COMPETITIVE ADSORPTION OF ALBUMIN AND MONOCLONAL IMMUNO γ-GLOBULIN MOLECULES ON POLYSTYRENE SURFACES

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
op vrijdag 18 mei 1990
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

Someday we'll look back on this
and it will all seem funny.

"Rosalita (come out tonight)", ©1974 Bruce Springsteen.

STELLINGEN

I

Maximale eiwitadsorptie vindt plaats rond het isoelektrisch punt van het eiwit-substraat complex.

Dit proefschrift, hoofdstuk 3

II

Vanuit een mengsel van eiwitten wordt de preferente adsorptie in de aanvangsfase bepaald door elektrostatische wisselwerkingen tussen eiwit en substraat.

Dit proefschrift, hoofdstuk 5

III

De beginsnelheid van eiwitadsorptie is niet voor elk eiwit bepalend voor de eindsituatie op het oppervlak.

L. van de Steeg, Doctoraalverslag, LUW (1989).

C. van Delden, Doctoraalverslag, LUW (1990).

Dit proefschrift, hoofdstuk 6

IV

Het door Feng *et al.* voorgestelde reaktiemechanisme van de graft copolymerisatie van acrylamide op polyetherurethanen met het Ce(IV)-ion als initiator, houdt onvoldoende rekening met homopolymerisatie van het acrylamide.

Feng, X.D., Sun, Y.H. and Qiu, K.Y., *Macromolecules* **18**, 2105 (1985).

V

In tegenstelling tot de geadsorbeerde hoeveelheid is, onder verzadigingsomstandigheden, de hydrodynamische laagdikte van geadsorbeerde homopolymeren onafhankelijk van de effectieve adsorptie-energie.

Beek, G.P. van der and Cohen Stuart, M.A., *J. Phys. France* **49**, 1449 (1988).

VI

Met de bewering dat de hydrofobe interaktie tussen apolaire aminozuren toeneemt als gevolg van een hydratatie-afname met toenemende temperatuur, raken Privalov en Gill het eiwitmolekuul in zijn hart.

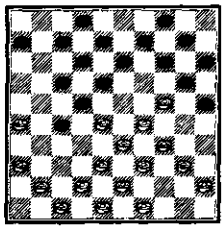
Privalov, P.L. and Gill, S.J., *Pure & Appl. Chem.*, **61**, 1097 (1989).

VII

Omdat een Japanner nauwelijks "nee" kent, heeft "ja" meerdere betekenissen.

VIII

Niet elke stelling is te verdedigen.



Stand na 17. 46-41

R. van de Beek–G. Leeflang, Kamp. v. Geld. '84-'85.

IX

Gezien het belang van de patient in de gezondheidszorg is het juist om met de toenemende vergrijzing deze mensen niet over één kam te scheren.

XIII

Elk bijgeloof is ongeloofwaardig.

Stellingen

behorende bij het proefschrift van A.V. Elgersma, Landbouwuniversiteit Wageningen.

18 mei 1990

aan mijn ouders

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 GENERAL

The topic of this thesis is proteins at interfaces.

There are numerous reasons to pay attention to this field, both because of its fundamental interest and its wide range of applications. Most proteins have a strong tendency to accumulate (adsorb) at interfaces and the final result is determined by many variables among which the pH, ionic strength, temperature, the properties of the protein molecules, the adsorbent surface, the solvent molecules and those of any other molecules in the medium. In nature, proteins are invariably encountered in very complex biological media, including body fluids like blood, lachrymal fluid, saliva and urine. These fluids contain various kinds of proteins and, therefore, the competitive adsorption from a solution containing a mixture of proteins is a relevant and challenging subject to study.

Proteins at interfaces are encountered in many disparate domains, such as biotechnology (immobilized enzymes), biochemistry (protein reactions), analytical chemistry (protein separation techniques), food processing industry (as fouling agents of equipment and as stabilizers of dispersions), cosmetic and pharmaceutic industries (emulsifiers and stabilizers) and in the biomedical field (fouling of intra- and extra-corporeal devices, drug delivery systems, biosensors and immunoassays).

Protein molecules are long polypeptides, containing several tens to hundreds of amino acid monomers. There are about twenty different amino acids and the sequence in which they appear in the polypeptide chain is highly specific. Upon biosynthesis, the polypeptide chain folds into its active conformation. The three-dimensional structure of a (globular) protein depends on interactions within the protein molecule and interactions between the protein molecule and its environment.

For protein molecules dissolved in an aqueous medium, the most relevant interactions determining the conformation are:

- electrostatic interactions in which charged side groups of the polypeptide chain (mostly residing at the exterior of the molecule) and low molecular weight ions are involved
- hydrogen bonds, involving groups of the protein molecule and water molecules
- van der Waals interactions
- hydrophobic interaction resulting in a tendency to bury the hydrophobic residues in the interior of the protein molecule, so that they are shielded from water

• covalent binding between two cysteine amino acids along the polypeptide chain to form a disulfide linkage.

When a protein molecule adsorbs from solution it undergoes an environmental alteration. Hence, its intra- and intermolecular interactions are affected. This may result in an adjustment of the three-dimensional structure. In view of the structure-function relations of proteins, the question of structure changes upon adsorption is of major interest. Structural changes themselves can even be the main driving force for proteins to adsorb spontaneously [1].

1.2 SYSTEMS INVESTIGATED

This study focusses on the (competitive) adsorption of proteins onto hydrophobic surfaces.

In most of the experiments latices are used as the adsorbent. Latices are polymer colloids in a liquid environment. The choice of polystyrene latices has been motivated by a number of reasons. In the first place, polystyrene latices can be prepared in such a way that important variables like the sign and magnitude of the surface charge can be controlled. Both negatively and positively charged latices (having a surface charge that is constant over a wide pH-range) have been used in this study. Second, latices have the advantage over macroscopic surfaces that they have a large surface to volume ratio. Therefore, simple analytical techniques can be used to determine the amount of protein adsorbed. This procedure obviates the necessity of marking the protein with an extrinsic label that itself might affect the structural properties of the protein, possibly influencing the adsorption behavior. Beside these motives the consideration has played a role that latices are often applied in systems where interaction with proteins is essential, e.g. in diagnostic test systems. The present study has great relevance for a better understanding of immunoassays as diagnostic test systems.

The proteins used in this study are Bovine Serum Albumin (BSA) and different monoclonal Immuno gamma Globulins (IgG's).

Serum Albumin is one of the most abundant proteins present in the blood of mammals. Its main functions are to regulate the osmotic pressure and the pH of the blood and to carry (metal)ions, phospholipids, hormones, etc. [2]. Albumin has been chosen as one of the adsorptives, because in practice this protein is frequently involved in some adsorption competition. Furthermore, in practical applications as diagnostic test systems BSA plays an important role.

Conventional samples of IgG are usually polyclonal, i.e. they consist of IgG molecules having different physico-chemical properties. Monoclonal antibodies are produced by a 'hybridoma-technique' [3], yielding IgG-samples which are highly specific, since they act against one single antigenic determinant. The four monoclonal IgG's used in this study were mouse-anti HCG's. Human Chorionic Gonadotropin (HCG) is a hormone produced by the placenta of pregnant women. The monoclonal antibodies (IgG's) differ in isoelectric point. This difference enables us to study the electrostatic contribution to the adsorption of IgG at a charged surface.

The main purposes of this work are to obtain more insight into the mechanism of adsorption of BSA and IgG individually as well as into the competitive adsorption between these two proteins. In view of practical applications another goal is to find optimum conditions for colloidal stability of latices coated with these proteins.

1.3 CONTENTS OF THIS THESIS

Recently some good reviews on protein adsorption have been written [1,4,5]. However, the literature lacks systematic overviews on competitive adsorption. Therefore, in **chapter 2** of this thesis we shall begin by reviewing the present state of competitive protein adsorption.

In studying protein adsorption the properties of the protein, the adsorbent surface and the medium must be carefully characterized. In **chapter 3** the purification and characterization of the materials together with the methods used in this study will be described in detail.

To interpret competitive adsorption behaviour of proteins it is required to know the affinity of each individual protein for the surface and therefore adsorption isotherms from single protein solutions are determined. The contribution of electrostatic interactions between the protein and the adsorbent is emphasized. This is done by varying the charge on the polystyrene latex surface and protein molecule independently. Electrokinetic measurements are performed to obtain information on the electrokinetic potential, which is related to the colloid stability of the (coated) latices. In **chapter 3** the results for the individual adsorption of BSA and the various monoclonal IgG's will be described.

Displacement of one protein by another implies desorption and automatically takes one to the issue of adsorption reversibility. In this respect distinction must be made between the 'reversibility' with respect to dilution, to pH-change, to exchange with dissolved protein molecules and to displacement by other proteins or substances. Displacement occuring upon sequential adsorption yields information about the binding strength of the proteins relative to each other. This matter will be presented in **chapter 4**.

Similar information can be inferred from studying the adsorption from a mixture of proteins. The contribution of electrostatic interaction to competitive adsorption will be discussed in **chapter 5**.

In **chapter 6** protein adsorption measurements on macroscopic polystyrene surfaces studied by reflectometry and streaming potential will be reported. The data are compared with those obtained with latices.

1.4 REFERENCES

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CHAPTER 2

SOME DYNAMIC ASPECTS OF PROTEIN ADSORPTION

2.1 INTRODUCTION

The literature on protein adsorption is to a large extent controversial. The main reason for this confusion is insufficient characterization of the systems studied. Systems encountered in practice, including such disparate biological fluids like blood, saliva, milk, fruit juices or sea water interacting with solid surfaces, are far too complex to be subjected to a systematic study of the principles of protein adsorption and its dynamics. For this reason, the present chapter will focus on relatively simple and well-defined systems.

Some trends and principles of protein adsorption have been reported in recent symposium proceedings [1,2] and review articles [3,4].

The emphasis of this chapter is on a systematic overviewing of sequential and competitive protein adsorption (section 2.4); such a compilation is not found in literature. Besides this noble motive it is a good introduction to the experimental part on sequential (chapter 4) and competitive adsorption (chapter 5). First, in section 2.2, some general features of single protein adsorption at solid surfaces are reviewed, followed by a discussion of the 'reversibility' of this adsorption in section 2.3. Finally, in section 2.5, the influence of an adsorbed protein layer on subsequent biological processes will be discussed. In view of the structure-function relations of proteins, special attention will be paid to antibody-antigen interaction.

2.2 SINGLE PROTEIN ADSORPTION

2.2.1 General

Protein adsorption is the net result of various interactions between protein, adsorbent surface and medium (involving (aqueous) solvent molecules and low-molecular weight (MW) ions) all of which may generally be dependent on ionic strength, pH and temperature. This section starts with a discussion on some static aspects of protein adsorption, whereas the remainder of this chapter reviews dynamic principles.

Adsorbed Amount

An important quantity in protein adsorption studies is the adsorbed amount. This can be determined by various methods. For finely dispersed adsorbents with a large surface to volume ratio, analytical methods have been applied in which the protein concentration before and after adsorption is compared [5,6,7,8]. For macroscopic surfaces the adsorbed

amount of protein has to be quantified directly on the surface. To that end, the protein may be marked with an extrinsic label, which may be radioactive [9,10,11] or fluorescing [12,13]. Whether or not there is a 'label-influence' on protein adsorption is still a matter of dispute [11,14,15]. To circumvent this uncertainty, methods based on intrinsic properties of the protein itself are more appropriate, such as Total Internal Reflection Fluorescence (TIRF) using the fluorescence of amino acid residues [16,17,18] or Attennuated Total Reflection-Fourier Transformed Infra Red Spectroscopy (ATR-FTIR) using IR vibrations of specific amide bands [19,20]. In addition to determining the adsorbed amounts, the last mentioned methods may also yield information about the conformation of the protein in the adsorbed state. Rather new techniques in the protein adsorption field are reflectometry [21,22,23,24], i.e. some modernized analog of ellipsometry [25], streaming potential measurements [24,26,27] and Enzyme-Linked Immuno Sorbent Assays (ELISA) [28,29].

Adsorption Isotherms

For globular proteins, adsorption isotherms (in which the adsorbed amount Γ is plotted versus the protein concentration in solution after adsorption) usually develop well-defined plateaus which are in the range of those calculated for a close-packed monolayer of native molecules, corresponding with a few mg per m^2. However, it must be noted that multilayer formation also has been reported [30,31].

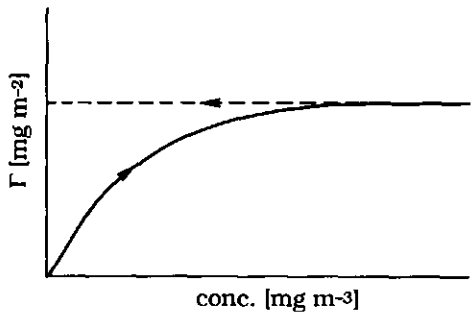

Figure 2.1 *Hysteresis between adsorption and desorption isotherms.*

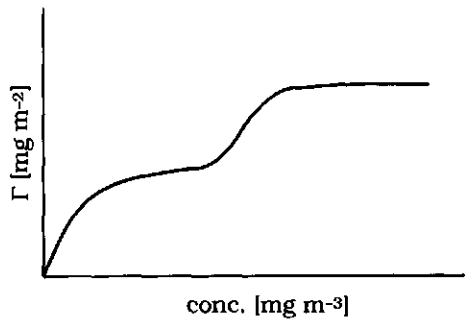

Figure 2.2 *Step-like adsorption isotherm.*

Linear polymer molecules attach via several contacts to an adsorbent surface. Therefore, the resulting free energy of adsorption per molecule is relatively large and the isotherms are of the high-affinity type, characterized by a very steep initial part. For globular protein

molecules the occurrence of isotherms having a finite initial slope is not exceptional. However, even when such non high-affinity *ad*sorption isotherms are found, the corresponding *de*sorption isotherms are usually of the high-affinity type, as indicated by the dashed line in Figure 2.1. This phenomenon suggests that, upon adsorption, proteins undergo a structural change enhancing the binding between the protein and the sorbent surface. Hysteresis will be further discussed in section 2.3.

Step-like adsorption isotherms, as shown in Figure 2.2, have been reported by various authors [6,7,30,32]. At rather high protein concentrations the formation of such steps can be attributed to bilayer formation, when the second layer is readily desorbable [30,33]. When no desorption takes place upon dilution, bilayer protein adsorption is not very likely. The 'kink' may then be related to a variation in the binding mode with increasing occupancy. For instance, for albumin and γ-globulin on polystyrene latices Fair and Jamieson [7] interpreted this phenomenon as a transition in the structure and/or organization of the adsorbed layer.

2.2.2 Factors that determine protein adsorption affinity

The adsorption affinity of a protein for a surface is characterized by the overall molar Gibbs energy of adsorption. The most important factors determining the adsorption affinity are: (i) electrostatic interaction between the participating components (including low-MW ions), (ii) hydrophobic dehydratation of parts of the adsorbent and/or protein molecules, (iii) structural stability and size of the protein molecule. These contributions will be discussed in some more detail below.

Effect of electrostatic interactions

In most cases both protein and adsorbent surface contain electrostatically charged groups. Although these charged groups are located at discrete sites, the surface charge is often treated as being smeared-out.

Upon adsorption, the electric double layers of both species overlap, resulting in attraction or repulsion. Many proteins spontaneously adsorb on like-charged adsorbent surfaces, even when these surfaces are hydrophilic (see below). Hence, in those cases electrostatic interactions are not to prevent proteins from adsorption. For the adsorption of many proteins, the 'conformational less stable ones' as albumin [6,11,34,35], immuno gamma globulins [36], fibrinogen [37], hemoglobin [38] and gelatin [39,40], a maximum plateau-value ($\Gamma_{pl,max}$) is found around their isoelectric point. The finding of bell-shaped Γ_{pl}(pH)-curves has been explained in terms of a decreasing structure stability of the globular protein with increasing net charge, rather than being the result of lateral repulsion between

adsorbed molecules [3,8,32,36,41,42]. Superimposed on the Γ_{pl}(pH)-trend caused by structural rearrangements in the protein, the influence of charge interaction between the protein molecule and adsorbent can be observed. The adsorbed amount of protein increases with increasing charge contrast as has been reported for albumin on polystyrene latices [5,6], fibrinogen on various polymeric surfaces [43], albumin, fibrinogen and IgG on platinum surfaces [44].

Concomitantly with protein adsorption, low MW-ions are incorporated in the contact region between the protein and adsorbent surface [5,45,46]. The ion uptake prevents a too high charge density in the low dielectric contact region, which, in turn, would result in an energetically unfavourable electrostatic potential. The uptake of ions upon adsorption is another indication that non-electrostatic interactions are important in the adsorption process.

Effect of hydrophobicity

Although globular protein molecules in an aqueous environment have the tendency to bury the hydrophobic amino acid residues in their interiors, some non-polar groups may reside at the outside of the molecule [42]. Dehydration of hydrophobic patches of both protein and adsorbent surface results in an entropy gain (i.e. a lowering of the Gibbs energy of the system) and therefore promotes protein adsorption. As a trend, the adsorption affinity and adsorbed amount are larger at more hydrophobic adsorbent surfaces [3,35,47,48,49]. Consistently, the desorbability of proteins tends to decrease in this direction. This will be further discussed in section 2.3. There are some indications that proteins have a maximum affinity for surfaces of intermediate polarity [50,51], although the opposite also has been reported [52].

Effect of structural stability of the protein

An elegant study has been undertaken by Norde *et al.* [6,23,27,45,53] in which the structural stability of proteins is related to their adsorption behaviour. The contribution of intramolecular hydrophobic bonding, relative to all other interactions determining the stabilization of a compact protein structure in solution, is relevant with respect to the occurrence of structural rearrangements upon adsorption. In fact, these structural alterations (involving an increased conformational entropy of the protein molecule) may be one of the major reasons for spontaneous adsorption. According to their resilience against such structural changes, proteins may be classified in two groups:

- proteins having a strong internal coherence, including ribonuclease, lysozyme, myoglobin. For this class, adsorption is mainly determined by the interaction between the

exterior of the protein and the adsorbent, and involves electrostatic and hydrophobic interaction.

• proteins having a weak internal coherence, so that upon adsorption they can undergo structural changes, which is the case for albumin, α–lactalbumin and IgG. For such proteins the rise in conformational entropy can dominate, outweighing unfavourable contributions due to hydrophilic dehydration or electrostatic repulsion. Typically, for such proteins the adsorption behaviour is not so sensitive to the nature of the adsorbent.

In Table 2.1 literature data are collected for a variety of proteins that undergo a conformational change upon adsorption. After desorption or displacement from the surface the native form is not always retained. Therefore one must be careful in applying reversible thermodynamics.

Table 2.1 *Structural changes observed upon adsorption for a variety of proteins on various adsorbents using different techniques.*

Protein	Adsorbate	Alteration	Technique	Ref.
Albumin	PHEMA, P(HEMA/MAA)	α–helix	ATR–FTIR	[54]
Albumin	copolypeptides, silicone	α–helix	desorption + CD	[55]
Albumin	PS–latices	n.d.	hydrogen ion titration	[56]
Albumin	different oxides	α–helix	displacement + CD	[57]
Albumin	poly ion–complexes	α–helix	Transmission CD	[58]
Albumin	hydrophilic silica	n.d.	TIRIF	[59]
γ–Globulin	glass	n.d.	Microcalorimetry	[60]
γ–Globulin	different hydrogels	β–sheet	ATR–FTIR	[19]
γ–Globulin	PS–latices	n.d.	potentiometric titration	[61]
γ–Globulin	copolypeptides, silicone	β–sheet	desorption + CD	[55]
Fibrinogen	copolypeptides, silicone	α–helix	desorption + CD	[55]
Fibrinogen	glass	α–helix	desorption + CD	[62]
Fibronectin	hydrophobic silica	n.d.	TIRF	[63]
Fibronectin	PS–latices	n.d.	sulfhydryl titration	[64]
Vitronectin	PS, oxidized PS	β–sheet	ATR–FTIR	[65]
Apomyoglobin	silica	n.d.	TIRF	[66]
Myoglobin	PDMS	n.d.	TIRF	[67]
A. Fact. III	silicon	n.d.	ELISA/Ellipsometry	[49]
Factor XII	quartz	n.d.	Transmission CD	[68]

Abbreviations: (n.d.) not distinguished, (PHEMA) poly-2–hydroxyethylmethacrylate, (MAA) methacrylic acid, (ATR–FTIR) attenuated total reflection–fourier transformed infrared spectroscopy, (CD) circular dichroism, (PS) polystyrene, (TIRIF) total internal reflection intrinsic fluorecence, (C. Fact.) Complement Factor, (PDMS) polydimethylsiloxane, (ELISA) enzyme-linked immunosorbent assay.

Effect of molecular size of the protein

Upon adsorption, proteins and other macromolecules form 'multiple contacts' with the surface. From studies with flexible homopolymers it is known that the adsorbed amount increases with increasing chain length (i.e. molecular mass) at least in not too good solvents. A fraction of the flexible polymer is actually attached, whereas the remainder protrudes into the solution as loops and tails. Depending on their structural stability, protein molecules may undergo (structural) rearrangements (see Table 2.1), but they do not unfold so far as to form a loose, highly solvated adsorbed layer. For proteins having a strong internal coherence, the bound fraction is independent of the degree of coverage of the surface [69]. On the other hand, by way of example, for the adsorption of IgG (being a conformationally less stable protein) on silica, a bound fraction of 0.02 in the plateau region of the isotherm as opposed to 0.20 when adsorption occurred from low protein concentrations was reported by Morrissey *et al.* [44,70].

It is difficult to study the exclusive influence of the molecular weight of proteins on the adsorption, since also other physical properties vary between different proteins. Establishing the influence of the molecular size of proteins on the adsorption can best be achieved by comparing the adsorption behaviour of monomers, dimers and oligomers of the same kind of protein. A preferential adsorption of the larger aggregates has been reported for albumin [71,72] and fibrinogen [73]. It can be generally stated that the larger MW-proteins (in accordance with the trend observed for synthetic flexible polymers) show larger adsorptions per unit area. Therefore, the adsorbed protein layer will also be thicker.

When the solution contains proteins of different size, their respective diffusities may play an important role as to the composition of the adsorbed layer. The smaller protein molecules, having a higher diffusion coefficient, are the first ones to arrive near a surface. The final composition of the adsorbed layer depends on the degree of exchange between the pre-adsorbed molecules and the later arriving ones. These kinetic aspects will be considered in section 2.4.

2.3 REVERSIBILITY ASPECTS

An appropriate definition [74] of a reversible process may read as "The transfer of a system from one state A to a different state B is reversible if in the opposite process (B to A) all the variables characterizing the state of the system return through the same values but in the opposite order, and if the exchanges of heat, matter and work with the surroundings are of the reverse sign and occur in the inverse order".

Changing medium conditions (i.e. increasing or decreasing protein concentration, pH, ionic strength, temperature etc.) followed by measurements of the adsorption may in principle provide information on the question whether a) desorption or b) additional protein adsorption takes place (see section 2.3.1). However, in my opinion a more realistic contribution to the reversibility issue is a study of the dynamics of the exchange of adsorbed proteins with similar protein molecules in solution, since no medium change is involved (see section 2.3.1c). In section 2.3.2 some concluding remarks will be drawn.

