ab)_2$) van een antilichaam groter is dan het Fc deel zal het Fc deel makkelijker in kleinere ruimten passen. Door op het oppervlak een 'moleculaire zeef' te maken met gaten waarin alleen het Fc deel past wordt bereikt dat de antilichamen alleen met dit deel kunnen hechten.. De grotere antigeenbindende delen steken op deze manier de oplossing in en blijven dus bereikbaar voor antigenen. Het resultaat is een gehechte laag van antilichamen die een gunstige oriëntatie hebben voor het binden van antigenen.



Figuur 2: Principe van een 'moleculaire zeef'.

Een mogelijk bijkomend voordeel van zo'n 'moleculaire zeef' is dat gehechte antilichamen minder structuur verliezen omdat de vrije ruimte op het oppervlak beperkt wordt. Structuuraanpassingen van gehechte eiwitten houdt in het algemeen spreiding over het oppervlak in en dat kan alleen als er voldoende vrije ruimte is.
Een 'moleculaire zeef' kan als volgt gemaakt worden: eerst worden op het oppervlak moleculen gehecht die delen van het oppervlak afschermen. Hierdoor worden die delen ontoegankelijk voor de hechting van later aangeboden antilichamen. De situatie moet zo gekozen worden dat in de ruimten tussen de voorgehechte moleculen antilichamen nog net met hun Fc deel kunnen adsorberen. Deze ruimte kan gevarieerd worden door meer of minder moleculen voor te hechten.

Resultaten

In modelsystemen is met verschillende technieken het hechtingsgedrag van antilichamen bestudeerd. Met behulp van reflectometrie wordt de gehechte massa van moleculen in de tijd gemeten. De antigeenbindingscapaciteit van een laag gehechte antilichamen wordt bepaald door een oplossing met antigenen aan de laag aan te bieden. De gebonden massa aan antigeen kan omgerekend worden naar het aantal antigeenmoleculen gebonden per antilichaam. De bindingscapaciteit weerspiegelt de oriëntatie van een antilichaam op het oppervlak. Infrarood-spectroscopie en circulair dichroisme spectroscopie zijn optische technieken die we hebben gebruikt om de vouwing/structuur van antilichamen te bepalen. Met deze technieken kunnen de veranderingen in de structuur door hechting op een oppervlak gevolgd worden.

Het effect van lading op de hechting van antilichamen is onderzocht op twee hydrofiele ('waterminnende') oppervlakken met verschillend ladingsteken. Uit de experimenten blijkt dat lading weliswaar een belangrijke rol speelt in het hechtingsgedrag van antilichamen, maar dat elektrostatische wisselwerkingen tussen antilichamen en dit type oppervlak niet zo sterk zijn dat een permanente hechting van de antilichamen wordt verkregen. Deze oppervlakken zijn dan ook niet geschikt om te gebruiken als dragermateriaal in testsystemen.

In het vervolg van het onderzoek is gebruik gemaakt van hydrofobe ('waterafstotende') oppervlakken omdat eiwitten zich wel sterk hechten aan dit type oppervlak. Op dit oppervlak is een 'moleculaire zeef' gemaakt met zogeheten triblok-copolymeren. Dit zijn moleculen die bestaan uit een lange keten waarvan het middendeel op het oppervlak hecht en de twee uiteinden de oplossing in steken. Zo vormen ze een soort spijkerbed op het oppervlak. De antilichamen moeten dan tussen de spijkers hechten. Met de verschillende genoemde technieken is gekeken naar de oriëntatie en de structuur van antilichamen op hydrofobe oppervlakken. Dit is gebeurd op een leeg oppervlak en op een oppervlak waarop zo'n 'moleculaire zeef' is aangebracht. Zo kon het effect van de zeef bepaald worden.

Uit de resultaten is gebleken dat op een leeg hydrofoob oppervlak antilichamen zich in allerlei oriëntaties hechten. De antilichamen die eerst eindstandig georiënteerd waren vielen na verloop van tijd om waardoor ze met hun zijkant op het oppervlak kwamen te liggen. Hierdoor waren de bindingsplaatsen minder goed bereikbaar. Ook ging door hechting op het oppervlak een deel van de structuur van

de antilichamen verloren. Door de ongunstige oriëntatie en de veranderingen in de structuur was de antigeenbinding van de gehechte antilichamen verre van optimaal.