2.3.1 Factors of consideration

a) **Desorption** of adsorbed protein molecules from a surface is influenced by many factors, among which:

Desorption time

An adsorbed protein molecule is attached via multiple contacts. The resulting activation Gibbs energy for desorption is much greater than for adsorption, and as a consequence the desorption process is many times slower. As a result, there may be differences between the adsorption and desorption isotherms that depend on the time of observation (Figure 2.1). When after prolonged observation times differences between adsorption and desorption persist, one can speak of true hysteresis [75]. True hysteresis implies that the Gibbs energy of the desorbed protein in solution is more positive than that of the (native) protein molecule before adsorption. This would result in a higher affinity of the desorbed molecule to re-adsorb [76]. The hysteresis loop is furthermore indicative of an entropy production. It has indeed been observed that desorbed protein molecules contain a reduced degree of secondary structure (see Table 2.1) and consequently a larger conformational entropy.

In the literature, the imporfance of the time in the desorption process is not always recognized.

Adsorption time

Literature data confirm the suggestion of an increasing protein-adsorbent interaction upon adsorption. Soderquist and Walton [55] found a decreased desorption rate with increasing residence time for albumin on siliconized glass. Bohnert and Horbett [77] reported a decreased elutability with adsorption time of albumin and fibrinogen from different polymeric surfaces. The desorption of IgG from silica showed at short adsorption times a single state, whereas at longer exposure times a 'biphasic desorption pattern' was observed [78]. In addition, it has been proved, using ATR-FTIR for albumin adsorbed on hydrophilic surfaces, that its α-helix content in the adsorbed state decreases with adsorption time [54].

Degree of occupancy

For the group of conformationally less stable proteins, the bound fraction decreases with increasing adsorbed amounts, see section 2.2.2. At a higher degree of occupancy, the conformation changes less and, therefore, desorption is more enhanced. Jennissen [75] found an increase of the desorption rate when the fractional saturation of phosphorylase b on covalently modified agarose gels was increased from 0.10 to 0.75.

However, a decreased desorbability from completely covered surfaces, e.g. albumin and IgG on polystyrene latices [7], has been observed and was interpreted in terms of intermolecular protein association on the surface. Moreover, a decreased desorption of albumin on glass around the isoelectric point of the protein compared to other pH-values was reported by Bull [79]. Whether or not protein association on surfaces occurs is still disputed [80,81].

Adsorbent characteristics

The hydrophobicity of an adsorbent surface determines the rate and amount of protein desorption [47,82,83]. For many proteins it has been observed that desorption (if any) from various hydrophobic surfaces upon dilution is extremely low [5,6,82,84]. As a peculiarity, it is mentioned that 80% desorption of IgG from polystyrene latices has been observed by adding dimethylsulfoxide [85]. On the other hand, partial desorption from hydrophilic surfaces upon dilution [43,70,86,87] or changing pH [57] has also been reported. From ATR-FTIR measurements it has been inferred that the extent of structural changes in an adsorbed protein molecule is larger on more hydrophobic surfaces and increases with time [54]. It can be suggested that the change in structure, which is related to the structure stability of the protein (see also section 2.2.2), results in a greater affinity for the surface and, as a result, retards the desorption process indefinitely and leads to real hysteresis.

Electrostatics

When a protein molecule appoaches a like-charged surface, an energy barrier (few kT-units) for deposition has to be overcome [88]. As a consequence, the adsorption is retarded, giving once attached molecules more time to adapt their structure before newly arriving molecules (can) adsorb. Electrostatic contributions to the sorption process are best shown for charged hydrophilic surfaces at which (partial) desorption occurs upon increasing the ionic strength [9,57].

b) **Additional protein adsorption** may occur upon changing medium conditions, as has been reported for step-wise addition of proteins [37,47,73]. An increase in the adsorbed amount can also be achieved by changing the pH or ionic strength to more favourable values (whereas the reverse change in conditions does not necessarily show any desorption)

[5,36,69]. However, the adsorbed amount thus obtained is often smaller as compared to that reached in a single step [5,36,55,69,73]. This is probably due to structural changes in the protein causing a larger surface area per molecule at lower surface coverage.

c) **Exchangeability**

Even in systems showing no desorption upon changing medium conditions, an exchange with similar or identical protein molecules in solution has been reported by various groups. A summary will be given below. The activation Gibbs energy for exchange is much smaller as compared to that for desorption by dilution and, as a consequence, the exchange rate will be much higher. An exchange process might be visualized by *d*etachment of parts of the adsorbed molecule in favour of *a*ttachment of newly arriving protein molecules. For proteins with a weak internal coherence, structural rearrangements in the adsorbed state may be the dominating contribution to the Gibbs energy of adsorption. When after displacement the native conformation is not (totally) regained, the adsorbent may be regarded as a catalyst facilitating structural transitions. It would be interesting to study the influence of 'exchange time' on the conformation of the protein in solution.

Brash and Samak [84] described the exchange of ^{125}I-albumin adsorbed on poly-ethylene against ^{131}I-albumin in solution. These authors established that the rates of desorption and adsorption are equal, proving a truly reversible exchange mechanism. Furthermore, both the extent and rate were found to increase with increasing shear rate and albumin concentration. The exchange of ^{125}I-fibrinogen adsorbed on glass for dissolved ^{131}I-fibrinogen has been reported by Chan and Brash [9]. Again, the exchanged fraction increases with protein concentration in solution, whereas in this case the shear rate (0–1600 s^{-1}) did not influence the extent and rate of exchange. Exchanging ^{125}I-fibrinogen from glass with non-labelled fibrinogen, Brash *et al.* [15] distinguished three populations of adsorbed fibrinogen, namely: non-, rapidly- and slowly exchanging fractions. The existence of a heterogeneous surface population has also been reported by Fraaye [89], who performed an exchange experiment with pre-adsorbed albumin on AgI and ^{14}C-iodoacetamide-labelled albumin in solution. Within one minute 30–40% rapidly exchanges, while 60–70% slowly exchanges in a few hours. Both the rate and extent of exchange were found independent of pH ranging from 4 to 6. Lok *et al.* [17,90], using TIRF, determined the exchange of both fluorescein isothiocyanate-labelled albumin and fibrinogen adsorbed on polydimethyl-siloxane with non-labelled protein in solution and vice versa. The rate for a partially exchangeable fraction, 40-50% in 100 hours, was lower as compared to the findings of Brash *et al.* [9,84]. Cheng *et al.* [91] extended the TIRF-experiments of Lok *et al.* [17,90], using

polymeric surfaces with varying surface properties. The exchange rate of adsorbed fluorescein isothiocyanate-labelled albumin was found to be transport limited, independent of the adsorbent. Chuang *et al.* [87] reported exchangeability under static conditions of ^{125}I-labelled albumin, fibrinogen and IgG from (hydrophilic) cuprophane with unlabelled protein. The extent of exchange was 38, 19 and 11 % in 24 hours, respectively. The rate of exchange from (hydrophobic) polyvinylchloride was found five times lower in the case of IgG. Bale *et al.* [92] also showed that the exchanged fraction of IgG is larger with increasing adsorbent hydrophilicity.

The general trend emerges that the exchangeability of proteins is reduced by increasing hydrophobicity of the adsorbent surface and furthermore by structural rearrangements that may have occurred in the adsorbed protein molecule.

2.3.2 Concluding remarks

Upon changing medium conditions, only a limited desorbable protein fraction is reported. Additional protein adsorption is found lower as compared to adsorption in one single step. The rate of protein exchange is larger at initial stages of the process, and only a limited exchangeable protein fraction is usually observed. From the above findings, it can be concluded that the adsorbed protein layer is heterogeneous with respect to its affinity for the adsorbent surface. Such a protein layer can be formed, because at low protein supply and hence low surface occupancies the proteins have more room and time for structural rearrangements. Obviously, the latest adsorbing proteins that are the least perturbed are more easily removed from the surface.

A dynamic exchange process, as evidenced in this reversibility section, must also be the underlying principle for sequential and competitive protein adsorption.

2.4 SEQUENTIAL AND COMPETITIVE PROTEIN ADSORPTION

2.4.1 General

Most investigations on competitive protein adsorption done so far involved biological fluids as a whole, such as, blood(plasma), saliva or milk. Adsorption studies using mixtures of the most abundant proteins, as models of these complex biological media, have been reported to a lesser extent. Most of these 'mixture-studies' simulate physiological conditions in blood(plasma), implying that: (i) the ionic strength is about 0.16M (suppressing electrostatic interactions), (ii) pH~7.4 (most proteins are negatively charged) and (iii) the protein concentrations or ratios are fixed.

In view of the dynamic exchange of adsorbed proteins with solute proteins, it is supposed that both **kinetic** and **thermodynamic factors** determine competitive adsorption. Most of the biological situations and practical applications involve adsorption from a flowing solution. In a laminarly flowing system a protein cannot reach the surface by convective transport only; it has to diffuse through a stagnant layer adjacent the surface (Nernst diffusion layer). The rate of transport towards the adsorbent surface is influenced by the protein concentrations, protein size (diffusity) and convectivity. Therefore, the initial state of an adsorbed layer will rather depend on kinetic factors, whereas both kinetics and factors that determine protein adsorption affinity (discussed in section 2.2) might determine the ultimate composition. Once a protein molecule is attached, its affinity for the surface can change. This surface-relaxation process, including exchange and displacement of adsorbed proteins, may proceed over a relatively long period (days), so that it is quite possible that kinetic factors are virtually frozen in, rendering the final result dependent on the history of the adsorption.

The continuation of this section is divided into a part on sequential (2.4.2) and one on competitive (2.4.3) protein adsorption. Special attention will be given to the time dependence of the composition of an adsorbed layer. The compositions of the adsorbed layer and (remaining) protein solution are not necessarily equal. Preferential adsorption of a given protein p is defined as the ratio between the fraction of p on the surface and that in solution.

2.4.2 Sequential adsorption

Sequential adsorption of proteins can be divided into two sub-processes. Adsorption of one protein is followed by the addition of a second (different) protein to the protein-adsorbent complex. Additional adsorption of the second protein may occur or the second protein may displace the pre-adsorbed protein, all of this depending on the relative affinities of the two proteins for the surface. The situation where an immobilized protein reacts with its complementary antigen or antibody will be described in section 2.5. Below, important features involving sequential adsorption will be discussed on the basis of data from the literature. The following notation is introduced: (1^{st}) stands for the pre-adsorbed protein and (2^{nd}) for the protein added in a later stage.

With increasing **preadsorbed amount** less additional adsorption occurs. This feature is frequently referred to as 'blocking' of the second protein. It has, for instance, been observed with albumin and fibrinogen (both 1^{st} and 2^{nd}) on different hydrophobic polymers [93]; albumin and IgG (both 1^{st} and 2^{nd}) on hydrophilic quartz [12] or hydrophobic polyurethane

[61]; IgG (1st) and albumin (2nd) on copolymeric latices of polystyrene [92]. In addition, the relative amount displaced decreases with decreasing surface coverage [92,93], indicating stronger interactions of the pre-adsorbed protein with the surface at low occupancy (cf. section 2.3.1). In the case of albumin (1st) and fibrinogen (2nd), the initial adsorption rate of fibrinogen linearly decreases with increasing amount of pre-adsorbed albumin (until 50% surface coverage), indicating a first order adsorption rate of fibrinogen with free surface area [93]. The reverse process does not show such a behaviour.

If displacement of a protein occurs, the displaced amount increases with increasing **concentration** of the second added protein [12]. However, complete displacement of adsorbed proteins from hydrophobic surfaces has never been reported. Complete displacement from hydrophilic sorbents was reported by Arai and Norde [53]: conformationally stable proteins could be displaced by a protein having a weak internal coherence.

To interpret the concentration dependency of the 2nd protein on the displacement of the 1st one, its **molecular size** must be considered. The following references all point to an increased displacement with increasing size of the 2nd protein. Among others, Bale *et al.* [92] found an increase in displaced amount of IgG in the order of the 2nd protein: fibrinogen>IgG (exchange)>albumin. This finding is supported by Koltisko and Walton [94], who showed that fibronectin displaced monomeric albumin much faster than adsorbed dimeric albumin. However, different proteins have different physical properties, e.g. structural stability, which may interfere with the influence of molecular size. In the chapters 4 and 5, examples will be discussed showing that molecular size alone is not sufficient to explain the results of sequential and competitive adsorption experiments.

Increasing **preadsorption time** leads to an optimalization of the protein-adsorbent attraction; see also section 2.3. Indeed, a reduction of the displaced amount is often found with increasing preadsorption time [5,92,93].

Displacement of pre-adsorbed proteins is easier when the adsorbent surface is **hydrophilic**. This has been observed by Shirahama *et al.* [23], and Arai and Norde [53] for a number of combinations of two proteins (both 1st and 2nd) viz. lysozyme, ribonuclease, myoglobin and α-lactalbumin on hydrophilic haematite and silica, hydrophobic polystyrene and hydrophobized silica. Accordingly, IgG (1st) was found to be more strongly displaced from more hydrophilic copolymer surfaces [92]. However, Arnebrandt and Nylander [95] found no influence of the hydrophilicity of the adsorbent: both on hydrophobic and hydrophilic surfaces additional adsorption of κ-casein leads to a small displacement of β-lactoglobulin. In the reverse sequential adsorption, no additional adsorption of β-lactoglobulin on pre-adsorbed κ-casein surfaces occured. The lower

structure stability and, therefore, higher adsorption affinity of κ-casein may account for the observed behaviour.

2.4.3 Competitive adsorption

Monomer versus adsorption of higher aggregates

One approach to study competitive protein adsorption makes use of protein solutions containing monomer (m), dimer (d) and oligomer (o) of one and the same protein. It is assumed that, execept for the molecular size, all other physical properties are identical. Preferential adsorption of (d) and (o) over (m) molecules from albumin solutions has been reported [71,72,94,96]. The preference is more pronounced when the albumin concentration/surface area ratio becomes larger [71,72]. The influence of electrostatic interactions between albumin and negatively charged polystyrene latices, together with the compositional change of the adsorbed layer with adsorption time was studied by Zsom [71]. Preferential adsorption of negatively charged (d) or (d+o) is enhanced on more negatively charged latices. Furthermore, the preferential adsorption of (d) or (d+o) on latices having σ_o<−11 μC cm^{-2} is already obtained in the initial stage of adsorption, because the composition of additional adsorption resembles the composition in solution! Moreover, on latices having σ_o>−4 μC cm^{-2} additional adsorption represents an increased preference of (d) and (d+o) of respectively 30% and even 74%. Koltisko and Walton [94] also found preferential adsorption of (d), which increases with adsorption time, on surfaces having a low negative surface charge density.

Adsorption from polydisperse protein samples shows a preferential adsorption of the higher aggregates. The same feature has been reported for the adsorption of polydisperse, flexible macromolecules [97].

Adsorption from mixtures of different proteins

The competitive adsorption from mixtures of different proteins can be studied by a number of techniques including experiments using labelled protein(s), depletion-HPLC or specific antibodies to the adsorbed protein. In Table 2.2 an overview is given of the adsorption from binary and tertiary blood protein mixtures after a relatively long adsorption time. It can be concluded that in general the adsorbed amount of a given protein in competition experiments is smaller as compared to that for the same protein on its own. In competitive adsorption studies involving fibrinogen, IgG and albumin, it is always observed that the preference for the adsorbent decreases in this order. This sequence is retrieved for a variety of surfaces and initial protein compositions. From Table 2.2 the

important conclusion can be drawn that the adsorption preference is more pronounced on hydrophilic surfaces [20,87,99,102].

Table 2.2 refers to the composition of layers adsorbed after a relatively long time. It would be interesting to also know how the composition of the adsorbed layer changes with time. To that end, kinetic competitive protein adsorption measurements are best suited. So far, only a limited number of such studies have been undertaken [12,17,20,99]. Beissinger and Leonard [12] observed that for albumin and IgG the surface composition after 20 min. is already obtained at 30 seconds for various protein concentrations in solution. The adsorbed amounts of both proteins increase rapidly during the first two min. to reach a plateau after about 20 min., whereas no displacement is observed. Lok *et al.* [17] found a slow increase of the amount of fibrinogen on silicon during adsorption, viz. the Alb/Fb ratio decreases from 83/17 to 75/25 and 65/35 for adsorption times of 30 sec., 4 and 6 hrs. respectively. No displacement of albumin was found. On the other hand, Gendreau *et al.* [99] reported a predominance of albumin over fibrinogen on germanium for $t_{ads}<7$ min., followed by displacement of albumin by fibrinogen. Probably, in this case displacement of albumin is promoted because of the hydrophilicity of the surface. The influence of shear rate on the composition of an adsorbed layer can be deduced from the data reported by Chittur *et al.* [20]. At a high shear rate, i.e. a thinner Nernst diffusion layer adjacent to the surface, the preference for protein molecules having the lowest diffusity (IgG) is somewhat enlarged, see Table 2.2.

Another kinetic factor, besides the effect of shear, on the composition of the adsorbed layer, has been described by Breemhaar *et al.* [28]. Using ELISA, they found, for a fixed initial protein composition in solution (Alb : IgG : Fb = 63 : 31 : 6 [wt%]) a major influence of the *total* protein concentration on the competitive adsorption behaviour. The preference for fibrinogen increases with increasing total protein concentration; under physiological conditions (c_{total} is about 75 mg cm^{-3}) the surface composition is 31 : 8 : 61 on polyvinylchloride and 21 : 10 : 69 on polystyrene. I shall return to this effect below.

To eliminate the influence of different protein diffusities, studies with proteins of equal size yield information, that is expected to be directly related to their relative affinities for the surface. To my knowledge, only two studies describe such a competitive adsorption. They refer to (i) κ-casein and β-lactoglobulin on hydrophobic and hydrophilic glass [95] and (ii) binary, tertiary and quartenary competitive adsorption of lysozyme, ribonuclease, myoglobin and α-lactalbumin in all possible combinations on different adsorbents [53]. The studies show preferential adsorption of κ-casein and α-lactalbumin, which could be attributed to their lower structure stability. The preference is again more pronounced on

Table 2.2 *Competitive adsorption of binary and tertiary protein mixtures. The composition of the initial protein solution and adsorbed layer after relative long adsorption times are given. If not stated otherwise the data refer to statical measurements. (pH_{ads}=7.4)*

Proteins	Adsorbent	Adsorption conditions I.S. [M]	time [h]	rate of shear [s^{-1}]	Composition of initial protein solution [wt%]	Composition of adsorbed layer [wt%]	Reference
Alb : IgG	PS-latex	0.17	4		17 : 83	1 : 99	[98]
	"	"	"		50 : 50	7 : 93	"
	"	"	"		99 : 1	75 : 25	"
	quartz	0.17	0.33	1400	28 : 72	11 : 89	[12]
	"	"	"	"	50 : 50	31 : 69	"
	"	"	"	"	89 : 11	75 : 25	"
	Polyvinylchloride	0.17	0.5		80 : 20	84 : 16	[87]
	Cuprophane	"	"		80 : 20	69 : 31	"
	Polyetherurethane	0.16	2	330	60 : 40	46 : 54	[20]
	Germanium	"	"	"	60 : 40	18 : 82	"
	"	"	"	1600	60 : 40	14 : 86	"
Alb : Fb	Germanium	0.15	0.5–3	?	50 : 50	not quantified	[99]
	Polyvinylchloride	0.17	2		50 : 50	29 : 71	[93]
	Polyethylene	"	"		50 : 50	40 : 60	"
	Silicon	"	"		50 : 50	46 : 54	"
	Silicon	0.17	20		50 : 50	24 : 76	[100]
	"	"	"		91 : 9	52 : 48	"
	PS-latex	0.17	4		91 : 9	26 : 74	[72]
	Silicon	0.20	6	58	93 : 7	65 : 35	[17]
	Polyvinylchloride	0.17	0.5		93 : 7	57 : 43	[87]
	Cuprophane	"	"		93 : 7	15 : 85	"
	Polyetherurethane	0.05	–		98 : 2	23 : 77	[101]
	Polystyrene	"	–		98 : 2	47 : 53	"
	Collagen	"	–		98 : 2	31 : 69	"
IgG : Fb	Silicon	0.17	20		50 : 50	8 : 92	[100]
	"	"	"		91 : 9	48 : 52	"
	Polyvinylchloride	0.17	0.5		77 : 23	15 : 85	[87]
	Cuprophane	"	"		77 : 23	16 : 84	"
	PS-latex	0.17	4		83 : 17	33 : 67	[72]
Alb : IgG : Fb	hydrophilic grafted Polyetherurethane	–	24		75 : 20 : 5	41 : 26 : 33	[102]
	Polyvinylchloride	0.17	0.5		75 : 19 : 6	21 : 25 : 54	[87]
	Cuprophane	0.17	0.5		75 : 19 : 6	18 : 6 : 76	"
	PS-latex	0.17	4		24 : 62 : 14	0 : 29 : 71	[72]

hydrophilic surfaces. In addition, electrostatic interactions are important in determining the (ultimate) composition of the adsorbed layer [53]. Competitive adsorption of three proteins having a rather strong internal coherence, viz. lysozyme, ribonuclease and myoglobin, show preferences which are electrostatically governed. With α-lactalbumin, a factor related to its low structure stability, largely contributes to preferential adsorption of this protein.

Adsorption from blood plasma

A behaviour similar to that observed by Breemhaar *et al.* [28], mentioned above, was observed by Vroman *et al.* [103,104,105] using diluted plasma. They concluded that the composition of the adsorbed protein layer varies with time and plasma concentration. Upon dilution a maximum in the adsorbed amount of fibrinogen occurs at a plasma concentration of about 1 vol%. The maximum decreases with increasing adsorption time. It is assumed that two processes interfere with each other in determining the amount of adsorbed fibrinogen. At high plasma concentration the fibrinogen adsorption is limited by its displacement by higher molecular weight proteins like Factor XII [105] and high molecular weight kininogen (HMWK) [105,106] or high density lipoprotein (HDL) [28], whereas in strongly diluted plasma solutions protein adsorption is restricted by its low concentration. The transient adsorption of fibrinogen is commonly known as the "Vroman-effect". Additional experiments to verify the "Vroman-effect" have been described, among which: (i) adding traces of radiolabelled albumin [107,108], IgG [108] or fibrinogen [108,109] to plasma, (ii) using plasma solutions lacking higher molecular weight proteins, i.e. HMWK [105,110] or fibrinogen [105] to interrupt the sequential pattern and (iii) characterization of the displaced protein layer, as a function of adsorption time, by sodium dodecyl sulfate (SDS) followed by polyacrylamide gel electrophoresis (PAGE) [111].

The exchange sequence (albumin, immunoglobulin, fibrinogen, fibronectin, Factor XII and high molecular weight kininogen (HMWK)) commonly reported for hydrophilic surfaces, like glass [105,109,111,112], does not apply to hydrophobic polystyrene [111] or polyethylene [112]. The sequence can probably be related to the higher affinity of adsorbed proteins on hydrophobic surfaces, leading to a lower ability for displacement from the surface. Cuypers *et al.* [113] proposed a tentative adsorption model for the "Vroman-effect", with protein concentrations in solution and affinities of the proteins for the surface as the model parameters. It follows that a diminishing difference between the affinities of the different proteins for the surface (as is the case with more hydrophobic surfaces) results in a

protein layer composition that is dominated by the solution concentrations of the proteins relative to each other.

Longer adsorption times result in larger conformational changes, which are more pronounced on hydrophobic surfaces (cf. sections 2.3.1 and 2.4.2). The occurrence of conformational changes upon adsorption on hydrophobic surfaces might be the main reason that the "Vroman-effect" in competitive adsorption is found on hydrophilic surfaces only.

2.5 INFLUENCE OF ADSORBED PROTEIN LAYERS ON BIOLOGICAL PROCESSES

In nature, one of the first events taking place when a biological fluid is exposed to a "foreign" material is the adsorption of proteins. As a consequence the interfacial properties of this material will be changed which, in turn, may affect subsequent events such as cell adhesion, microbiological growth and activation of solute proteins (e.g. intrinsic coagulation in blood). To prognosticate ultimate situations on a surface, it is mandatory to understand initial interactions between surface and protein. For example, surfaces which preferentially adsorb immuno gamma globulins or fibrinogen out of (blood)plasma adhere more blood platelets than albumin coated-surfaces [114]. When adhered blood platelets aggregate and the aggregates are subsequently released from the surface, thrombi are formed and a life-threatening situation may occur. The platelet adherence is probably due to the formation of a complex of an enzyme of the platelet membrane (glycosyl transferase) with carbohydrate chains of adsorbed glyco-proteins [115]. It is not surprising that the search for biocompatible materials is mainly directed by the occurence of protein adsorption.

Another important issue is the formation of (immune) complexes between an antibody and an antigen when one of them is immobilized on a surface. Antibodies are immuno gamma globulins, containing a specific region, viz. the hypervariable domains on the F_{ab}-part, which specifically interact with an epitope on their complementary antigen (see also Figure 2.3). Non-covalent intermolecular forces are thought to be responsible for the formation of an antigen-antibody complex. Association constants are found in the range of 10^5–10^{11} $[M^{-1}]$ [116] and they are usually influenced by ionic strength, temperature and pH [117].

The fact that one of the interacting proteins is adsorbed might also affect its association behaviour. Orientational and conformational alterations may influence the accessibility of the antigen binding site(s). Moreover, non-specific binding of the antigen with the adsorbent surface may occur possibly displacing pre-adsorbed antibodies, see chapter 4. Finally, at relatively high surface occupancy the IgG-molecules might sterically hinder

each other. Using monoclonal antibodies against different epitopes of myoglobin, Darst *et al.* [87] showed a specific loss of certain antigenic determinants on the adsorbed myoglobin, indicating a non-random orientation and/or conformation on the surface.

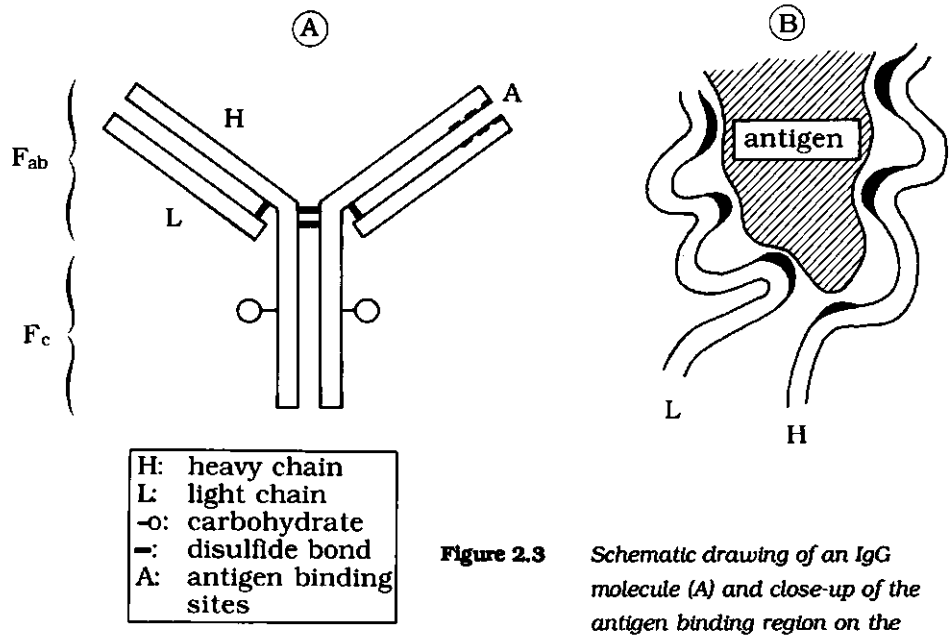

Figure 2.3 *Schematic drawing of an IgG molecule (A) and close-up of the antigen binding region on the antibody (B).*

When IgG molecules are adsorbed, as e.g. in diagnostic test systems, the antigen binding site should be directed towards the solution. The orientation of the antibody can be directed by pre-adsorption of Protein A, a protein which specifically binds an IgG molecule in its non-antigen binding part (F_c-unit) [118]. Introduction of a spacer on a surface chemically coupled to an antibody, results in an increased immunological sensitivity [119]. In section 3.2 more information is presented on the adsorption of monoclonal IgG's.

2.6 CONCLUSIONS

In this chapter an overview of protein adsorption with special emphasis on dynamic aspects is given. In our group, the structural stability of a protein is considered to be as one of the most important parameters influencing its adsorption behaviour. For proteins with a

'weak' internal coherence, structural alterations upon adsorption are thought to be a major reason for adsorption. The ensuing conformational entropy gain may dominate the Gibbs energy of adsorption, so that it may overcompensate for unfavourable contributions, e.g. electrostatic repulsion and/or hydrophilic dehydration. For the same reason differences in structural stability may play a decisive role as to the adsorption preference in sequential and competitive experiments.

From literature dealing with the reversibilty of protein adsorption it was concluded that the adsorbed protein layer is heterogeneous. Structural changes upon adsorption tend to decrease with increasing degree of occupancy on the surface. Consequently, the 'last' adsorbing proteins will be easier desorbed (section 2.3.1a), exchanged (section 2.3.1c) or displaced (section 2.4.2). The extent of these three different processes are smaller on hydrophobic surfaces, so that the influence of structural alterations is more pronounced on these surfaces. For competitive protein adsorption the initial composition of the adsorbed layer (section 2.4.3) is mainly determined by kinetic factors. Subsequent surface-relaxation, including exchange and displacement of adsorbed proteins, are more pronounced on hydrophilic surfaces. The finding of a "Vroman-effect", the transient adsorption of fibrinogen from plasma on hydrophilic adsorbents, illustrates this feature.

Literature on the influence of electrostatic interactions on competitive protein adsorption is virtually absent. However, such interactions probably play an important role. In the forthcoming chapters 3, 4 and 5 of this thesis single, sequential and competitive adsorption of albumin and IgG on polystyrene latices will be discusssed. In addition, in chapter 6 a study on the adsorption kinetics of these proteins on macroscopic surfaces will be described.

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CHAPTER 3

SINGLE PROTEIN ADSORPTION ON DIFFERENTLY CHARGED POLYSTYRENE LATICES

3.1 ADSORPTION OF BOVINE SERUM ALBUMIN &)

ABSTRACT

The present study deals with the electrostatic aspects of the interaction between monomeric Bovine Serum Albumin (BSA) and polystyrene latex particles. Adsorption and electrophoresis experiments have been performed as a function of pH, using both negatively and positively charged latices with different surface charge densities.

The adsorption isotherms develop well-defined plateaus. These plateaus vary with pH, showing a maximum at the isoelectric point of the protein-covered polystyrene particles, rather than that of the isoelectric point of the protein itself.

Co-adsorption of low molecular weight ions is shown to be an important parallel process. Studying it helps to discriminate between electrical and chemical contributions to the binding affinity.

3.1.1 INTRODUCTION

The adsorption of proteins onto (polymeric) surfaces has been extensively studied over the last decades. The state of the art is well documented in references [1,2,3]. Especially the interaction of whole blood or plasma proteins with surfaces of artificial materials is of prime interest in various biomedical applications, e.g. in artificial tissues and organs [4], drug delivery systems [5], biosensors [6] and immunoassays [7]. In biological systems usually protein mixtures are encountered that are often too complex to allow for unambiguous conclusions to be drawn. An alternative approach to unravel overall adsorption mechanisms is to separate the participating components and study the more 'simple' systems obtained in this way. It should be realized however that adsorption from a protein mixture may not be the sum of the adsorptions of the individual proteins as shown in the respective single protein solutions. Many of the protein adsorption studies are still disputable. One reason is that the materials used are not sufficiently purified and characterized. For example, it is known that from solutions of BSA [8] and HSA [9] the dimers and oligomers preferentially adsorb to polystyrene surfaces.

&) Accepted for publication in the "Journal of Colloid and Interface Science"

In addition, small amounts of impurities of high molecular weight proteins can perturb the protein adsorption behaviour and hence subsequent events like cell adhesion [10]. Another shortcoming of several studies is that an insufficient number of variables is studied. Protein adsorption is the intricate result of a combination of a number of contributions and the measurement of just a few adsorption isotherms does by no means suffice to give significant insight.

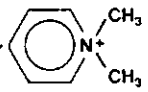
In the present study the electrostatic contribution to the adsorption of monomeric BSA on polystyrene latices is investigated. Available data of albumin adsorption on polystyrene latices [9,11,12,13,14,15] are extended and improved. The interference of protein associates is excluded and the influence of the surface charge on the latex is more systematically studied. It will be demonstrated that some generally accepted interpretations require adjustment.

3.1.2 EXPERIMENTAL

Materials

Polystyrene latices were prepared emulsifier-free. Negatively charged latices were supplied by AKZO-CR (Arnhem, The Netherlands); positively charged latices were prepared following a procedure described by Blaakmeer and Fleer [16]. Table 3.1 gives the surface charge density (σ_o) determined by conductometric titration together with the diameters of the latex particles derived from electron microscopy (EM).

Table 3.1 *Charged surface groups, surface charge density, and diameter of the various polystyrene latices. In addition, the uniformity coefficient of the two positively charged latices is given (see text). The diameter is determined with [a]SEM and [b]TEM.*

Code	Surface group	σ_o [μC cm^{-2}]	Diameter [nm]	U
N	~ SO_3^-	−5.5	501[a]	–
NN		−11.6	577[a]	–
P	~ 	+7.8	751±38[b]	1.0046
PP		+13.2	999±22[b]	1.0010

The particle size distribution of the positively charged latices is given. The particle size distribution is characterized by the uniformity coefficient, $U=d_{32}/d_{10}$, where d_{32} is the volume-surface average diameter and d_{10} the number average diameter. At least 150

particles were sampled. An EM photo of a typical latex sample is shown in Figure 3.1. As seen, the latex is almost homodisperse.

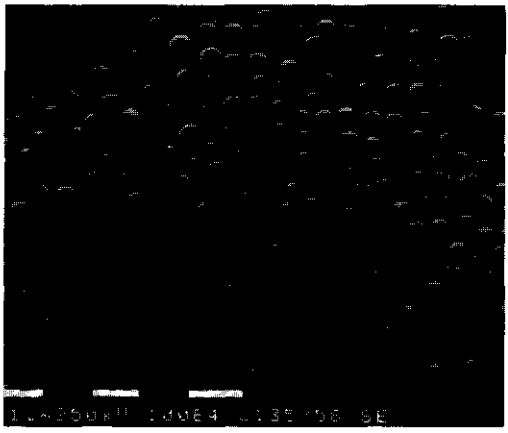


Figure 3.1 *SEM-picture of latex N (σ_o=-5.5 $\mu C\ cm^{-2}$) showing the homodispersity of the sample.*

BSA (Boseral 30TA) was donated by Organon Teknika (Boxtel, The Netherlands). It is a sterile solution of 300 g dm^{-3} BSA in 0.15 M sodium citrate. No stabilizers are added and the BSA is caprylate-free. Boseral 30TA was purified by HPLC Size Exclusion Chromatography (HP-SEC). The conditions for preperative HP-SEC, using Waters 6000A pumps and a Rheodyne valve 7010, were: Gel: 740x25 mm Fractogel TSK HW-55s (Merck); Eluents: 0.10 M NaH_2PO_4/NaOH/pH=5.0; Flow rate: 2.0 $cm^3\ min^{-1}$; Sample: 2 cm^3 75 mg cm^{-3} BSA; Detection: Pye Unicam LC-UV 280 nm; Fraction size: last part (8.5 min.) from each run was collected and pooled. By removing 11.0% dimer and 0.2% oligomer BSA from the solution, a monomer content of at least 99.5% was obtained, as analytically determined using a BIO-sil TSK 250 column (Merck) with UV monitoring at 214 nm (Pye Unicam LC). The monomeric BSA solution was stored in small volumes at -20 ^oC and thawed shortly before use. The BSA concentration in these samples (in 0.1 M NaH_2PO_4, pH=7) is 4.0 mg cm^{-3}, based on an exctinction coefficient ε(280 nm) of 0.661 $cm^2\ mg^{-1}$ [17]. Prior to every adsorption measurement the concentration of monomer and dimer has been determined. No reaggregation of the monomeric BSA was observed upon standing at different pH-values during several days.

The isoelectric point of BSA has been determined by both Iso Electric Focussing (IEF) and Two Dimensional-IEF (FBE-3000 and ECPS 3000/150 (Pharmacia)). Its value varied between 4.7 (IEF) and 5.0 (2D-IEF).

To trace the influence of the phosphate buffer on the BSA adsorption, some experiments were performed in 5 mM NaCl instead of phosphate buffer. For that purpose the purified monomeric BSA solution was ultrafiltrated with 0.1 M NaCl pH=7 using a 10 ml ultrafiltration cell (Amicon) with a 25 mm XM50 filter (Diaflo) and stored as above.
Water was purified by reverse osmosis and subsequently passed through a Super-Q system (Millipore). All other chemicals were of analytical grade and used without further purification.

Adsorption isotherms

A typical adsorption experiment was carried out in a 10 cm^3 polycarbonate tube (Nalgene, Sybron) to which 7 cm^3 BSA solution with a concentration varying between 0–0.20 mg cm^{-3} of a given pH was added. Subsequently 1 cm^3 latex of the same pH and representing about 0.2 m^2 surface area was mixed with the BSA solution. The final buffer concentration in all experiments was 5 mM NaH_2PO_4. The experiments were performed in phosphate buffer to maintain constant pH during the protein adsorption process. The buffer concentration was kept low to allow for relatively strong electrostatic interactions between adsorbent and protein.
The tubes were rotated gently (to avoid dimerization of the BSA molecules) for 2.5–3 hours at 21 ^oC. During this incubation time the change in pH did not exceed 0.2 units. About 7 ml of the solution was passed through a 0.2 µm filter (Acrodisc, Gelman Sciences). No protein adsorption to the filter was detected by comparing filtered samples with centrifuged ones (15 min. at $4.71x10^4$ m s^{-2} (Sarstedt MH2)). The remaining part of the solution was used for electrophoresis experiments which were performed within two days. The BSA concentration in the filtrate was determined by FPLC®-SEC, using a Superose-12 column (Pharmacia) with UV monitoring at 206 nm (Uvicord S, LKB). The detection limit is $1x10^{-3}$ mg cm^{-3}. Most adsorption isotherms were duplicated and every concentration determination was performed twice. The BSA concentration was related to a calibration curve using the peak height as a measure. Calibration curves at different pH showed a correlation coefficient of 0.9998. The reproducibility between two concentration determinations is within 2%, indicating an uncertainty of about 0.02 mg m^{-2} for the initial part of the adsorption isotherm and increasing to 0.05 mg m^{-2} at plateau-values.

The reversibility of BSA adsorption with respect to changes in the protein concentration in solution was tested in the following way. To determine whether desorption took place upon lowering the protein concentration in solution, samples were centrifugated after adsorption under saturation conditions (Sarstedt MH2, 15 min. at $4.71x10^4$ m s^{-2}). The

supernatant was replaced by a 5 mM buffer solution of the same pH. After gently rotating for about 16 hours, leading to complete resuspension of the latex, the BSA concentration in solution was determined. This 'dilution'-procedure was repeated once. In view of the detection limit, changes in the surface concentration beyond 0.05 mg m^{-2} were considered to be significant.

BSA adsorption reversibility as a function of pH was studied as follows: a) *additional adsorption*: after adsorption under saturation conditions, with sufficient protein remaining in solution to allow additional adsorption, the pH was varied by adding concentrated HCl or NaOH. b) *desorption*: after adsorption at the plateau value, samples were once diluted (following the 'dilution'-procedure mentioned above) and subsequently adjusted to a different pH.

Electrophoresis

All experiments were performed with a MK2 microelectrophoresis Zeta Sizer (Malvern Instruments Ltd.) at 25 ^{o}C. Prior to a measurement about 0.3 cm^3 (BSA-coated) latex was mixed with 14.7 cm^3 5 mM electrolyte solution (buffer or NaCl) of the same pH, so that the final solution contained about 5.10^8 particles per cm^3. The obtained mobilities were transformed into zeta potentials using the procedure of O'Brien and White [18]. The accuracy in the mobility measurements was typically $\pm$ 0.1x10^{-8} $m^2.V^{-1}.s^{-1}$ leading to an uncertainty in the zeta potential of 2 mV.

3.1.3 RESULTS AND DISCUSSION

Adsorption isotherms

Adsorption isotherms, in which the adsorbed amount (Γ) is plotted versus the protein concentration in solution after adsorption (c_{BSA}), are shown in Figure 3.2 for various latices at different pH. Even when the protein has the same charge sign as its sorbent (isoelectric point of BSA is about 4.7-5.0) adsorption occurs spontaneously. For all latices the adsorption isotherms show well-defined saturation plateaus at higher c_{BSA}.

The initial slope of the adsorption isotherm reflects the interaction between protein and adsorbent. As a trend for both negatively charged latices the initial slopes decrease with increasing pH, i.e. with increasing electrostatic repulsion between protein and latex. When the initial slopes at three pH-values (6.0, 7.0, 8.0) for the latex with σ_o=–11.6 μC cm^{-2} are divided by the corresponding initial slopes for the latex with σ_o=–5.5 μC cm^{-2} a mean ratio of 1.8 $\pm$ 0.2 is obtained. Along the lines of electrostatic interaction it can not easily be understood that the negatively charged BSA molecule has a somewhat higher affinity for

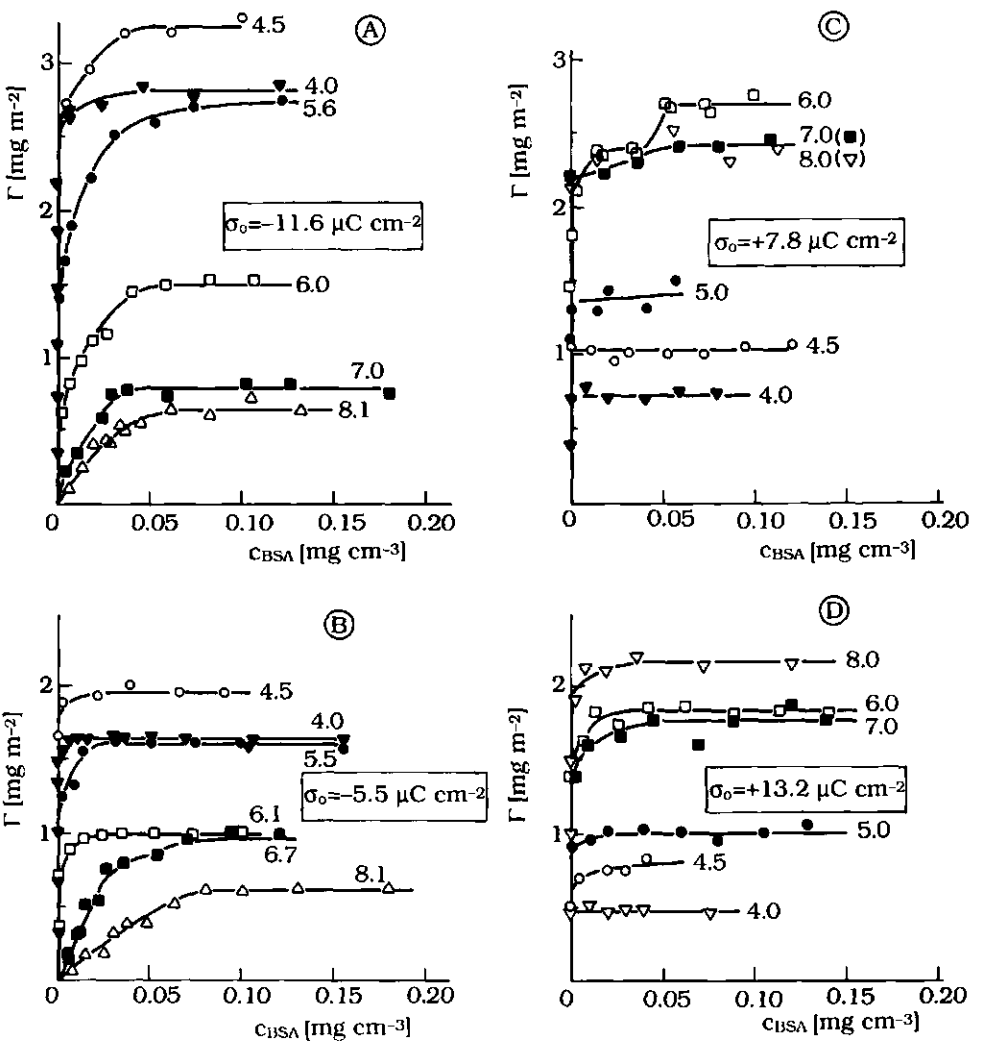


Figure 3.2 *Adsorption isotherms for BSA on PS latices at various pH and surface charge densities of the adsorbent.*

the more negatively charged PS surface. On positively charged latices the isotherms show a high affinity character under all circumstances, even when the protein and the adsorbent have the same charge sign. The difference with respect to the affinities between positively and negatively charged latices illustrates again that interpretation on the basis of overall-electrostatic interaction only is not appropriate. For the latex with σ_o=+7.8 μC cm^{-2} at pH=6.0 a 'step' in the adsorption isotherm is observed. Since no desorption upon dilution was found, this 'kink' is more likely due to conformational optimalization of the adsorbed protein molecules rather than the formation of a second layer. Other authors [14] have observed similar 'steps' under other conditions.

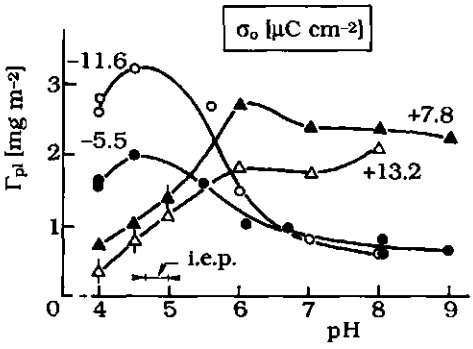


Figure 3.3 *Plateau adsorption of BSA on PS latices with different surface charge densities.*

Figure 3.3 shows the plateau values of adsorption (Γ_{pl}) as a function of the pH. On both negatively charged PS-latices the plateau value has a maximum ($\Gamma_{pl,max}$) around pH=4.7, i.e. near the i.e.p. of the dissolved BSA molecule. However, for the two positively charged latices no maximum in Γ_{pl} is found around this pH; instead, Γ_{pl}(pH) tends to show a maximum at pH=6 which is most pronounced for the latex with σ_o=+7.8 μC cm^{-2}. Superimposed on the influence of the latex surface charge density on the shape of the Γ_{pl}(pH) curve, the influence of the overall electrostatic interaction between the latex and the protein is obvious. Furthermore, the plateaus of the isotherms tend to increase with increasing 'negative character' of the polystyrene surface.