Hierna is het hechtingsgedrag van de triblok-copolymeren bestudeerd. De polymeren hechten snel en sterk aan het hydrofobe oppervlak. Wanneer antilichamen op de 'moleculaire zeef' (de voorgehechte laag polymeren) gehecht zijn is hun antigeenbindingscapaciteit groter. De fractie gunstig georiënteerde antilichamen neemt toe wanneer zich op het oppervlak meer voorgehechte moleculen bevonden, dus wanneer de ruimten tussen die moleculen kleiner is. De polymeermoleculen voorkomen bovendien het ongunstige omvallen van antilichamen. Het totale aantal gunstig georiënteerde antilichamen neemt echter af met de bezettingsgraad van de voorgehechte moleculen. Dit komt doordat er minder hechtingsplaatsen beschikbaar zijn; het oppervlak is bijna vol. Bij hoge concentraties aan voorgehechte polymeren wordt zelfs een antilichaam afstotend oppervlak gevormd. De antilichamen die gehecht zijn op de 'zeef' behouden voor een groot deel hun originele structuur. Dit gunstige effect neemt toe naarmate er meer voorgehechte moleculen aanwezig waren.

De 'moleculaire zeef' is ook toegepast in een industrieel vervaardigbare test. Om de hechting en bindingscapaciteit van de antilichamen in dit systeem te kunnen volgen zijn de antilichamen en het antigeen radioactief gemerkt (gelabeld). Deze labeling heeft geen effect op het hechtingsgedrag en de bindingsactiviteit van de antilichamen. De effecten zijn kwalitatief in overeenstemming met de resultaten in de modelsystemen. De antilichamen gehecht op de 'moleculaire zeef' hebben een veel hogere bindingscapaciteit dan antilichamen gehecht op een leeg oppervlak. Voordelen van deze nieuwe adsorptiemethode zijn onder andere dat het hechtingsproces beter te sturen is en dat kleinere hoeveelheden kostbaar antilichaam gebruikt hoeven te worden om toch een goed werkende test te maken. Dit biedt weer de mogelijkheid om in een en hetzelfde testsysteem antilichamen tegen verschillende antigenen te gebruiken.

Uit het onderzoek beschreven in dit proefschrift weten we nu hoe we de oriëntatie en structuur, en daarmee de antigeenbindingsactiviteit, van antilichamen op een oppervlak kunnen manipuleren. Het is gebleken dat dit mogelijk is door een soort 'moleculaire zeef' op het oppervlak aan te brengen door eerst polymeermoleculen op het oppervlak te hechten. Het voordeel van deze methode is dat het eenvoudig en op grote schaal toe te passen is in bestaande industriële testsystemen.

CURRICULUM VITAE

Maria Gabriëlle Eleonore Gerarda Bremer werd geboren op 15 augustus 1971 te Randwijk. In 1989 behaalde zij het VWO-diploma aan het 'Heldring College' te Zetten. Daarna begon zij aan de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit Wageningen. Tijdens de doctoraalfase volgde zij de fysisch-chemische oriëntatie met de afstudeervakken *Kolloïd- en Grensvlakchemie* en *Natuurkunde.* Een stage van 6 maanden werd afgelegd bij het Ian Wark Research Institute van de University of South Australia te Adelaide. In september 1995 studeerde zij af en begon in november van dat jaar aan een promotieonderzoek bij de vakgroep Fysische- en Kolloïdchemie van de Landbouwuniversiteit. Het onderzoek werd uitgevoerd in samenwerking met Organon Teknika BV te Boxtel, en heeft geleid tot dit proefschrift. Vanaf 1 april 2001 is zij werkzaam als wetenschappelijk onderzoeker bij de afdeling Radiochemie van het Interfacultair Reactor Instituut van de Technische Universiteit Delft.

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Monique