When the adsorption on a latex with σ_o=+7.8 μC cm^{-2} was performed in 5 mM NaCl instead of phosphate buffer and the initial pH was between 6.0 and 8.5, the pH after adsorption was around 6.0 for all these cases. This demonstrates the necessity of an additional buffer to maintain the pH constant during adsorption. The amount adsorbed in the plateau was about the same as in the presence of phosphate buffer, Γ_{pl}=2.7 mg m^{-2}, indicating that $\Gamma_{pl,max}$

around pH=6.0 for positively charged latices are not specifically affected by the type of electrolyte in the system.

The occurence of a maximum in the Γ_{pl}(pH)-curves may have several reasons. The most important contribution is the decreasing conformational stability of the protein with increasing net charge on the molecule. This implies a greater tendency to structural rearrangements of the adsorbing molecules, resulting in a larger surface area per molecule and therefore a smaller adsorbed amount [19,20,21]. In addition, it is not completely ruled out that at pH-values away from the isoelectric point of the protein an increase of lateral electrostatic repulsion between adsorbed molecules may lead to a smaller adsorbed amount. These considerations may be an explanation for the maximum in Γ_{pl}(pH) around the i.e.p. of the protein molecule as is found with BSA adsorbed on negatively charged latices. However, for the positively charged latices the maximum in Γ_{pl}(pH) has shifted to pH-values to the alkaline side of the i.e.p. of the solute protein.

In literature the occurrence of maximum protein adsorption around the isoelectric point of the protein has been reported for e.g. albumine [11,12,13,15], immuno gamma globulins [19], fibrinogen [22], hemoglobin [23] and gelatin [24,25], but no such maximum has been found for the so-called 'conformationally stable' proteins like cytochrome C and RNase [13]. However, it must be noted that these data are dealing with negatively charged surfaces. The scarce data referring to protein adsorption on positively charged surfaces do indeed point to an alkaline shift in the pH for which Γ_{pl} attains its maximum value [26], confirming our observation. Below, after presenting electrophoresis data this issue is reconsidered.

Reversibility aspects

Apart from its intrinsic interest it is important for the electrophoresis experiments to know whether proteins desorb upon dilution with buffer, since electrophoresis is performed with very dilute solutions. We never found such desorption at any system subjected to electrophoresis.

Reversibilty with respect to pH-change has been tested using the highly negatively charged latex. The adsorption was found to be semi-reversible: (i) changing the pH from a state where Γ_{pl} had attained a maximum value (pH≈4.5) no desorption occurred, but (ii) starting at a pH where Γ_{pl} was not a maximum, changing it to 4.5 lead to additional adsorption, although the adsorption was smaller than that obtained at pH=4.5 in a single step.

Electrophoresis

Electrophoresis experiments were carried out to derive additional information on the electrostatic interaction between the protein and the latex and on the co-adsorption of low molecular weight ions. In addition, it allows us to relate the mobility of (BSA-coated) PS-particles to the colloidal stability of the latices.

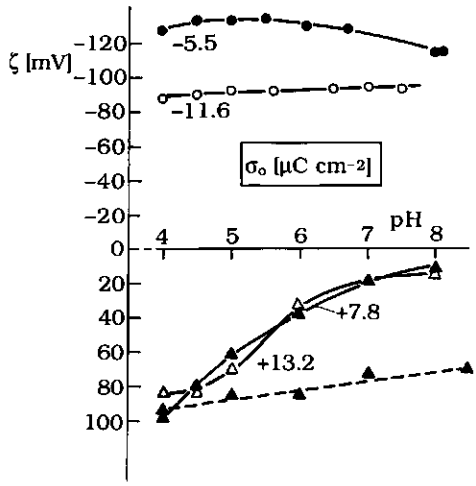


Figure 3.4 *The ζ-potential of bare PS latices in 5 mM NaH_2PO_4. The ζ-potential of the latex with σ_0=+7.8 μC cm^{-2} in 5 mM NaCl is also given, (dashed curve).*

Figure 3.4 shows the ζ-potential of bare PS-particles as a function of pH. A very small increase in the ζ-potential with pH was observed for the negatively charged latex with σ_0=–11.6 μC cm^{-2}. For the latex with σ_0=–5.5 μC cm^{-2} a slight maximum in the ζ-potential as a function of pH has been found. The absolute value of the ζ-potential was larger for the lower negatively charged PS-particle. This finding is (electrostatically) in agreement with the earlier observation of a somewhat higher affinity of negatively charged BSA for the more negatively charged PS-latex. For the positively charged latices a dramatic decrease of the ζ-potential and, hence, the electrokinetic charge density was observed with increasing pH. As the positively charged latices have a surface charge density which is virtually independent of the pH in the range 4 to 10 [16], the decrease in ζ-potential is ascribed to specific phosphate ion adsorption No such pronounced decrease has been observed when the mobility was examined in 5 mM NaCl. The influence of specifically adsorbed phosphate ions on the adsorption will be discussed below.

The occurrence of a maximum in the ζ-potential as a function of the salt concentration, i.e. ionic strength, has often been ascribed to some kind of hairiness of the PS particles [27,28,29]. Also the combined effects of counterion and co-ion adsorption are proposed as an

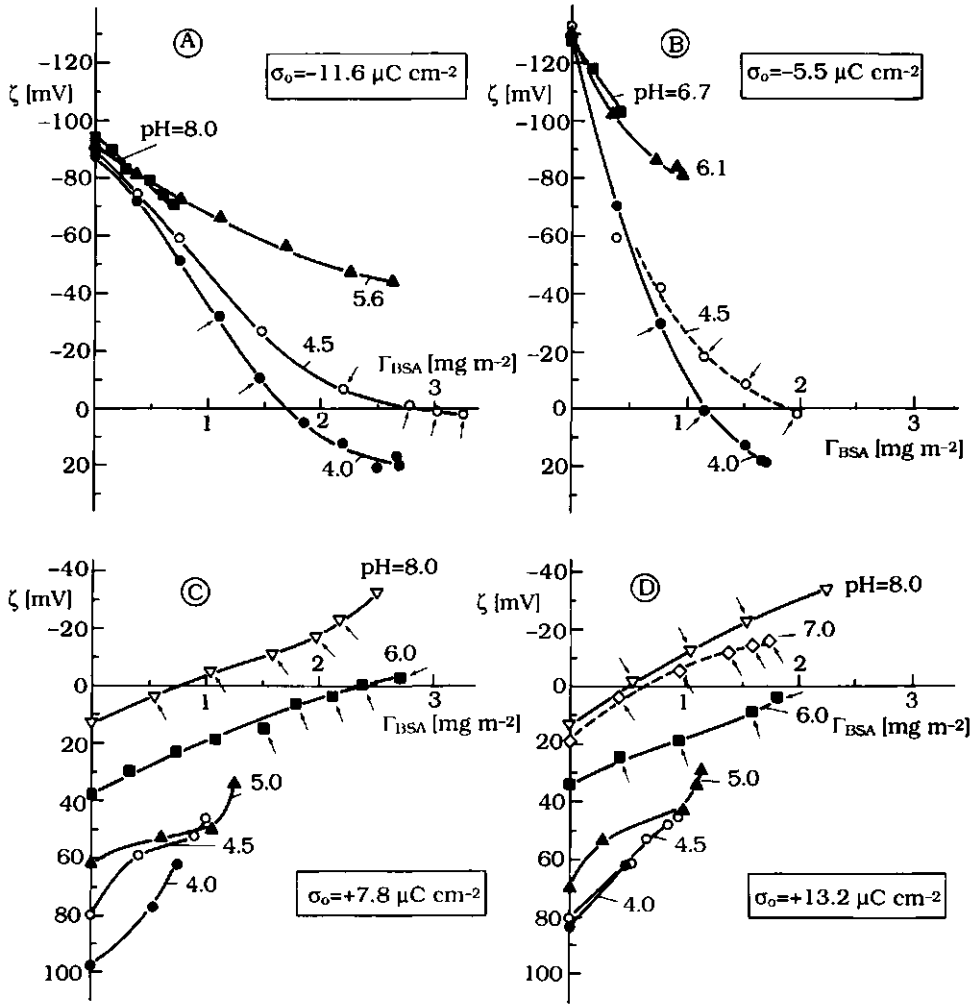


Figure 3.5 *The ζ-potential as a function of the adsorbed amount BSA on different PS lattices. The pH is indicated.*

explanation of the ζ-maximum [30,31]. In our system, with increasing pH of the buffered medium, $H_2PO_4^-$ ions are titrated into HPO_4^{2-}, so that the ionic strength increases.

In Figure 3.5 the ζ-potentials of the BSA-PS complexes are given as functions of the amount of adsorbed BSA. With increasing Γ the absolute value of the ζ-potential decreases. This decrease is dependent on the pH, i.e. on the charge of the BSA molecule. For the negatively charged latices the decrease in ζ-potential becomes less strong with increasing negative charge on the BSA molecule. As expected, the variation of the ζ-potential with Γ is for the latex with σ_o=–5.5 μC cm^{-2} more pronounced than that for σ_o=–11.6 μC cm^{-2}. For the two positively charged latices there is almost no difference between the curves for ζ(Γ). At pH=6.0 a reversal of the ζ-potential occurs at a high adsorbed amount, wheras ζ already reverses at a lower coverage in the case of pH-values 7.0 and 8.0. This last observation can be easily understood in electrostatic terms.

Under conditions of low absolute values for ζ, flocculation of the latices is often observed; it is indicated by arrows in Figure 3.5. Electrokinetic charge reversal by BSA leads to restabilization. This restabilization has also been demonstrated for systems with negatively charged latices beyond half saturation and pH=4.0 where flocculation occurs. Changing the pH towards pH>5.5 caused restabilization of the systems. If flocculation had occurred by 'bridging' between BSA molecules and PS particles, restabilization by pH-change were possible only if partial desorption of BSA molecules from one particle took place. However, as shown before (reversibility part), upon changing the pH to more alkaline values, desorption of pre-adsorbed BSA molecules from the surface was not found. Therefore, it is inferred that flocculation of PS latices by BSA adsorption is the result of charge neutralization.

Figure 3.6 gives the ζ-potential at complete coverage ($\Gamma=\Gamma_{pl}$) as a function of pH for the different latices. The ζ-potential of free HSA (Human analog of BSA) is also indicated in the same figure. An identical electrophoretic behaviour of BSA and HSA is presumed. To convert mobilities into ζ-potentials, the BSA molecule is assumed to be a sphere with a radius of 3.4 nm, having the same molecular volume as its ellipsoid form. As expected in view of the charge on the BSA molecule, ζ-potentials of BSA-covered latices become more negative with increasing pH, although there are quantitative differences.

The isoelectric points of BSA-covered negatively charged latices and free BSA in solution fairly coincide. This implies that the negative charge of the underlying PS-surface must be esentially compensated by co-adsorption of counterions other than those associated with the protein in solution. The isoelectric pH for the BSA-covered positively charged latices is

situated around pH=6, indicating that the positive charge of the PS-surface compensates, at least partly, negative charged groups of the protein.

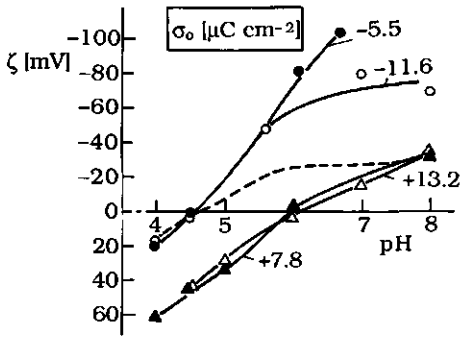


Figure 3.6 *The ζ-potential of PS latices completely covered with BSA ($\Gamma=\Gamma_{pl}$). The ζ-potential of HSA in solution is represented by the dashed curve, (values are derived from data given in reference [32]).*

Interpretation of adsorption maxima as a function of pH

The maximum in Γ_{pl}(pH) occurs at the isoelectric pH of the BSA-latex complex. In Figure 3.7 this coincidence is illustrated. It applies to all systems studied. For negatively charged latices the i.e.p. of the protein-covered particles does not significantly differ from the i.e.p. of the protein molecules in solution. As noted before, this is attributed to the co-adsorption of positive ions from solution to compensate for the negative charges of the latex.

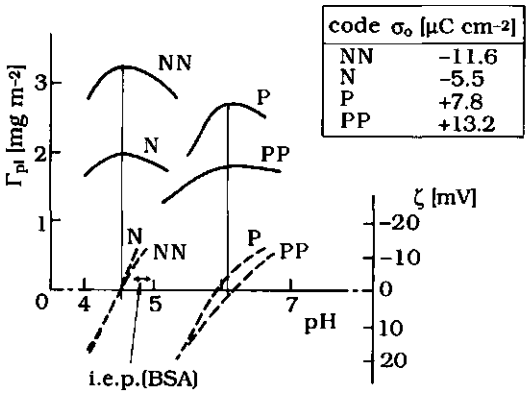


Figure 3.7 *Plateau-adsorption and ζ-potential as a function of pH, showing the coincidence of the pH where Γ_{pl} has a maximum and the i.e.p. of the BSA-covered PS particles.*

On the other hand, for BSA on positively charged latex the i.e.p. is situated at more alkaline values which agrees with the earlier finding that the PS surface charge compensates, at least partly, the negative protein charge.

As to these observations literature data are scarce. Abramson observed a coincidence between the isoelectric points of Horse Serum Albumin adsorbed on negatively charged quartz and collodion particles and in the dissolved state at pH=4.8, at which Γ_{pl} also has its maximum [33]. A similar behaviour has been reported by Norde *et al.* for Human Serum Albumin adsorption onto negatively charged polystyrene latices [26,32]. More recently, a shift of the isoelectric point of the protein covered negatively charged PS-particles due to the binding of anions, such as SCN^-, to BSA to a more acidic value has been observed by Shirahama *et al.*. The pH at which Γ_{pl} passes through a maximum varied correspondingly [34]. The same authors reported a coincidence between the isoelectricity of dissolved Bovine Hemoglobin (pH is about 6.8) and the complex of Bovine Hemoglobin with negatively charged PS-latices. At this pH Γ_{pl} also has its maximum [23]. When BSA is adsorbed under plateau conditions on positively charged Calcium Oxalate Monohydrate the i.e.p. of the complex is about 5.4, which is alkaline relative to the i.e.p of the protein in solution. Unfortunately, Curreri *et al.* did not report the pH at which Γ_{pl} attains its maximum in these systems [35].

Ion participation in the BSA adsorption process

Comparing the electrokinetic charges before and after adsorption, one acquires information on the participation of low MW ions in the adsorption of BSA onto PS latices [32,36]. From Figures 3.4 and 3.6, ζ-potentials of the bare and covered PS particles together with the dissolved BSA molecules can be converted into electrokinetic charges using diffuse double layer theory. A numerical solution for spherical double layers was used, kindly set at our disposal by Dr. A. de Keizer. In Table 3.2 the obtained data are tabulated. The charge uptake by the adsorption process can be expressed as:

$$\Delta_{ads}\sigma_{ek}=\sigma_{ek}(PS/BSA)-\sigma_{ek}(PS)-\sigma_{ek}(BSA).\Gamma.A \qquad [3.1]$$

Where σ_{ek} is the charge density per unit surface area at the slipping layer of resp. PS/BSA (covered PS particles), PS (bare PS particles) and BSA (dissolved protein). Γ is the amount of BSA adsorbed in mass per unit latex area and A is the surface area of BSA per unit mass; $A=4\pi r^2.N_{av}.(MW)^{-1}$ with r(BSA)=3.4 nm, MW=67000 g mol^{-1} and N_{av} is Avogadro's number. Because of overall electroneutrality $\Delta_{ads}\sigma_{ek}$ represents the charge transfer between solution and the adsorbed layer. Since all adsorption experiments are performed in 5 mM NaH_2PO_4

Table 3.2 *Data for the calculation of $\Delta_{ads}\sigma_{ek}$ using Eq. [3.1] (see text).*

		σ_0=−11.6 μC cm^{-2}			σ_0=+7.8 μC cm^{-2}			σ_0=+13.2 μC cm^{-2}		
pH	σ_{ek}(BSA) [μC cm^{-2}]	Γ_{pl} [mg m^{-2}]	σ_{ek}(PS) [μC cm^{-2}]	σ_{ek}(PS/BSA) [μC cm^{-2}]	Γ_{pl} [mg m^{-2}]	σ_{ek}(PS) [μC cm^{-2}]	σ_{ek}(PS/BSA) [μC cm^{-2}]	Γ_{pl} [mg m^{-2}]	σ_{ek}(PS) [μC cm^{-2}]	σ_{ek}(PS/BSA) [μC cm^{-2}]
4.0	0.60	2.80	−2.25	0.30	0.72	2.80	1.30	0.47	2.10	1.30
4.5	0.10	3.25	−2.35	0.05	1.00	2.00	0.88	0.80	2.15	0.85
5.0	−0.25	3.15	−2.40	−0.30	1.35	1.35	0.60	1.00	1.65	0.50
5.5	−0.75	2.55	−2.50	−0.78	2.00	1.10	0.33	1.55	1.28	0.25
6.0	−1.00	1.50	−2.55	−1.25	2.70	0.90	−0.05	1.80	0.75	0.05
6.5	−1.10	1.10	−2.83	−1.68	2.65	0.70	−0.23	1.80	0.60	−0.15
7.0	−1.20	0.85	−3.10	−1.95	2.45	0.50	−0.40	1.75	0.50	−0.35
8.0	−1.45	0.65	−3.65	−2.45	2.40	0.35	−0.80	2.10	0.45	−0.90

as the additional buffer Δ_{ads}pH is about zero so that $\Delta_{ads}\sigma_{ek}$ is only caused by the transfer to or from solution of ions other than protons.

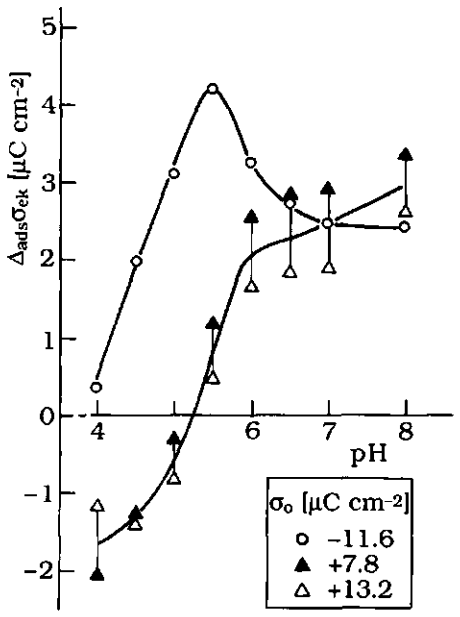


Figure 3.8 *Variation in electrokinetic charge density with pH for PS latices completely covered with BSA.*

In Figure 3.8 $\Delta_{ads}\sigma_{ek}$ is given as a function of pH for the latex with σ_o=−11.6 μC cm^{-2} and the mean of the two positively charged latices. For the negatively charged latex $\Delta_{ads}\sigma_{ek}$ has a positive value throughout, indicating uptake of cations. This is even so at pH=4 where the BSA molecules are positively charged. Based on proton titration data of Human Serum Albumin on negatively charged polystyrene particles Norde *et al.* [37] concluded that in adsorbed HSA the average position of the carboxyl groups is relatively close to the adsorbent, probably because of non-electrostatic interactions. Positive ions from solution must be incorporated in the contact region between the protein and the surface to prevent an accumulation of net negative charge [38]. The present data are in good agreement with earlier work by Norde and Lyklema [32]. Namely, the adsorption of HSA on a PS-latex with σ_o=−15.5 μC cm^{-2} in 0.05 M KNO_3, indicating that there is no specific influence of the (buffered) medium on ion transfer.

For the positively charged latices $\Delta_{ads}\sigma_{ek}$ is negative at low pH, but assumes a positive value at pH≥5.2. Above, it was concluded that negative charges of the BSA molecule interact with the positively charged surface groups. At low pH the proteins do not bear sufficient negative

charge to compensate the positive surface charge. Then anions from solution will be incorporated. At higher pH-values the excess negative charge of the BSA molecules is compensated by an uptake of cations in the contact region, so that $\Delta_{ads}\sigma_{ek}>0$. It is possible that the corollary for negatively charged latices (anion uptake at very low pH) could also be realized, but our measurements did not extend below pH 4.0.

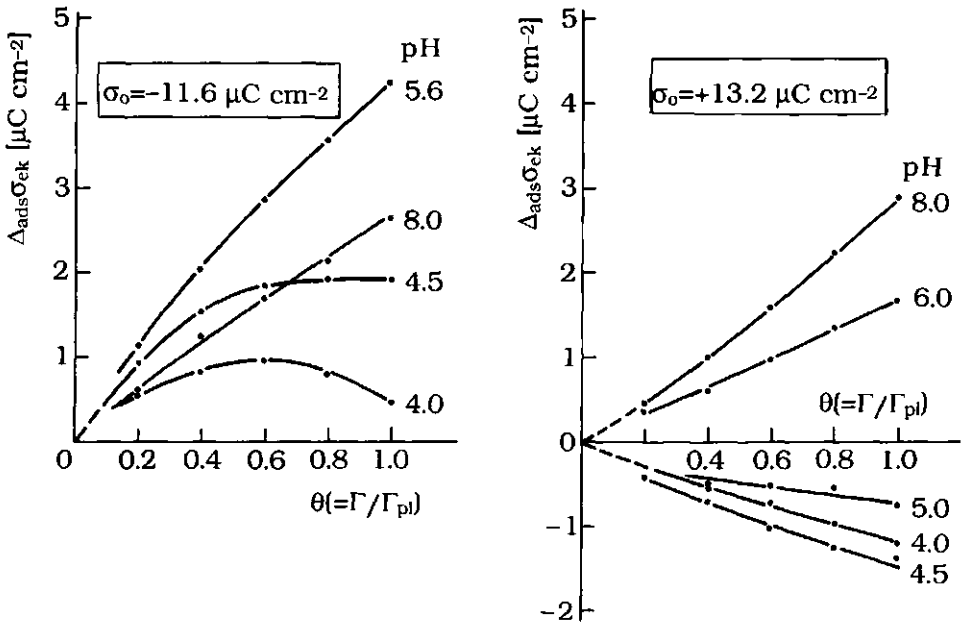


Figure 3.9 *Variation in electrokinetic charge density of PS latices as a function of the degree of coverage with BSA. The pH is indicated.*

Figure 3.9 shows $\Delta_{ads}\sigma_{ek}$ as a function of the surface coverage, $\theta=\Gamma/\Gamma_{pl}$, for two latices. As can be seen, the variation of $\Delta_{ads}\sigma_{ek}$ is proportional with θ, except for the negatively charged latex at pH=4.0 and 4.5. This proportionality indicates that the BSA molecules adsorb in a same fashion, irrespective of the surface coverage. The maximum in $\Delta_{ads}\sigma_{ek}(\theta)$ around θ=0.6 for the latex with σ_o=−11.6 μC cm^{-2} at pH=4.0 coincides with the coverage where reversal of the ζ-potential takes place, see Figure 3.5a. Beyond this charge reversal further adsorbing positively charged BSA molecules experience an electric repulsion from the covered surface, resulting in a decreased uptake of cations. Although such charge

reversals also occur with positively charged latices no maximum in $\Delta_{ads}\sigma_{ek}(\theta)$ has been observed. In the case of pH=4.5, at low coverage the proteins are compensating the PS surface charge by an uptake of cations whereas at higher coverages the BSA molecules adsorb without affecting $\Delta_{ads}\sigma_{ek}$.

A question still remains about the influence of the pre-adsorbed phosphate ions at the positively charged PS-particle on the protein adsorption process. Are they replaced by the (more) negatively charged adsorbing protein or will additional cations be incorporated upon adsorption? Both these events give a positive value of $\Delta_{ads}\sigma_{ek}$. It has been established [39] that the adsorption of phosphate ions to BSA is negligible. From this it may be inferred that phosphate ions are replaced from the surface. Moreover, it has been stated that interactions at a Human Serum Albumin/Calcium hydroxyapatite interface depend on the surface charge rather than on the electrokinetic charge [40]. Finally, from a study [41] of an 'opposite-like' situation, i.e. negatively charged PS-particles and BSA molecules in the presence of divalent cations (Ca^{2+}, Mn^{2+}), it was concluded that the pH where Γ_{pl} is maximal (pH=4.5) did not shift due to the binding of these cations to both latex and protein. Therefore, one might suggest that the influence of the phosphate ions on the adsorption of BSA is not determining the position of $\Gamma_{pl,max}$.

3.1.4 CONCLUSIONS

Although the information presented in this section generally confirm existing ideas about protein adsorption, it appears that some trends are quantitatively different. In particular, it is found that maximum adsorption as a function of pH is not determined by the i.e.p. of the protein but rather by that of the protein and particle together. This trend is not specific for the albumin-polystyrene latex system. It is more pronounced for the adsorption of immuno gamma globulin on polystyrene latices, as will be demonstrated in section 3.2.

Adsorption of proteins involves co-adsorption of low MW ions. This co-adsorption is needed to screen any excess potentials that may develop in the contact region between the protein and the charged latex surface, due to the tendency of the protein to expose certain groups to the latex. As a concequence, a study of this co-adsorption is essential to understand the overall protein adsorption process.

3.2 ADSORPTION OF DIFFERENT TYPES OF MONOCLONAL IMMUNOGLOBULIN [&]

ABSTRACT

Electrostatic aspects of the interaction between monoclonal mouse-anti-Human Chorionic Gonadotropin Immuno gamma Globulins and polystyrene latex particles are reported. Adsorption and electrophoresis experiments have been performed as a function of pH, using both negatively and positively charged latices and monoclonal Immuno gamma Globulins having different isoelectric points.

Adsorption isotherms show high affinity character and well-defined plateaus. The plateau-values vary with pH, showing a maximum at the isoelectric point of the protein-covered polystyrene particles, rather than at the isoelectric point of the Immuno gamma Globulin in solution Since a similar behaviour has been reported for Bovine Serum Albumin in section 3.1, this observation might be a more general feature in protein adsorption.

3.2.1 INTRODUCTION

The adsorption of immuno gamma globulins (IgG's) (antibodies) onto polymeric microspheres as an application for diagnostic test systems was first reported by Singer and Plotz [42]. The basic principle of such a test system is the agglutination of antibody-coated particles due to their immunological reaction with complementary antigen in solution (e.g. urine or plasma).

A main problem to solve in such applications is the orientation or, for that matter, the conformation of the antibodies on the microsphere. More specifically, the specific antigen combining site located in the so-called F_{ab}-part of the IgG molecule should be directed toward the solution. Furthermore, non-specific interactions of the antigen (and other species) with the IgG-coated microsphere and leakage of IgG molecules from the surface should be precluded. To overcome the above mentioned problems, different approaches dealing with the improvement of surface properties have been reported, e.g. optimization of the polarity (balance) of the substrate [43], application of spacers for covalent coupling of IgG molecules on the suface [44], immobilization of antibodies in a gel by radiation polymerization of hydrophilic monomers [45].

Besides optimization of adsorbent properties to control IgG adsorption, recent developments have led to the preparation of better defined protein samples. In 1976 Milstein and Köhler [46] discovered the 'hybridoma-technique', a method to produce

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[&] Accepted for publication in "Colloids and Surfaces"

monoclonal antibodies on a large scale. The antibodies from one clone are highly specific, since they are active against one single antigenic determinant (epitope). So far, most IgG adsorption studies used polyclonal antibodies [10,19,42,43,44,45,47], which are in fact mixtures of IgG molecules with different physical properties.

In this section the influence of the surface charge of (hydrophobic) polystyrene latices on the adsorption of monoclonal IgG's having different isoelectric points is reported. The use of such monoclonal antibodies is a convenient means to study the electrostatic contribution to the IgG-latex interaction.

3.2.2 EXPERIMENTAL

Materials

The used polystyrene latices were described in section 3.1.2.

Monoclonal immunogamma globulins (IgG's) were kindly donated by Organon Teknika (Boxtel, The Netherlands). Before receiving, the samples were Na_2SO_4 precipitated, dialyzed against 9 g dm^{-3} NaCl and stored at –20 °C. All IgG-samples were mouse-anti-HCG (Human Chorionic Gonadotropin) from isotype IgG-1.

The isoelectric points (i.e.p.'s) of the IgG's as were determined by Organon Teknika using Iso Electric Focussing (IEF), are tabulated in Table 3.3. In addition, we subjected the proteins in 5 mM phosphate buffer to Two Dimensional–IEF (polyacrylamide gel, Servalyte® 3–10 (Serva), FBE-3000 and ECPS 3000/150 (Pharmacia)). The resulting i.e.p.'s are also given in Table 3.3. For IgG-6A a lower i.e.p. was observed, probably caused by specific phosphate ion binding; for the others the two determinations agreed satisfactorily.

Table 3.3 *Isoelectric points of the monoclonal IgG's, the pH of performing HP-IEC and the monomer content of the purified samples determined by HP-SEC. In addition the IgG concentration in 0.05 M NaH_2PO_4 during storage is given.*

CODE	IEP (IEF)	IEP (2D-IEF)	pH (HP-IEC)	PURITY [%]	CONC. [mg cm^{-3}]
IgG-2B	4.8-5.1	4.9-5.2	4.0	99	5.9
IgG-7B	5.2-6.0	-	4.0	99	13.6
IgG-4F	6.9-7.3	6.8-7.0	4.5	97	12.0
IgG-6A	8.7-9.3	7.9-8.1	5.0	99	10.8

After receiving, the monoclonals were purified by High Performance Ion Exchange Chromatography (HP-IEC) using a Fractogel® TSK SP-650(S) column (Merck). The pH-

values at which the HP-IEC purifications were performed are given in Table 3.3. After purification the samples were analytically checked by High Performance Size Exclusion Chromatography (HP-SEC) and HP-IEC, using respectively a Biosil TSK 250 (Merck) and Biosil TSK SP-5(PW) (Merck) column. It was shown that they were almost mono-peak with respect to HP-SEC, of which the fraction is included in Table 3.3. However, IgG-2B and IgG-7B remain heterogeneous when analyzed by HP-IEC. In Figure 3.10 the existence of three different fractions is shown. The occurence of three different fractions has been confirmed by 2D-IEF (Pharmacia); the electrophoresis pattern shows three bands, having an identical i.e.p.~5. Also for IgG-4F and IgG-6A two or three bands on a 2D-IEF gel could be distinguished for pH≥i.e.p.. However, from adsorption experiments (see below) it can be concluded that no preferential adsorption of either one of these fractions took place. Therefore, the monoclonals purified as described above have been used.

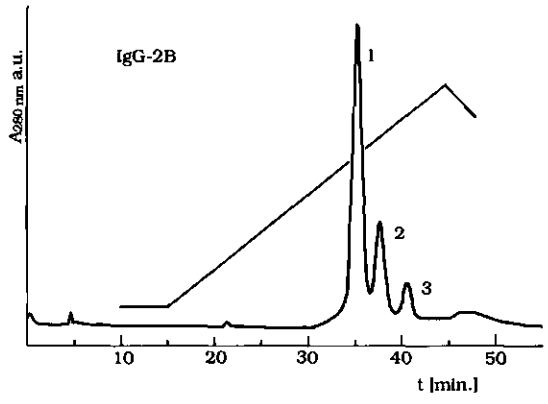

Figure 3.10 *Analytical Ion Exchange Chromatogram, showing three different fractions within one monomeric monoclonal (IgG-2B, i.e.p.~5). Analysis was performed at pH=4.5.*

After being analyzed, the samples were ultrafiltrated with 0.05 M NaH_2PO_4 at pH=6.5 using a 50 cm^3 ultrafiltration cell (Amicon) with a 43 mm XM50 filter (Diaflo®). The concentrations of the purified monoclonals (see Table 3.3) were determined by UV-spectroscopy at 280 nm (Hitachi 150-20 Spectrophotometer), using an extinction coefficient $\varepsilon(280\ nm)=1.45\ cm^2\ mg^{-1}$ [48]. The IgG solutions were divided into small volumes, stored at -20 °C and thawed once shortly before use.

Titrating each monoclonal in 5 mM NaH_2PO_4 from pH 4 to 10 with 2N NaOH (and 1 M HCl from pH 10 to 4) and measuring the absorbance at 280 nm resulted only for IgG-6A in a turbid solution between pH-values of 5.5 and 7.5.

In Table 3.4 the amino acid composition of the different monoclonals is given. As can be seen, the mutual differences are small which underlines that different monoclonals of the

same IgG-isotype consist of a large constant part (including the F_c-unit) and a small variable parts in the two F_{ab}-fragments.

Water was purified by reverse osmosis and subsequently passed through a Super-Q system (Millipore). All other chemicals were of analytical grade and used without further purification.

Table 3.4 *Amino acid analysis (AAA) of the four monoclonals. Hydrolysis is performed in 5.7 N HCl at 105 °C during 24 hours. The results are expressed in mg amino acid per 100 mg protein.* [a] *Due to hydrolysis it is not possible to discriminate between ASN and ASP and between GLN and GLU.*

	Monoclonal:			
Amino Acid	IgG-2B	IgG-7B	IgG-4F	IgG-6A
ASN/ASP [a]	9.26	9.62	10.35	9.61
THR	8.17	8.59	7.62	8.21
SER	11.00	9.65	10.30	10.12
GLN/GLU [a]	11.50	12.67	11.61	11.05
PRO	7.88	5.60	4.89	5.67
GLY	2.82	3.12	3.21	3.24
ALA	3.91	3.74	3.17	3.09
$(CYS)_2$	1.74	1.90	1.90	1.74
VAL	6.34	6.34	6.73	6.26
MET	1.70	1.36	1.63	1.49
ILE	3.46	3.85	3.89	3.74
LEU	5.34	6.59	6.58	7.59
TYR	6.31	5.99	6.21	5.04
PHE	5.18	5.14	5.76	5.94
LYS	7.60	7.83	8.58	8.32
HIS	2.53	2.69	2.14	3.08
ARG	4.09	3.84	4.02	4.14
TRP	1.15	1.48	1.40	1.66

Adsorption isotherms

A representative adsorption experiment was started with a 2 cm^3 polystyrene tube (Greiner) containing 1.65 cm^3 IgG solution with a concentration varying between 0–0.50 mg cm^{-3} of a given pH. To this solution 0.25 cm^3 polystyrene latex of the same pH and representing about 0.08 m^2 was added. The final buffer concentration in all experiments was 5 mM NaH_2PO_4. The buffer concentration was kept low to allow for relatively strong electrostatic interactions between adsorbent and protein.

The tubes were rotated for about 20 hours at 21 °C. During this incubation time the change in pH did not exceed 0.2 units. The samples were centrifugated for 15 min. at 4.71×10^4 m s^{-2} (Sarstedt MH2). The supernatant was taken from the sample to determine the protein concentration; the sediment was resuspended overnight in 5 mM NaH_2PO_4 of the same pH and used for electrophoresis experiments within one day.

The concentration of IgG in the supernatant was determined by different methods:

a) FPLC®-SEC, using a Superose-12 column (Pharmacia) with UV monitoring at 206 nm (Uvicord S, LKB). The IgG concentration was related to a calibration curve using the peak height as a measure. Column interactions may occur when proteins (like IgG) have an i.e.p. comparable or larger than the pH of the eluents, in those cases method b) is used as an alternative.

b) Sol Particle Immuno Assay (SPIA®), using a Reader Microelisa® system with a 540 nm filter (Organon Teknika). The method is well documented in references [49,50]. It is a very sensitive method, allowing the detection of concentrations down to 30 ng cm^{-3}.

c) FPLC®-IEC, using a Mono S™ column (Pharmacia) with UV monitoring at 206 nm. This method was used to verify whether preferential adsorption of one of the fractions of monoclonal 2B took place. To that end, adsorption experiments were carried out with solutions by pooling different fractions obtained during the preparative purification step. Its composition was 42% fraction 1, 38% fraction 2 and 20% fraction 3. As a reference a solution consisting of 97% fraction 1 and 3% fraction 2 was used. Both on negatively (σ_o=-11.6 μC cm^{-2}) and positively (σ_o=+7.8 μC cm^{-2}) charged latices this adsorption experiment was performed at pH=4.0 with an adsorption time of 16 hours. No preferential adsorption of any fraction was observed.

Electrophoresis

All experiments were performed with a MK2 microelectrophoresis Zeta Sizer (Malvern Instruments Ltd.) at 25 °C. Prior to a measurement about 0.3 cm^3 (IgG-coated) latex was mixed with 14.7 cm^3 5 mM NaH_2PO_4 of the same pH. The final solution contained about 5.10^8 particles per cm^3. Using SPIA as the detection method, desorption of IgG upon dilution with buffer was never found in any system subjected to electrophoresis. The influence of centrifugation, followed by resuspension, on the mobility of the (IgG-coated) particles was checked by comparing with 'non-centrifuged' samples. No difference in mobility was observed.

The obtained mobilities were transformed into zeta potentials using the procedure of O'Brien and White [18].

3.2.3 RESULTS AND DISCUSSION

Adsorption experiments

The adsorption isotherms of every possible IgG-latex combination have the following features in common:

(i) In all but one (IgG-6A on positively charged latex at pH 4.0) case the initial part of the isotherm merges with the Γ-axis. Because the initial part of the isotherm reflects the interaction between the protein and the adsorbent surface it is concluded that the proteins adsorb with a high affinity. In this context it is repeated that the SPIA-technique allows detection of very low IgG concentrations. As these virtually infinitely steep initial slopes were observed irrespective of the sign of the electrostatic interaction between protein and substrate (as judged by the signs of the electrophoretic mobilities and the i.e.p.'s of the proteins) it is concluded that the overall interaction between the protein and polystyrene surface is not dominated by electrostatic forces. Less high affinity adsorption have been reported for the adsorption of goat and rabbit IgG on anionic polyvinyltoluene latices [19] and human IgG on negatively charged polystyrene latices [51,52]. It might be speculated that this is a result caused by using polyclonal IgG samples.

(ii) In all cases the adsorption isotherms show well-defined plateaus. In Figure 3.11 these plateau values (Γ_{pl}) are given as a function of pH for the various combinations of monoclonals and PS-latices. All Γ_{pl}(pH)-curves exhibit a maximum ($\Gamma_{pl,max}$). The pH where Γ_{pl} attains its maximum increases with increasing i.e.p. of the monoclonal. These pH-values are the same for the two negatively charged latices, although $\Gamma_{pl,max}$ is larger for the more negatively charged latex. Furthermore, the difference between $\Gamma_{pl,max}$ between the two negatively charged latices is about 1.5 mg m^{-2} for all monoclonals. It suggests that the adsorption behaviour around this maximum is rather similar for the various monoclonals on these surfaces. IgG-2B and IgG-7B having a 'low' i.e.p. in solution show maximum adsorption on negatively charged latices around this i.e.p.. A similar observation was made with the adsorption of Bovine Serum Albumin (i.e.p.=4.7-5.0) on the same latices [53]. With increasing i.e.p. of the monoclonal (IgG-4F and IgG-6A), the pH of maximum adsorption onto negatively charged latices shifts to the acid side of the i.e.p. of the solute protein.

For the latex having σ_o=+7.8 μC cm^{-2} $\Gamma_{pl,max}$ occurs about one to two units more alkaline as compared to the negatively charged latices. Furthermore, it seems that the value of $\Gamma_{pl,max}$ increases with the 'negative character' of the adsorbent surface, although in this respect IgG-2B adsorption on the positively charged latex deviates. The last could possibly be ascribed to the higher proline content in the monoclonal, see Table 3.4. It is well known that this amino acid causes a decrease in the stability of the (secondary) structure of

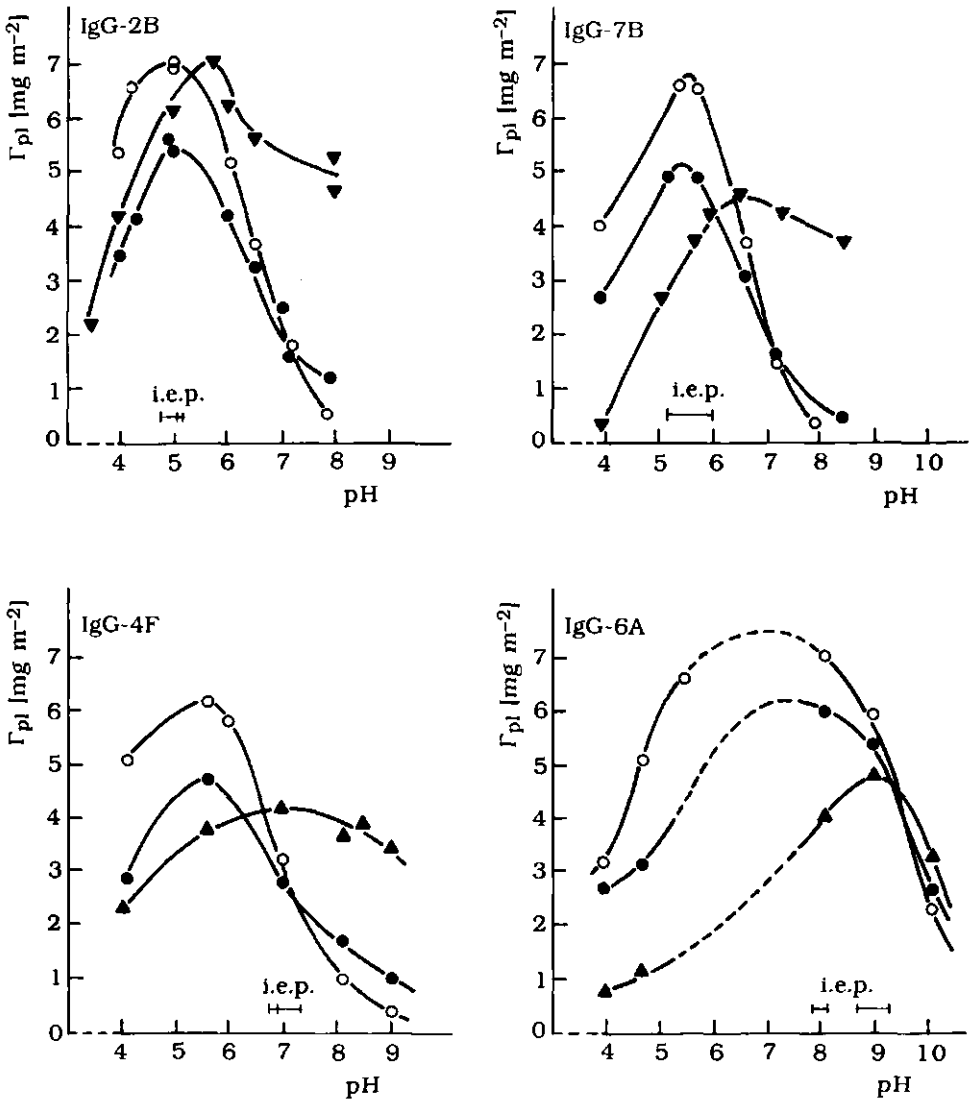

Figure 3.11 *Plateau adsorption of four monoclonal IgG's on PS latices with different surface charge densities. Because of instability of monoclonal IgG-6A in the pH region between 5.5 and 7.5 the corresponding parts of the curves are interpolated (dashed lines in Figure 3.11d). The i.e.p.'s of the monoclonals are indicated.* (○): σ_o=−11.6 μC cm^{-2} (●): σ_o=−5.5 μC cm^{-2} (▼,▲): σ_o=+7.8 μC cm^{-2}

proteins [54]. It could therefore be that the monoclonal 2B adapts its structure more easily to the (positively charged) surface.

For a number of other proteins, evidence is available to indicate that the most important contribution to the occurence of a maximum in the Γ_{pl}(pH)-curves is the decreasing conformational stability of the protein with increasing net charge on the molecule [2]. This results in a larger surface area per molecule due to a greater tendency to structural rearrangements of the adsorbing molecules. In the literature maximum protein adsorption around the isoelectric point of the protein has been reported for various proteins, amongst others immuno gamma globulins [19], adsorbed on negatively charged surfaces. The scarce data referring to protein adsorption on positively charged surfaces do indeed point to an alkaline shift in the pH for which Γ_{pl} attains its maximum value [26], confirming the results described in this paper.

In view of the foregoing it can be stated that electrostatic interactions are not decisive as to the occurrence of IgG adsorption onto PS particles, but that they nevertheless influence the interaction significantly. After presenting the electrophoresis data we shall return to this matter.

Electrophoresis

The electrophoresis experiments were performed to obtain information on the electrostatic interaction between the IgG molecules and the PS latices. Furthermore, it allows one to relate the mobility of (IgG-coated) PS particles to the colloidal stability of these latices. In Figure 3.4 the ζ-potential of bare PS particles has already been presented as a function of pH. The most striking feature is the dramatic decrease of the ζ-potential of the positively charged latex with increasing pH due to specific phosphate ion adsorption. As suggested from literature data [53], specifically adsorbed phosphate ions are not affecting $\Gamma_{pl,max}$.

In Figure 3.12 ζ-potentials of the IgG-PS complexes for IgG-2B on three latices are given as a function of the amount adsorbed IgG. With increasing Γ the absolute value of the ζ-potential decreases to reach more or less a plateau value. This decrease is dependent on the pH, i.e. on the charge of the IgG molecule. For all monoclonal IgG's the variation of the ζ-potential with Γ for the latex with σ_o=–5.5 μC cm^{-2} is more pronounced than for σ_o=–11.6 μC cm^{-2}. The same trend was found for Bovine Serum Albumin [53].

The encircled points in Figure 3.12 indicate flocculation of the (coated) particles. Flocculation of IgG-covered PS particles already occurs at much higher ζ-potentials compared to BSA adsorption on the same latices [53]. The flocculation of negatively charged

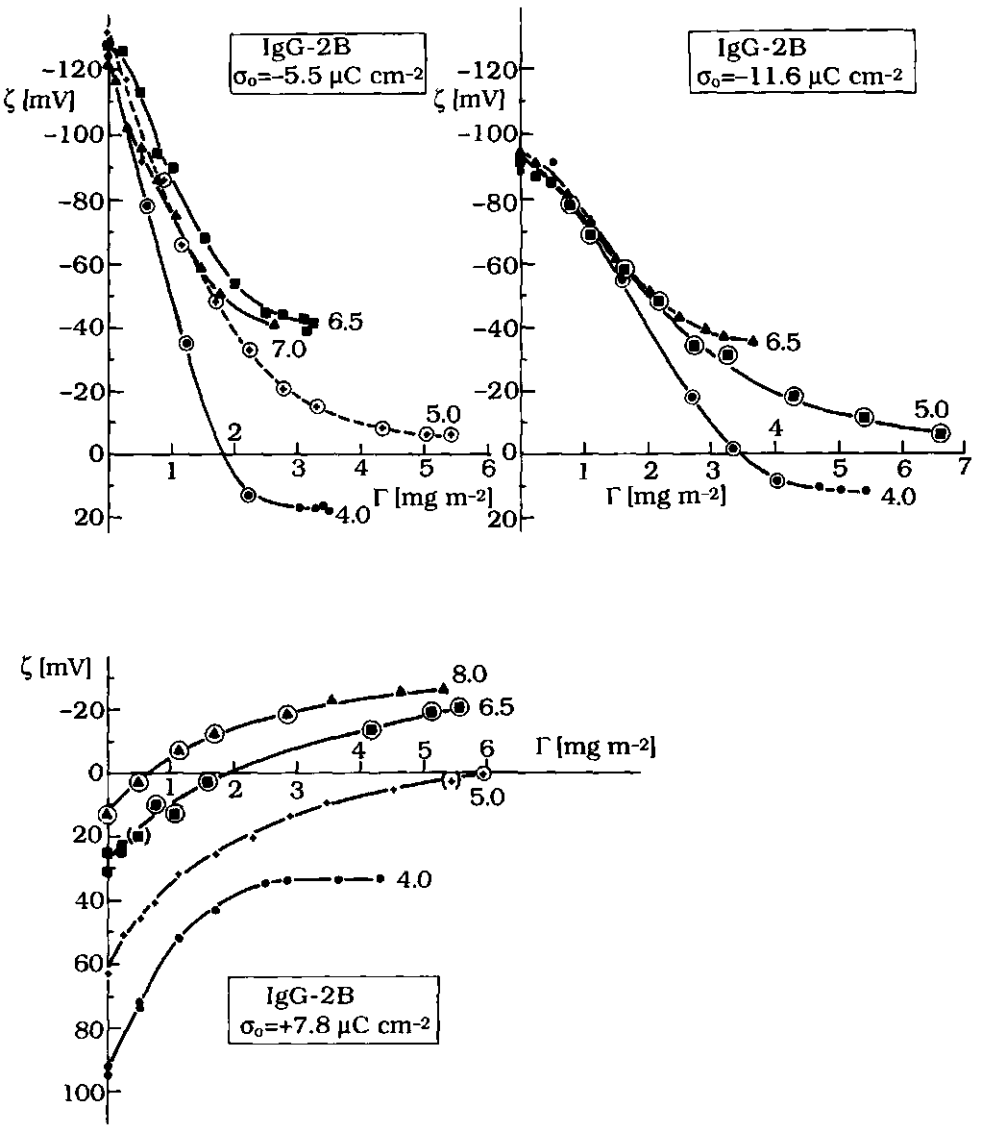

Figure 3.12 *The ζ-potentials of the IgG -PS complexes as a function of the adsorbed amount of monoclonal IgG-2B on three latices. The pH is indicated. Encircled points indicate flocculation.*

latices by monoclonal IgG's at low surface coverage is probably the result of a 'bridging'-mechanism, whereas flocculation of BSA/PS-particles has been ascribed to charge neutralization. In the literature, the occurrence of a 'bridging'-mechanism for the adsorption of (polyclonal) IgG on negatively charged PS-latices was concluded from QELS [51] and turbidity measurements [55].

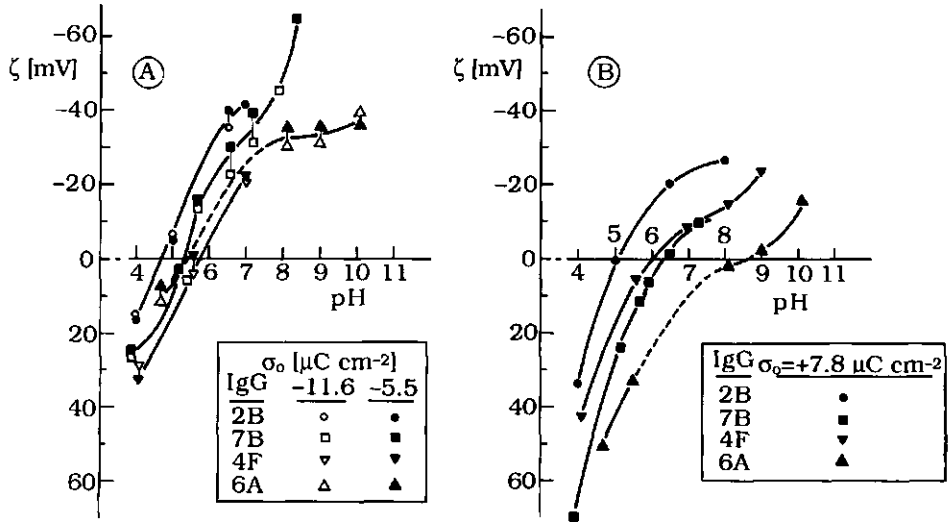

Figure 3.13 *The ζ-potentials of PS latices completely covered with IgG ($\Gamma=\Gamma_{pl}$) for A) negatively charged latices and B) positively charged latex.*

Figure 3.13 shows the ζ-potentials at complete coverage ($\Gamma=\Gamma_{pl}$) as a function of pH for both the negatively charged latices and the positively charged latex. As expected in view of the charge on the monoclonals, ζ-potentials of IgG-covered latices become more negative with increasing pH. However, there is a significant difference whether IgG is adsorbed on a negatively or a positively charged surface. This difference can be related to electrostatic interactions, including co-adsorption of low MW ions, between protein and latex [36].

Because electrokinetic data for the solute monoclonals are not at our disposal, the electrokinetic charge densities of the dissolved IgG molecules can not be established. Consequently, it is not possible to derive the participation of low MW ions in the overall adsorption process by comparing the electrokinetic charges before and after adsorption, as has been done for Bovine Serum Albumin [53].

Adsorption maxima as a function of pH

Combining the findings of the two foregoing sections, it is concluded that the maximum in Γ_{pl}(pH) occurs at the isoelectric pH of the IgG-latex complex. In Figure 3.14 this coincidence is illustrated. This observation applies to all IgG monoclonals and all latices studied, as well as for BSA (also indicated in the figure).

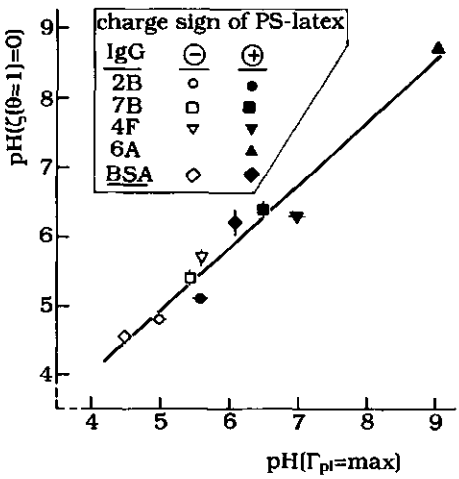

Figure 3.14 *Relation between the isoelectric point of the protein-PS latex complex ($\Gamma=\Gamma_{pl}$) and the pH where Γ_{pl} reaches a maximum value. Data of BSA are taken from section 3.1.*

For systems where the i.e.p. of the dissolved protein and the protein-latex complex coincide, the latex surface charge must be fully compensated by the co-adsorption of low MW ions. This co-adsorption is needed to screen any excess charge that may develop in the contact region between protein and latex and it depends on the sign of the surface charge. When a difference is observed between the isoelectricity of the protein itself and its complex with latex particles, the PS surface charge must compensate (at least partly) the charge on the protein. The consequence is that less co-adsorption of low MW ions is required to compensate excess charge in the contact region between protein and latex.

In literature the pH-coincidence between maximum protein adsorption and the isoelectricity of the protein-latex complex has, to our knowledge, only been reported for protein adsorption on negatively charged surfaces, as described previously [53].

3.2.4 CONCLUSIONS

The use of SPIA and FPLC-SEC as detection methods gave comparable (and complementary) results for the adsorption of IgG on PS latices, as calculated from depletion measurements. The main result of this study is the observation of maximum protein

adsorption as a function of pH around the isoelectricity of the protein-latex complex, rather than at the i.e.p. of the solute protein. This trend has also been observed for Bovine Serum Albumin [53] and may therefore also apply to other proteins and other substrates. It would be interesting to check the general validity of this observation.

Comparing the i.e.p. of the dissolved protein with that of the protein/PS complex indicates co-adsorption of low MW ions. In studying these effects the use of monoclonal IgG's having a well-defined isoelectric point, rather than polyclonal IgG exhibiting a range of i.e.p.'s, is mandatory.

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CHAPTER 4

SEQUENTIAL PROTEIN ADSORPTION ON DIFFERENTLY CHARGED POLYSTYRENE LATICES

4.1 INTRODUCTION

Sequential protein adsorption is a two-step process. First, one type of protein is adsorbed onto a surface, followed by the adsorption due to addition of a second protein to this protein-adsorbent complex. Adsorption of the second protein may involve displacement of the pre-adsorbed protein. From the literature it is inferred that many variables are relevant to describe sequential protein adsorption, including the amount and residence time of the pre-adsorbed protein [1,2], as well as conditions at which the second protein is supplied (e.g. pH, adsorption time, concentration) [1,3].

Sequential adsorption has to be considered in the development of diagnostic test systems in which immunologically active proteins are pre-adsorbed on a carrier. To suppress non-specific interactions of the complementary antigen, non-occupied parts of the adsorbent surface have to be covered with a second protein [4,5,6]. Besides this sequential adsorption, partial displacement of pre-adsorbed protein could be desirable to obtain a (homogeneous) population of immunoglobulins that are strongly attached to the surface.

This chapter deals with sequential adsorption of albumin and immunoglobulins emphasizing the role of electrostatic interactions. Therefore, experiments are performed at various pH-values, i.e. different charges on the protein molecules, using differently charged polystyrene latices.

4.2 EXPERIMENTAL

MATERIALS

Polystyrene latices with surface charge densities (σ_0) of −11.6, −5.5, +7.8 μC cm^{-2} have been used. The proteins studied are Bovine Serum Albumin (BSA) and monoclonal Immuno gamma Globulins 2B, 4F and 6A (Organon Teknika, Boxtel, The Netherlands); their isoelectric points (i.e.p.'s) are given in Table 4.1.

Table 4.1 *Isoelectric points of the proteins used.*

Protein	BSA	IgG-2B	IgG-4F	IgG-6A
i.e.p.	4.7-5.0	4.9-5.2	6.8-7.0	7.9-8.1

A few experiments were performed with BSA containing 97 wt% dimers. This fraction has been obtained by collecting heads during the preparative BSA purification (as described in chapter 3). All experiments were performed in low ionic strength (electrolyte 5 mM NaH_2PO_4), to prevent suppression of the electrostatic component of the interaction. Water was purified by reverse osmosis and subsequently passed through a Super-Q system (Millipore). All other chemicals were of analytical grade and used without further purification. In chapter 3 more characteristics of the latices and the proteins are presented.

METHODS

Procedure for sequential adsorption

The procedure for the sequential adsorption experiments is as follows:

1 The first protein (1) is adsorbed under saturation conditions ($\Gamma=\Gamma_{pl}$) at pH_1 during a period $t_{ads,1}$. In chapter 3 the Γ_{pl}-values as a function of pH for single adsorption of BSA and IgG's are given. A typical adsorption experiment was performed in a 2 cm^3 polystyrene tube (Greiner) containing 1.65 cm^3 protein solution to which 0.25 cm^3 polystyrene latex, representing about 0.08 m^2 was added.

2 After $t_{ads,1}$, the sample was centrifuged during 10-15 min at 4.71×10^4 m s^{-2} (Sarstedt MH2) and the supernatant replaced by a 5 mM NaH_2PO_4 solution of the same pH. After gently resuspending the latex sediment for about 20-30 min., the sample was centrifuged again. The replacement step with buffer was necessary to reduce the concentration of non-adsorbed protein (1), since these protein molecules might interfere with the adsorption of the secondly supplied protein. Desorption upon dilution was never observed.

3 The latex sediment, precoated with protein (1), was resuspended in a solution of protein (2) of concentration c_2 and pH_1 and incubated for a period of $t_{ads,2}$. The initial concentration of protein (2) c_2 is expressed in mg per latex surface area. The latex surface area was somewhat increased to 0.050 m^2 cm^{-3} to improve the accuracy of the analytical determination of the displaced amount.

4 After $t_{ads,2}$ the sample was centrifuged, as described in step **2**, followed by the determination of the concentration of both proteins in the supernatant. To that end two analytical methods were used: (i) SPIA for the determination of (low) concentrations of displaced protein (1) and (ii) FPLC-SEC for the determination of the additionally adsorbed amount of protein (2). Both methods are described in chapter 3. In the case where monoclonal 6A was the secondly supplied protein, the concentrations of BSA and IgG-6A were both determined by SPIA.

The influence of the one protein on the SPIA determination of the other has been tested. Concentrations up to 0.50 mg cm^{-3}, which are beyond the highest concentration c_2 used, did not affect the agglutination of protein (1) in a SPIA-test. In addition, displacement of protein (1) has been checked qualitatively with FPLC-SEC, since the concentrations are rather small.

Two-Dimensional Iso Electric Focussing (2D-IEF)

To quantify the influence of electrostatic interactions between protein (2) and the protein (1)-latex complex, the electrokinetic charge of solute proteins should also be known. However, electrophoretic mobilities of dissolved proteins are hard to obtain. Instead, 2D-IEF measurements were performed to obtain information on the electrostatic state of the proteins. These electrophoretic displacement (d) data are used as a semiquantitative measure of electrophoretic mobilities. In Figure 4.1 a 2D-IEF (polyacrylamide gel, Servalyte 3-10 (Serva) using a FBE-3000 and ECPS-3000/150 (Pharmacia)) picture of four proteins is given.

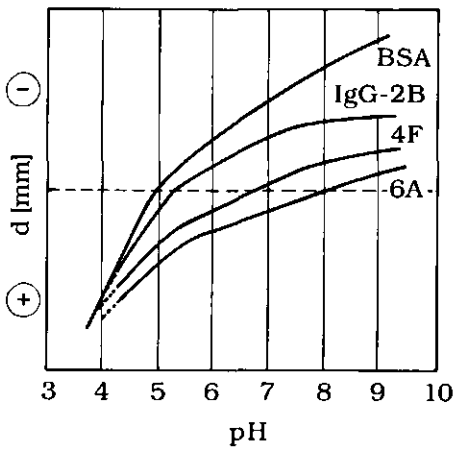

Figure 4.1 *Separate 2D-IEF experiments of BSA and monoclonals 2B, 4F and 6A are combined in one drawing. Migration of the proteins took place at 500 V during 30 min.*

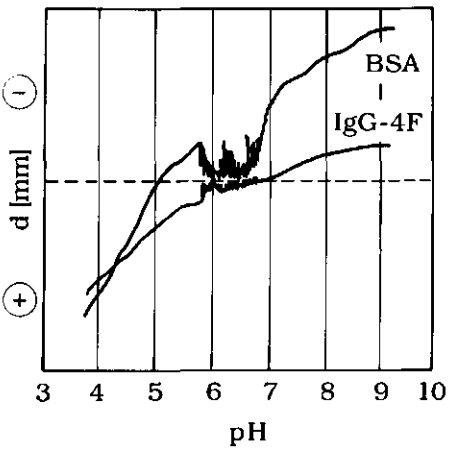

Figure 4.2 *A 2D-IEF picture of a solute mixture of BSA and IgG-4F (see text for further details).*

Furthermore, to obtain information on electrostatic interactions between dissolved BSA and IgG, a solution of BSA (0.50 mg cm^{-3}) and IgG-4F (0.60 mg cm^{-3}) at pH=5.7 was kept at rest for 2 hrs. and subsequently subjected to 2D-IEF. From Figure 4.2 it can be inferred that

under conditions of electrostatic attraction between BSA and IgG aggregation in solution occurs. It is expected that such aggregation will take place during the sequential adsorption experiments.

Electrophoresis

Electrophoretic mobility measurements of (coated) polystyrene particles have been described in section 3.1 and 3.2.

4.3 RESULTS AND DISCUSSION

4.3.1 COMPETITION BETWEEN BSA AND PRE-ADSORBED IgG

Because the systems studied are very complex and the process of sequential adsorption intricate, the effects of the experimental variables are strongly interrelated.

Effect of adsorption time ($t_{ads,1}$ and $t_{ads,2}$)

FPLC-SEC analysis has been shown that, at room temperature, BSA in aqueous solution starts to degrade after ca. 45 hrs.. For this reason the adsorption time $t_{ads,2}$ of BSA should be kept within that period of time. Furthermore, one must keep in mind that $t_{ads,2}$ indirectly influences $t_{ads,1}$; longer $t_{ads,2}$ give pre-adsorbed molecules more time to optimize their interaction with the surface. Figure 4.3 shows the fraction IgG displaced from the surface $(\Delta\Gamma/\Gamma)_{IgG}$ by BSA as a function of its adsorption time ($t_{ads,1}$) for two different situations, namely:

#1: IgG-2B (i.e.p.=4.9-5.2) is adsorbed on the latex having σ_o=−11.6 μC cm^{-2} at pH=5.0 and

#2: IgG-4F (i.e.p.=6.8-7.0) is adsorbed on the positively charged latex at pH=8.5.

The amounts of IgG adsorbed after $t_{ads,1}$ are denoted in Figure 4.3.

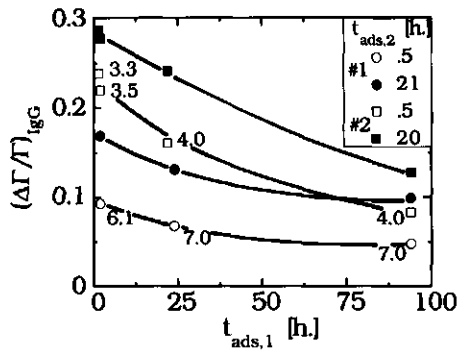

Figure 4.3 *Fraction of IgG displaced by BSA as a function of its pre-adsorption time. For two situations (#1 and #2, see text) c_2=3.5 mg m^{-2}; $t_{ads,2}$ and the adsorbed amount of IgG are indicated.*

BSA is able to displace a fraction of the pre-adsorbed IgG. A decreased displaced fraction is found with increasing $t_{ads,1}$, indicating stronger attachment (structural or orientational rearrangement) of the IgG molecules at the surface. In situation #2 displacement is larger than that in situation #1. It is shown below that this may be related to the larger adsorbed amounts of pre-adsorbed protein. An indication for rearrangement of the pre-adsorbed molecules is obtained for situation #2 by comparing the amount displaced after $t_{ads,2}$=0.5 hr. with that after $t_{ads,2}$=20 hrs. For short pre-adsorption times ($t_{ads,1}$=1.5–2.0 hrs.) the ratio $(\Delta\Gamma/\Gamma)_{IgG}(t_{ads,2}$=0.5 hr.$)/(\Delta\Gamma/\Gamma)_{IgG}(t_{ads,2}$=20 hrs.) is 0.80, meaning that 80% of the displacement over 20 hours has already taken place within the first 30 min.. For longer pre-adsorption times ($t_{ads,1}$=22–94 h.) this ratio is significantly smaller, i.e. ca. 0.65, indicating that the molecules had more time to rearrange at the surface and, consequently, are more difficult to displace from the surface. For situation #1 the ratio is nearly invariant with $t_{ads,1}$, which may, again, be explained in terms of a larger adsorbed IgG amount.

Effect of BSA concentration on IgG displacement

The influence of the BSA concentration (c_2) with $t_{ads,2}$=21 hrs. on the displacement of pre-adsorbed IgG has been investigated for three sets of conditions. The conditions are tabulated in Table 4.2.

Table 4.2 *Pre-adsorption of different monoclonals, $t_{ads,1}$=23 hrs.*

situation	IgG code	IgG i.e.p.	latex σ_0 [μC cm^{-2}]	pH_{ads}	Γ_{pl} [mg m^{-2}]
#1	2B	4.9-5.2	−11.6	5.0	7.0
#2	4F	6.8-7.0	+7.8	8.5	4.0
#3	6A	7.9-8.1	+7.8	5.5	2.6

In Figure 4.4 the displaced fraction of IgG is plotted against c_2 [mg m^{-2}]. Increasing c_2 results in a larger $(\Delta\Gamma/\Gamma_{pl})_{IgG}$, which seems to approach a plateau. The increase of $(\Delta\Gamma/\Gamma_{pl})_{IgG}$ with c_2 is in accordance with the dynamic behaviour of adsorbed proteins (section 2.3). The probability of desorption of IgG molecules increases when more BSA molecules are available to adsorb. The low value for $(\Delta\Gamma/\Gamma_{pl})_{6A}$ may be explained by its rather low pre-adsorbed amount (Γ_{pl}=2.6 mg m^{-2}) involving a larger fraction of each adsorbed IgG molecule in contact with the surface. Such a variation of the attached fraction has also been found by Morrissey [8] for IgG on silica. It is furthermore interesting to

compare the sequential adsorption of monomeric BSA with dimeric BSA, as has been done for situations #1 and #3. Figure 4.4 shows that at a given mass concentration, dimeric BSA displaces more IgG than monomeric BSA does (even though the molar concentration of the BSA dimer is half of that of the monomer).

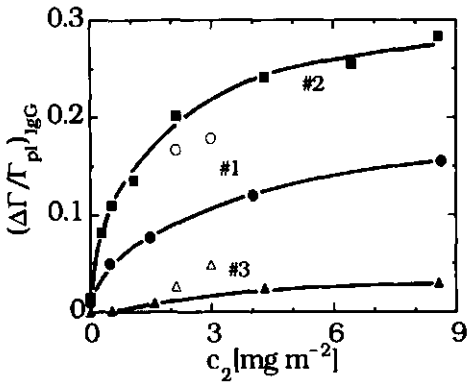

Figure 4.4 *Fraction of IgG displaced as a function of the concentration of added BSA for three situations (see text). Open symbols indicate dimeric BSA.*

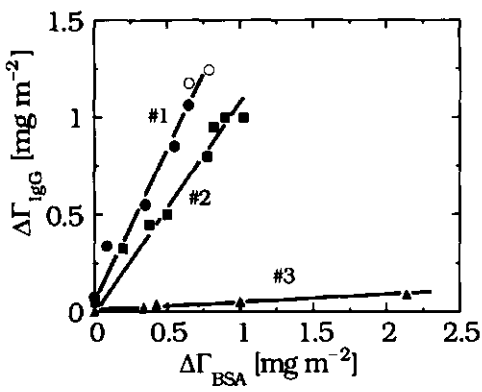

Figure 4.5 *Displaced amount of IgG as a function of additionally adsorbed amount of BSA for three situations, as indicated in the text.*

In Figure 4.5 the displaced amount of IgG ($\Delta\Gamma_{IgG}$) is plotted against the pertaining sequentially adsorbed amount of BSA ($\Delta\Gamma_{BSA}$). All three situations show a linear relationship. In situation #3, a large additional adsorption of the second protein does not involve displacement of the pre-adsorbed one. In view of the 2D-IEF results for oppositely charged BSA and IgG-4F, the formation of a protein complex between negatively charged BSA and positively charged IgG molecules on the surface is very probable. The slopes of the lines in Figure 4.5 increase with increasing pre-adsorbed amounts, indicating that fewer BSA molecules are needed for displacement when more IgG molecules are pre-adsorbed.

Effect of the amount of pre-adsorbed IgG

Above, the influence of the adsorption times ($t_{ads,1}$ and $t_{ads,2}$) and c_2 on the extent of displacement of IgG and additional adsorption of BSA was examined. Now the influence of the amount of pre-adsorbed IgG will be discussed. Sequential adsorptions are studied for a set of different situations, i.e three IgG's and three latices at various pH-values. The

displaced amount of IgG ($\Delta\Gamma_{IgG}$) and additional BSA adsorption ($\Delta\Gamma_{BSA}$) are given in Table 4.3.

Table 4.3 *Competition of BSA with pre-adsorbed monoclonals ($t_{ads,1}$=$t_{ads,2}$=21 hrs. and c_2=3.5 mg m^{-2}).*

IgG	σ_0 (latex) [μC cm^{-2}]	pH_{ads}	Γ_{pl}(IgG) [mg m^{-2}]	ζ_{PS} [mV]	$\zeta_{IgG/PS}$ [mV]	d_{BSA} [nm]	d_{IgG} [nm]	$\Delta\Gamma$ [mg m^{-2}] IgG	BSA
2B	−11.6	4	5.3	−88	+15	+27	+28	0.46	0.65
		5	7.0	−92	−6	0	+5	1.20	0.56
		6	5.1	−94	−30	−13	−7	0.26	0.56
		8	0.4	−96	−45	−31	−14	0	0.69
	−5.5	4	3.5	−128	+16	+27	+28	0.12	1.20
		5	5.6	−134	−5	0	+5	0.75	0.35
		6	4.4	−132	−30	−13	−7	0.13	0.39
		8	1.4	−120	−45	−31	−14	0	0.48
	+7.8	4	3.3	+95	+33	+27	+28	0.09	0.02
		5	6.4	+60	+1	0	+5	0.65	0.09
		6	6.5	+40	−20	−13	−7	0.46	0.28
		8	5.1	+10	−26	−31	−14	0.26	0.65
4F	−11.6	4	5.1	−88	+28	+27	+29	0.19	0.74
		5.5	6.0	−93	+4	−7	+9	0.39	0.35
		7	3.2	−95	−20	−22	−1	0.04	0.04
		8.5	0.6	−96	?	−35	−9	0	?
	−5.5	4	2.9	−128	+32	+27	+29	0.04	?
		5.5	4.5	−133	−1	−7	+9	0.22	0.22
		7	2.7	−125	−21	−22	−1	0	?
		8.5	1.4	−112	?	−35	−9	0	0.26
	+7.8	4	2.2	+95	+42	+27	+29	0	0.14
		5.5	3.7	+50	+5	−7	+ 9	0.10	1.06
		7	4.1	+20	−9	−22	−1	0.74	0.65
		8.5	3.9	+20	−18	−35	−9	0.78	0.97
6A	−11.6	4	3.1	−88	+19	+27	+33	0	0.13
		5.5	6.5	−92	0	−7	+14	0.39	0.22
		9	6.0	−94	−31	−38	−4	0	?
	−5.5	4	2.7	−128	+15	+27	+33	0	0.09
		5.5	4.1	−133	0	−7	+14	0.21	0.13
		9	5.4	−108	−35	−38	−4	0	?
	+7.8	4	0.8	+95	+60	+27	+33	0	0.14
		5.5	1.3	+50	+34	−7	+14	0	1.20
		9	4.8	+3	−2	−38	−4	1.22	0.88

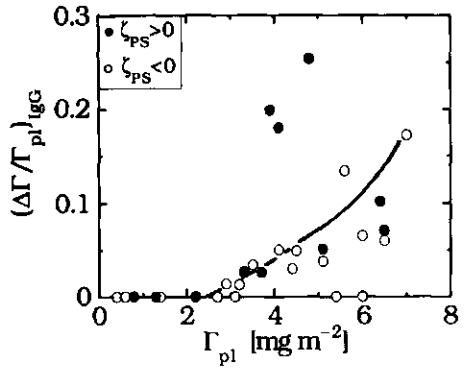

Figure 4.6 *Displaced fraction of IgG as a function of its pre-adsorbed plateau amount. In addition, the sign of the bare latices is given, see inset.*

In Figure 4.6 the fraction displaced IgG is plotted as a function of the plateau-value of pre-adsorbed IgG. Displacement of IgG occurs only when pre-adsorbed amounts are larger than about 3 mg m^{-2}. Easier displacement at higher surface coverage may be the result of a smaller adsorbent surface area occupied per adsorbed protein molecule [1,2]. From single protein adsorption (chapter 3) it was concluded that Γ_{pl} reaches a maximum at isoelectric conditions for the protein-latex complex. This is the reason that displacement of pre-adsorbed IgG is larger around the isoelectric state of the protein-latex complex. Furthermore, for the additional adsorption of BSA no simple correlation is found with Γ_{pl} of IgG, indicating that factors other than those related to the pre-adsorbed amount of IgG, such as electrostatic interactions, play a role in sequential adsorption.

Effect of electrostatics

In view of the foregoing, we now discuss the influence on the displacement of IgG by BSA in further detail. If displacement of IgG were electrostatically influenced, it should be expected that the extent of displacement increases if the interaction between BSA and the latex is electrostatically more favourable than that between IgG and the latex surface. The electrostatic interaction energy between two charged species contains the product of their surface potentials [8]. As mentioned before, ζ-potentials of dissolved IgG's are not available; as a measure for that quantity the electrophoretic displacement (d_{BSA}, d_{IgG}) in 2D-IEF of the proteins, derived from Figure 4.1, are used. The ζ-potentials and d-values are tabulated in Table 4.3. The product of the ζ-potential of (coated) polystyrene particles and the d_{BSA}-value or d_{IgG}-value is taken as a measure for the electrostatic interaction.

In Figure 4.7A the displaced fraction of IgG is plotted as a function of the product of the ζ-potential of the bare PS-latex and the difference between the electrophoretic displacements of BSA and IgG: $\zeta_{PS}.(d_{BSA}-d_{IgG})$; this product will be further denoted as F_{el}. A negative value of F_{el} thus indicates that the electrostatic interaction of the latex with BSA is more favourable compared to that with IgG. Figure 4.7A shows that displacement tends to be enhanced by a more favourable electrostatic interaction of BSA with the PS-latex, i.e. $(\Delta\Gamma/\Gamma_{pl})_{IgG}$ increases when F_{el} becomes less positive. Unfortunately, data points at very negative values of F_{el} are not available so that continuation of the trend into that region cannot be tested. Hence, it can be concluded that, besides by its pre-adsorbed amount, displacement of IgG is affected by electrostatic interactions. In Figure 4.7B the pertaining additional BSA adsorption is plotted as a function of F_{el}. As expected from the foregoing, $\Delta\Gamma_{BSA}$ increases with a decreasing positive value of F_{el}.

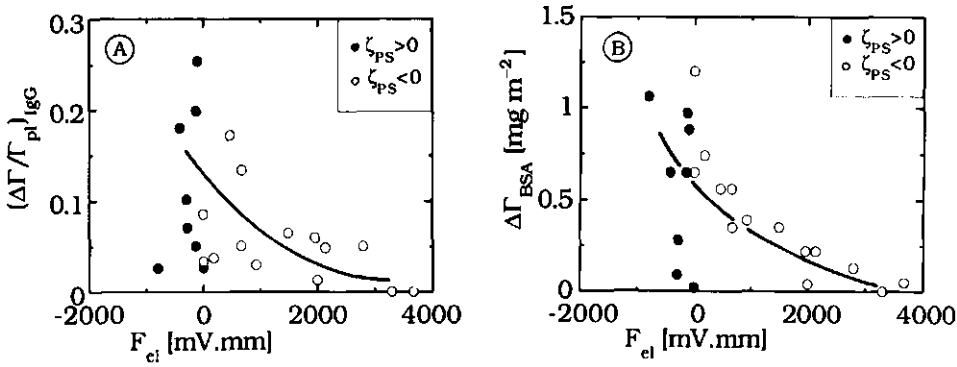

Figure 4.7 *Effect of electrostatic interaction of the proteins with the latex on A) the displaced fraction of IgG and B) the additionally adsorbed amount of BSA. For all data points Γ_{pl}>3 mg m^{-2}.*

No displacement of IgG is obtained when $\Gamma_{pl}(IgG)$<3 mg m^{-2}, although additional BSA adsorption does take place. Large values of $\Delta\Gamma_{BSA}$ are found under electrostatically attractive conditions between BSA and IgG-coated latices. If additional BSA adsorption on IgG-coated latices would be electrostatically governed, $\Delta\Gamma_{BSA}$ should correlate with $\zeta_{PS/IgG}.d_{BSA}$. However, such a correlation is not found, indicating that additional adsorption of BSA is not determined by electrostatics only. Hence, it can be concluded that additional adsorption of a second supplied protein does not necessarily imply displacement of the pre-adsorbed protein.

An important finding in this chapter is the capability of BSA molecules to partially displace pre-adsorbed IgG from (charged) hydrophobic surfaces. To my knowledge, a similar study has only been performed by Bale *et al.* [2]. They reported a fraction of (polyclonal) bovine gamma globulin of about 0.30 displaced by BSA from a negatively charged polystyrene latex at pH=7.4. At this pH we found no displacement of IgG. The different result may be caused by the choice of conditions, i.e. in the sequential adsorption study by Bale *et al.* [2] they were: $t_{ads,1}$=4 hrs., $t_{ads,2}$=24 hrs. and c_2=2500 mg m^{-2}. The low value for $t_{ads,1}$ and (extremely) high value for c_2 in combination with the use of BSA containing some 10 wt% dimer (Sigma) may be responsible for the partial displacement of IgG. Different adsorption conditions, as in our study, give different results, namely partial displacement of IgG from a positive latex surface at pH≈7 and from negatively charged latices at lower pH.

If pre-coated latices are electrostatically unstable, i.e. if they flocculate readily (see chapter 3), additional adsorption of BSA does not provide restabilization of the complex. This feature may have its implication for the practice of the development of diagnostic test systems.

4.3.2 COMPETITION BETWEEN IgG AND PRE-ADSORBED BSA

Sequential adsorption with BSA as protein (1) and IgG as protein (2) was also studied at various conditions of electrostatic interactions, using different monoclonal IgG's. In Table 4.4 the displaced amount of BSA ($\Delta\Gamma_{BSA}$) and additional IgG adsorption ($\Delta\Gamma_{IgG}$) are given, as well as ζ-potentials of the (BSA-coated) latices and electrophoretic displacements in 2D–IEF of BSA and IgG's, as obtained by interpolating in Figure 4.1.

The most striking observation from Table 4.4 is that under most conditions IgG is hardly able to displace pre-adsorbed BSA. In some cases large additional IgG adsorptions are found. As has already been concluded from the reverse sequential situation, additional adsorption of the protein (2) does not always imply displacement of protein (1).

For pH=4.0 and 4.5 no displacement of BSA occurs. Additional IgG adsorption follows an electrostatically determined sequence ($\Delta\Gamma_{2B}>\Delta\Gamma_{4F}>\Delta\Gamma_{6A}$), i.e. less positively charged IgG molecules adsorb more strongly on a BSA-latex complex having a positive ζ-potential. Small changes in ζ-potentials of the complexes support this finding (results not shown). For pH=6.0 addition of IgG-4F (d_{4F}=+16 mm) to the BSA-coated latex with $\zeta_{PS/BSA}$=–60 mV results in a large additional amount ($\Delta\Gamma_{4F}$=3.16 mg m^{-2}) without displacement of BSA. The ζ-potential of the stable complex changes from –60 mV to –20 mV leading to flocculation of the system. Additional adsorption may involve the formation of an IgG-BSA complex on

the surface, as has also been found for the additional adsorption of BSA on an oppositely charged IgG-latex.

From the positively charged latex BSA is displaced only at pH=6.0, which is around the isoelectric state of the BSA-latex complex. At pH=6.0 the adsorption isotherm of BSA on the latex having σ_o=+7.8 μC cm^{-2} (see section 3.1) shows a 'step'. The amount of BSA displaced by (positively charged) IgG-4F just equals the difference between the two adsorption plateaus. This suggests that the second plateau represents BSA molecules that are less tightly bound. Using (negatively charged) IgG-2B as protein (2), the displaced amount of BSA is larger than with IgG-4F.

Table 4.4 *Competition of solute IgG with pre-adsorbed albumin ($t_{ads,1}$=5 hrs. and $t_{ads,2}$=21 hrs.)*

	σ_o	Γ_{pl}		c_2	ζ_{PS}	$\zeta_{PS/BSA}$	d_{IgG}	d_{BSA}	$\Delta\Gamma$ [mg m^{-2}]	
pH_{ads}	[μC cm^{-2}]	[mg m^{-2}]	IgG	[mg m^{-2}]	[mV]	[mV]	[nm]	[nm]	BSA	IgG
4.0	−11.6	2.7	2B	5.6	−88	+20	+28	+27	0	0.64
			4F	5.6	−88	+20	+29	+27	0	0.33
			6A	5.6	−88	+20	+33	+27	0	0
	+7.8	0.8	2B	5.6	+95	+62	+28	+27	0	0.54
			4F	5.6	+95	+62	+29	+27	0	0.26
			6A	5.6	+95	+62	+33	+27	0	0
4.5	−11.6	3.2	2B	6.6	−90	+2	+17	+12	0.01	2.14
			4F	5.4	−90	+2	+22	+12	0.01	0.1
			6A	4.6	−90	+2	+26	+12	0.01	0.2
	+7.8	1.4	2B	5.2	+80	+45	+17	+12	0.03	1.16
			4F	2.7	+80	+45	+22	+12	0.01	0.1
			6A	1.0	+80	+45	+26	+12	0.02	0.1
6.0	−11.6	1.5	2B	5.1	−94	−60	−16	−13	0	0
			4F	5.8	−94	−60	+16	−13	0	3.16
	+7.8	2.7	2B	6.5	+40	0	−16	−13	0.72	3.19
			4F	3.9	+40	0	+16	−13	0.40	0.97

pH_{ads}=4.0: for all situations c_2=5.6 mg m^{-2}, which is for each beyond Γ_{pl}(IgG).
For the other two pH-values $c_2=\Gamma_{pl}$.

If displacement of BSA were electrostatically influenced, it is expected that the displacement increases when the interaction between IgG and the latex is electrostatically more favourable than that between BSA and the latex surface. Then, the amounts of BSA being displaced by IgG should increase with more negative values for $F_{el}=\zeta_{PS}.(d_{IgG}-d_{BSA})$. With $F_{el}=-120$ mV nm IgG-2B is able to displace 27% of pre-adsorbed BSA. With $F_{el}=1160$ mV nm the fraction of BSA displaced by IgG-4F amounts to 0.15. These data show a larger fraction of displaced BSA when F_{el} becomes more negative, as expected. In view of the influence of electrostatic interaction on the displacement of BSA by IgG and that of IgG by BSA (section 4.3.1), it may more generally be stated that if displacement of a protein occurs, its extent is enlarged when the electrostatic interaction of the secondly supplied protein with the surface is more favourable than that of the pre-adsorbed protein.

Some attention was paid to other effects influencing the sequential adsorption. Below, the observations are briefly summarized.

- Results for $t_{ads,1}=1$ hr. are not different from those for $t_{ads,1}=5$ hrs., indicating that the resistance against displacement of BSA molecules is already determined in the initial stage of adsorption. However, for pre-adsorbed IgG a continuing decrease in displacement with increasing $t_{ads,1}$ was observed, as shown in Figure 4.3.
- A lowering of c_2 results in a decreased additional adsorbed amount of the IgG's, as expected. A similar effect has been described for the reverse sequential adsorption.

4.4 CONCLUSIONS

Polyclonal IgG consists of many different IgG molecules, having distinct properties, e.g. isoelectric points, hydrophobicities and/or structural stabilities. In competitive and sequential adsorption there is a great probability for preferential adsorption of some of these IgG molecules. In section 5.3.2 adsorption from mixtures of monoclonals having different isoelectric points will be described. To study the electrostatic aspects of sequential adsorption of BSA and IgG the use of monoclonal IgG having a well-defined isoelectric point is strongly recommended.

The BSA samples used in this study were essentially fatty acid 'free' [10]. Removal of fatty acids reduces the conformational stability of the BSA molecule. Because structural rearrangements in the adsorbing protein molecule significantly contributes to the adsorption affinity, it is to be expected that defatted BSA is more strongly adsorbed to the latex surface. Consequently, it would act as a more effective displacer and would be less easily displaced by other proteins. Pitt *et al.* [10] indeed found an increase in the initial

adsorption rate of delipidized albumin on alkylized polyurethanes, but the desorption behaviour was not affected by removing the fatty acids. However, Chuang *et al.* [11] did not find an alteration in the adsorption affinity of delipidized albumin on cuprophane and polyvinylchloride.

The presence of higher aggregates in a protein sample also is an important factor influencing the (sequential) adsorption behaviour. Dimeric BSA molecules are more capable to displace IgG molecules from the surface than monomeric BSA under the same conditions, see Figure 4.4. This observation supports the idea of a dynamic behaviour of (pre-)adsorbed protein molecules. See also section 2.3.

If displacement of albumin or monoclonal IgG (for Γ_{pl}>3 mg m^{-2}) occurs, its extent depends on the electrostatic interaction between the respective proteins and the surface. The additional adsorbed amounts of IgG or BSA are also governed by electrostatics.

If no displacement occurs, additionally adsorbed amounts of different IgG's follow electrostatic interaction between the solute monoclonal and the BSA-covered polystyrene surface. Additional BSA adsorption on different IgG-coated latices does not show such a trend. This absence for BSA might be due to its larger structural adaptability which overrules the electrostatic contributions to the adsorption affinity. Under attractive conditions between the secondly supplied and the pre-adsorbed protein, large additional adsorbed amounts are measured. This may (partly) be ascribed to the formation of a protein-protein complex on the surface, similarly to the aggregation in solution which has been demonstrated to occur between oppositely charged BSA and monoclonal 4F.

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CHAPTER 5

COMPETITIVE ADSORPTION BETWEEN ALBUMIN AND IMMUNO-GAMMA GLOBULINS ON POLYSTYRENE LATICES

5.1 INTRODUCTION

Biological fluids, like blood, saliva, tear fluid, milk and sea water, contain many different proteins. When such fluids come in contact with a 'foreign' surface, a cascade of events takes place, with protein adsorption being one of the first. Adsorption of single proteins is already a complex process in which several physico-chemical factors are involved. The phenomena become even more complicated when different types of protein are in solution.

Competitive adsorption involves both kinetic and thermodynamic factors. It has been shown that the initial composition of an adsorbed protein layer is, in part, kinetically determined. If later arriving protein molecules have a higher higher affinity for the adsorbent, they tend to displace the pre-adsorbed ones. However, because of the limited desorbability of proteins the ultimate composition of the adsorbed layer is generally determined both by thermodynamic and kinetic factors. In chapter 2 some aspects of competitive and sequential protein adsorption have been reviewed. From the literature, the following trend emerges: Immuno gamma Globulin (IgG) adsorbs preferentially over albumin under many different adsorption conditions [1,2,3,4].

Since both BSA and IgG may undergo structural alterations upon adsorption (see for example Table 2.1 or the results in chapter 3) and hence adsorb tenaciously, the initial situation might be virtually frozen in, fixing the ultimate result. Apart from contributions due to the structural stability of the proteins, there will be electrostatic factors to the (initial) composition of the adsorbed layer. In the present chapter these last features will also be studied. To that end different types of monoclonal IgG's, having different isoelectric points, and differently charged latices were used.

5.2 EXPERIMENTAL

MATERIALS

Polystyrene latices with surface charge densities (σ_o) of −11.6 and +7.8 μC cm^{-2} were used. Monomeric Bovine Serum Albumin (BSA) and monoclonal Immuno gamma Globulins 2B, 7B, 4F and 6A (Organon Teknika, Boxtel, The Netherlands) were used in this study; their isoelectric points are given in Table 5.1. All experiments were performed in

media of low ionic strength (5 mM NaH_2PO_4), to allow for relatively strong electrostatic interactions. Water was purified by reverse osmosis and subsequently passed through a Super-Q system (Millipore). All other chemicals were of analytical grade and used without further purification. More characteristics of the latices and proteins are presented in Tables 3.1 and 3.3.

Table 5.1 *Isoelectric points (i.e.p.'s) of the proteins used.*

Protein	BSA	IgG-2B	IgG-7B	IgG-4F	IgG-6A
i.e.p.	4.7-5.0	4.9-5.2	5.2-6.0	6.8-7.0	7.9-8.1

METHODS

Two-Dimensional Iso Electric Focussing (2D-IEF)

In chapter 4 it was concluded that aggregation in solution between different proteins occurs under conditions of electrostatic attraction. To avoid such aggregation, competitive adsorption experiments were performed at pH-values where the proteins present in solution have the same charge sign. The pH range where this condition applies for IgG-4F and BSA can be read from Figure 4.2.

Competitive protein adsorption

Adsorption experiments were typically carried out in a 2 cm^3 polystyrene tube (Greiner) containing 1.65 cm^3 of the protein mixture to which 0.25 cm^3 polystyrene latex was added (V_o=1.90 cm^3). Unless otherwise stated, the surface area of the added latex (S_o) was about 0.08 m^2. In the literature [5] it is reported that preferential adsorption of dimeric albumin over monomeric molecules is more enhanced when the albumin concentration/surface area ratio becomes larger. In the present study the effect of S_o on the competitive adsorption of BSA and IgG-7B has been verified. A major part of this chapter deals with the influence of electrostatics on the competitive adsorption. For both proteins the initial concentration (c_o) in the mixture is chosen sufficiently large to reach its plateau value (Γ_{pl}) as it would occur in single protein adsorption (chapter 3), i.e. for a given pH and protein-latex combination $c_o.V_o/S_o > \Gamma_{pl}$. After a fixed incubation time ranging from 2-3 min. (the shortest time posible in our set-up) to 40 hrs., the sample was centrifuged during 10-15 min. at 4.71×10^4 m s^{-2} (Sarstedt MH2). Subsequently, the concentrations of the proteins in the supernatant were determined. To that end, three analytical methods were applied:

a) For the competitive adsorption of BSA and monoclonal (2B, 7B, 4F) FPLC®-SEC with a Superose-12 column (Pharmacia) and UV monitoring at 206 nm (Uvicord S, LKB) was used.

The concentrations of BSA and IgG were linearly related to the peak height. In Figure 5.1 an example is given where the shaded areas represent the amounts of protein adsorbed.

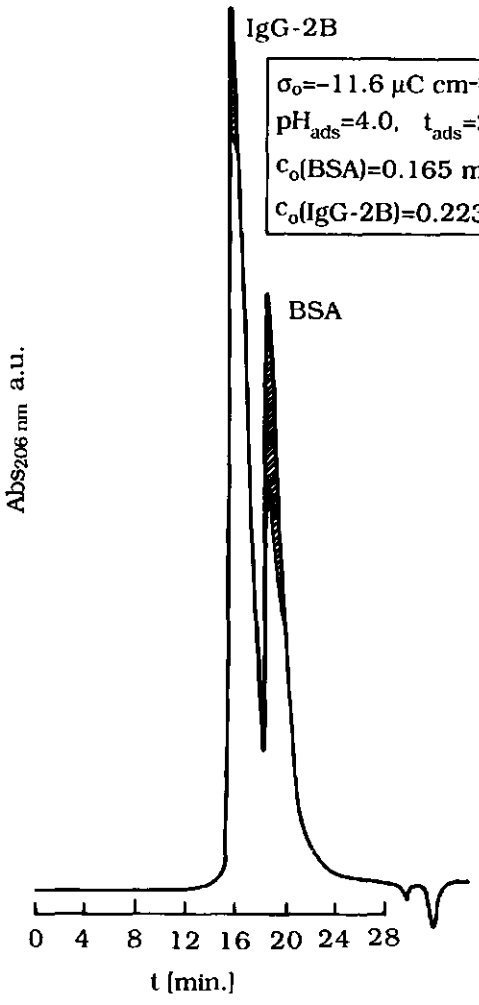

Figure 5.1 *Analytical Size Exclusion Chromatogram of BSA and monoclonal 2B.*

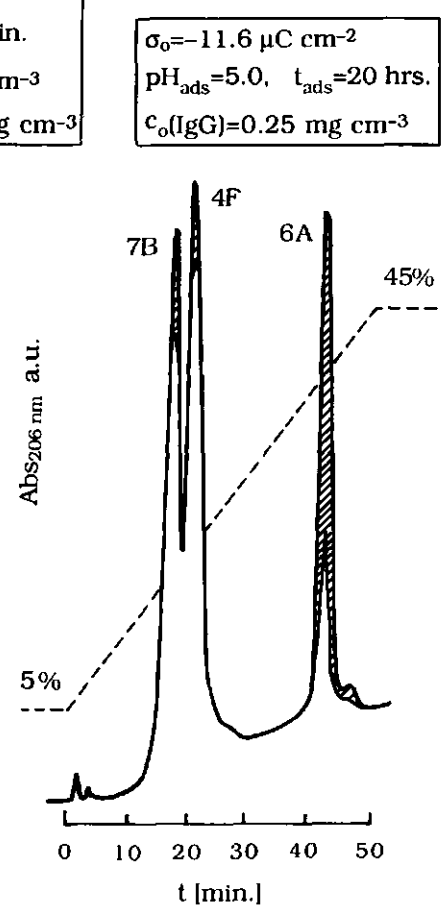

Figure 5.2 *Analytical Ion Exchange Chromatogram of a mixture of the monoclonals 7B, 4F and 6A.*

b) For the competitive adsorption of BSA and IgG-6A method a) is not applicable for IgG-6A due to column interactions. Hence, a Sol Particle Immuno Assay (SPIA®) was used for both proteins. A Reader Microelisa® system with a 540 nm filter (Organon Teknika) was applied. The method is well documented in references [6,7].

c) To study competitive adsorption between different IgG's FPLC®-Ion Exchange Chromatography was used. The conditions for FPLC®-IEC were: Gel: Mono S™ (Pharmacia); Eluens: A) Milli Q, B) 0.25 M Na_2HPO_4/pH=5.0/H_3PO_4; Gradient: 5%B→45%B (50 min.); Flow rate: 0.5 cm^3 min^{-1}; Sample: 200 µl; Detection: Uvicord S (LKB) UV 206 nm. The concentration after adsorption of the IgG's was linearly related to the peak height. Each data point in Table 4.5 is the result of duplicate experiments. In Figure 5.2 an example of the adsorption from a tertiary mixture is given (shaded areas indicate adsorbed amounts). The baseline drift is due to the increasing absorbance of the eluens with increasing salt gradient.

5.3 RESULTS AND DISCUSSION

5.3.1 ADSORPTION FROM MIXTURES OF ALBUMIN AND IMMUNO GAMMA GLOBULIN

Influence of adsorption time

For two combinations (#1: BSA and IgG-2B at pH=4.0, #2: BSA and IgG-7B at pH=6.5) the effect of adsorption time on the surface composition is determined. By using two lattices of different charge sign, both electrostatically attractive and repulsive conditions between proteins and adsorbent surface were studied. The surface composition may be expressed as the mass fraction of each protein in the adsorbed layer, i.e. for IgG: $\phi_{IgG}=\Gamma_{IgG}/(\Gamma_{IgG}+\Gamma_{BSA})$, where Γ is the adsorbed amount in mg m^{-2}. The initial concentrations (c_o) of BSA and IgG were chosen such, that initially the mass flux towards the surface is equal for both proteins. The flux equation for a diffusion-controlled transport towards an empty surface is given by [8]

$$dn_i/dt=c_{o,i}\,(D_i/\pi)^{0.5}\,t^{-0.5} \qquad [5.1]$$

where dn_i/dt is the mass flux of protein i arriving at one unit area of the surface, i refers to BSA or IgG, c_o is the initial concentration of protein and D_i is the diffusion coefficient of protein i; $D_{IgG}=4.0\ 10^{-11}$ m^2 s^{-1} [9] and $D_{BSA}=7.3\ 10^{-11}$ m^2 s^{-1} [10,11].

When the adsorption rate is diffusion-controlled and if the adsorbent surface acts as a protein sink (i.e. all arriving molecules adsorb immediately) at initial stages of adsorption (i.e. very low surface coverage), the rate of adsorption $d\Gamma_i/dt$ equals the rate of arrival dn_i/dt. From Eq. [5.1] it follows that an equal initial adsorption rate for BSA and IgG,

$d\Gamma_{BSA}/dt = d\Gamma_{IgG}/dt$, is attained if $(c_{o,IgG})/(c_{o,BSA})=1.35$. Furthermore, c_o of both proteins is chosen large enough to reach the plateau value as obtained in single protein adsorption. To compare the obtained ϕ_{IgG}-values one may relate the adsorbed amounts from the competition experiment to the plateau value of single protein adsorption. Under conditions of equal mass fluxes, identical affinities of BSA and IgG for the surface would result in a surface composition that can be directly related to the adsorption from the corresponding single protein solutions, meaning that $\phi_{IgG}=\phi_{pl,IgG}$, with $\phi_{pl,IgG}=\Gamma_{pl,IgG}/(\Gamma_{pl,IgG}+\Gamma_{pl,BSA})$. Deviation of ϕ_{IgG} from $\phi_{pl,IgG}$ indicates preferential adsorption of one of the proteins. In Table 5.2 the surface composition for four different situations as a function of time are tabulated.

Table 5.2 *The surface composition, expressed as the fraction of IgG (ϕ_{IgG}) in the adsorbed layer, as a function of adsorption time.*

# (IgG, pH)	σ_o [$\mu C\ cm^{-2}$]	c_o [$mg\ cm^{-3}$] IgG	BSA	ϕ_{IgG} (2 min.)	ϕ_{IgG} (10 min.)	ϕ_{IgG} (3 hrs.)	ϕ_{IgG} (24 hrs.)	$\phi_{pl,IgG}$
1 (2B, 4.0)	+7.8	0.144	0.107	0.87	0.76	0.48	0.22	0.85
(2B, 4.0)	−11.6	0.223	0.165	0.43	0.33	0.25	0.19	0.65
2 (7B, 6.5)	+7.8	0.171	0.126	0.69	0.65	0.64	0.64	0.81
(7B, 6.5)	−11.6	0.152	0.112	0.96	0.94	0.90	0.87	0.60

For combination #1 a distinct decrease of ϕ_{2B} with time is observed on both latices. As will be shown below, this decrease is both caused by additional adsorption of BSA and displacement of IgG-2B. For combination #2 the surface fraction of IgG-7B remains almost constant with time on both latices. Comparing ϕ_{IgG} with $\phi_{pl,IgG}$ leads to the conclusion that in most cases BSA is preferentially adsorbed, only for IgG-7B and BSA at pH=6.5 on the negatively charged latex IgG is preferentially adsorbed. The IgG preference might be ascribed to the low affinity of BSA for the negatively charged latex at pH=6.5, as illustrated in Figure 3.2.

Effect of surface area (S_o)

For the combination BSA and IgG-7B at pH=6.5 the influence of S_o of two differently charged latices on the composition of the adsorbed layer is investigated. Adsorption was performed under conditions of equal initial mass flux of BSA and IgG-7B towards the surface. In Figure 5.3 the surface fraction of IgG-7B (ϕ_{7B}) is plotted as a function of S_o. For

both latices ϕ_{7B} decreases when the available area becomes smaller. For the positively charged latex a prolonged adsorption time does not influence the $\phi_{7B}(S_o)$-curve. However, for the negatively charged latex ϕ_{7B} decreases with adsorption time, which can again be attributed to the low affinity of BSA for the negatively charged latex at pH=6.5 (cf. Figure 3.2). Moreover, the decrease is larger for smaller S_o-values. Since depletion becomes smaller with decreasing S_o, the protein concentrations that after adsorption remain in solution are relatively high. This could help to explain why additional BSA adsorption and displacement of IgG-7B (resulting in the lowering of ϕ_{7B}) is larger at smaller S_o-values.

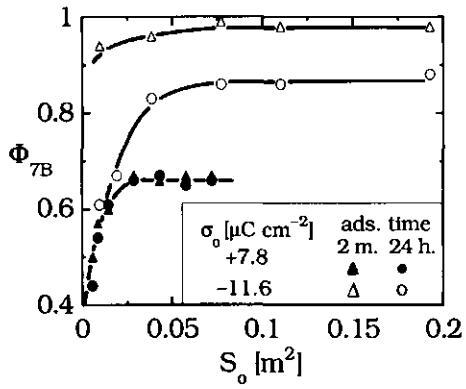

Figure 5.3 *Adsorbed fraction of IgG-7B as a function of surface area in the system, pH_{ads}=6.5. Open symbols: c_o(IgG-7B)= 1.35xc_o(BSA)=0.324 mg cm^{-3}. Closed symbols: c_o(IgG-7B)= 1.35xc_o(BSA)=0.171 mg cm^{-3}.*

The other competitive adsorption experiments described in this chapter were all performed with S_o=0.08 m^2, leading to plateau regions of $\phi_{7B}(S_o)$.

Effect of initial concentrations of BSA and IgG

To compare different IgG-BSA-polystyrene systems, the adsorbed amount of protein in the competitive experiment (Γ_i) may be related to the plateau value of single adsorption ($\Gamma_{pl,i}$), where i stands for BSA or IgG. Therefore, a normalized adsorbed amount is defined as:

$$\theta_i=\Gamma_i/\Gamma_{pl,i} \qquad [5.2]$$

An additional advantage of this normalization is that the effect of the structural stability of proteins to the adsorption is eliminated, so that the influence of electrostatic interaction shows up more clearly.

Furthermore, the initial protein concentrations in the mixture (which are large enough to yield plateaus if the proteins adsorb on their own) were related to each other by

$$Z_{BSA}=(c_o/\Gamma_{pl})_{BSA}/((c_o/\Gamma_{pl})_{BSA}+(c_o/\Gamma_{pl})_{IgG}) \qquad [5.3]$$

with $Z_{IgG}=1-Z_{BSA}$.

If both proteins would have the same affinity for the surface, one can deduce from Eqs. [5.2] and [5.3] that for any value of Z_i:

$$Z_i = \theta_i \qquad [5.4]$$

For example, when a protein mixture contains an initial BSA concentration corresponding to three times its single plateau value and an IgG concentration of twice its single plateau value, it follows from Eq. [5.3] that $Z_{BSA}=0.6$. If both proteins had the same affinity for the surface one might apply Eq. [5.4] resulting in a surface composition of $\theta_{BSA}=0.6$ and $\theta_{IgG}=0.4$. Because Γ_{pl} for BSA and IgG are different a larger adsorbed amount of one of the proteins does not automatically imply preferential adsorption. The definition is based on the tacit assumption that $\theta_{BSA}+\theta_{IgG}=1$, meaning that no cooperative effects exist between the adsorption of the two proteins.

Hence, deviations from Eq. [5.4] point to preferential adsorption of one of the proteins.

Influence of electrostatics

To elucidate the electrostatic contribution to competitive protein adsorption, both repulsive and attractive conditions between proteins and adsorbent were studied. Below, these two conditions are discussed, thereby relating Z_{BSA} to θ_i as introduced above. The adsorption times were 2-3 min. (initial situation) and about 24 hours ('equillibrium' situation).

- **repulsive conditions**

Table 5.3 summarizes experimental data for single adsorptions obtained at two different electrostatically repulsive conditions, (i) all proteins and adsorbents positive (#1 and #2) and (ii) all proteins and adsorbents negative (#3, #4 and #5).

From Eq. [5.4] it was deduced that under conditions of equal affinities between the two proteins, θ_{BSA} versus Z_{BSA} should be a straight line with unit slope, whereas for the pertaining θ_{IgG} the slope would be minus one. In Figure 5.4 the initial (t_{ads}=2 min.) surface composition θ_i is given as a function of Z_{BSA} for these five situations. As expected, θ_{IgG} decreases and θ_{BSA} increases with increasing Z_{BSA}. Furthermore, large θ_{IgG}-values seem to correspond with low θ_{BSA}-values. In Figure 5.4A the product of the electrophoretic displacement d of the IgG molecule in 2D-IEF and the ζ-potential of the latex is indicated.

Table 5.3 *Summary of experiments conducted under electrostatically repulsive conditions for different proteins and lattices. The ζ-potentials of the latices are taken from Figure 3.4, the plateau values (Γ_{pl}) of the proteins from Figures 3.3 and 3.11 and the electrophoretic displacements (d) of the proteins in the 2D-IEF gels are derived from Figure 4.1.*

#	symbol	IgG	pH	ζ_{PS} [mV]	Γ_{pl} [mg m^{-2}] IgG	BSA	d [mm] IgG	BSA
1	+	2B	4.0	+95	3.90	0.75	+28	+27
2	□	6A	4.0	+95	0.75	0.75	+33	+27
3	O	7B	6.5	−93	4.00	1.10	−6	−17
4	Δ	2B	7.0	−94	2.30	0.80	−13	−22
5	◊	4F	7.5	−94	2.30	0.65	−4	−27

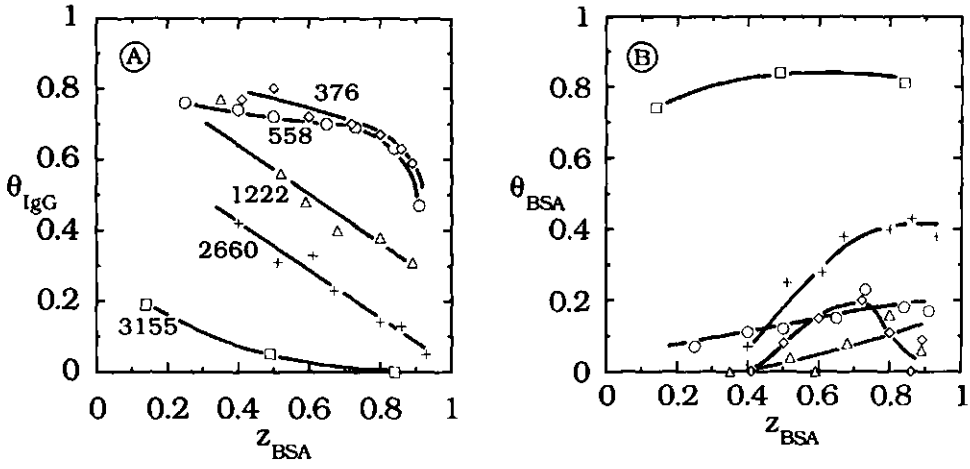

Figure 5.4 *Normalized adsorbed amounts under electrostatically repulsive conditions as a function of Z_{BSA} (t_{ads}=2 min.). A) θ_{IgG} and B) θ_{BSA}. Symbols as in Table 5.3.*

This product may be considered as a measure of the electrostatic interaction. It can be seen that with increasing value for $\zeta_{PS}.d_{IgG}$ the normalized adsorbed amount of IgG decreases. Low values of θ_{6A} correspond to large values of $\zeta_{PS}.d_{IgG}$, reflecting the non-high affinity nature of the protein in single adsorption (cf. section 3.2). The non-high affinity behaviour of BSA on negatively charged latices for 6.0<pH<8.0 (see Figure 3.2) is in

accordance with the low θ_{BSA}-values in Figure 5.4B. No clear trend of θ_{BSA} with $\zeta_{PS}.d_{BSA}$ is found.

The change in adsorbed amounts with time is given in Figure 5.5, where $\Delta\theta_i=\theta_i$(24 hrs.)–θ_i(2 min.) is plotted as a function of Z_{BSA}. The various IgG's behave differently: small increments in the adsorbed amounts are observed for IgG-2B on both latices, $\Delta\theta_{6A}$ decreases with Z_{BSA} and for IgG-7B and IgG-4F displacement of adsorbed molecules occurs. In Figure 5.5B an unambiguous trend can be observed: with increasing Z_{BSA} the additionally adsorbed amount of BSA increases for all situations. Sequential adsorption data from chapter 4 do not show displacement of IgG-7B and IgG-4F by BSA under the same conditions. This suggests that in the competitive adsorption process, where molecules of IgG and BSA adsorb simultaneously, the adsorbed IgG molecules have less possibilities to adapt themselves optimally to the adsorbent surface so that they are more easily displaced.

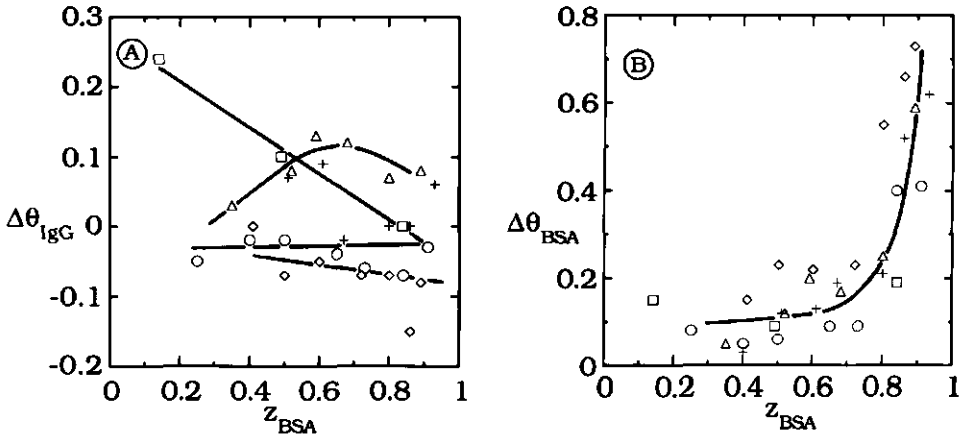

Figure 5.5 *Comparison of normalized amounts at two different times, $\Delta\theta_i=\theta_i$(24 hrs.)–θ_i(2 min.), as function of Z_{BSA} under electrostatically repulsive conditions for A) IgG and B) BSA. Symbols as in Table 5.3.*

Preferential adsorption of one of the proteins in the 'equilibrium' situation, as indicated by a deviation from Eq. [5.4], can be deduced by adding $\Delta\theta_i$-data from Figure 5.5 to θ_i-values in Figure 5.4. Preferential adsorption is found for IgG on the negatively charged latex. For the positively charged latex BSA preferentially adsorbs from a mixture of IgG-6A and BSA, whereas no preference is found for adsorption from a solution containing BSA and IgG-2B. Hence, preferential adsorption of the one protein is found when in the single adsorption isotherm the 'other' protein shows less affinity. One can predict preferential adsorption by

comparing $\zeta_{PS}.d_{IgG}$ with $\zeta_{PS}.d_{BSA}$ (see Table 5.3). This finding leads to the important conlusion that the initial situation to a large extent dictates the occurence of preferential adsorption.

• **attractive conditions**

In Table 5.4 data for two electrostatically attractive conditions are collected, namely for positively charged proteins adsorbing onto a negatively charged latex (#6 and #7) and negatively charged proteins on an oppositely charged latex (#8, #9 and #10).

In Figure 5.6 the initial (t_{ads}=2 min.) surface composition θ_i is given as a function of Z_{BSA} for these five situations. The decrease in θ_{IgG} and increase in θ_{BSA} with Z_{BSA} is more pronounced as compared to the repulsive case (Figure 5.4), indicating that under attractive conditions the initial composition of the adsorbed layer is directly related to the protein concentrations. For none of the proteins could any correlation between θ_i and $\zeta_{PS}.d_i$ be observed.

Table 5.4 *Summary of experiments conducted under electrostatically attractive conditions for different proteins and latices. The ζ-potentials of the latices are taken from Figure 3.4, the plateau values (Γ_{pl}) of the proteins from Figures 3.3 and 3.11 and the electrophoretic displacements (d) of the proteins in the 2D-IEF gels are derived from Figure 4.1.*

#	symbol	IgG	pH	ζ_{PS} [mV]	Γ_{pl} [mg m^{-2}] IgG	BSA	d [mm] IgG	BSA
6	⊞	2B	4.0	–89	5.50	2.70	+28	+27
7	■	6A	4.0	–89	3.15	2.70	+33	+27
8	●	7B	6.5	+27	4.50	2.60	–6	–17
9	▲	2B	7.0	+16	5.50	2.40	–13	–22
10	♦	4F	7.5	+13	4.20	2.30	–4	–27

The change in the amounts of the proteins adsorbed as a function of time is given in Figure 5.7, where $\Delta\theta_i=\theta_i$(24 hrs.)–θ_i(2 min.) is plotted as a function of Z_{BSA}. Figure 5.7A shows that $\Delta\theta_{IgG}$ is rather small, which implies that the final amount of adsorbed IgG is already reached at short adsorption times. In Figure 5.7B again no displacement of BSA is observed, whereas additional adsorption of BSA from solutions with IgG-2B and IgG-4F shows a maximum at intermediate Z_{BSA}-values. This maximum may be explained as follows: for large Z_{BSA}-values initially a relatively large amount of BSA is adsorbed,

resulting in low $\Delta\theta_{BSA}$-values and for small Z_{BSA}-values initially a large amount of IgG is adsorbed, also leading to low $\Delta\theta_{BSA}$-values.

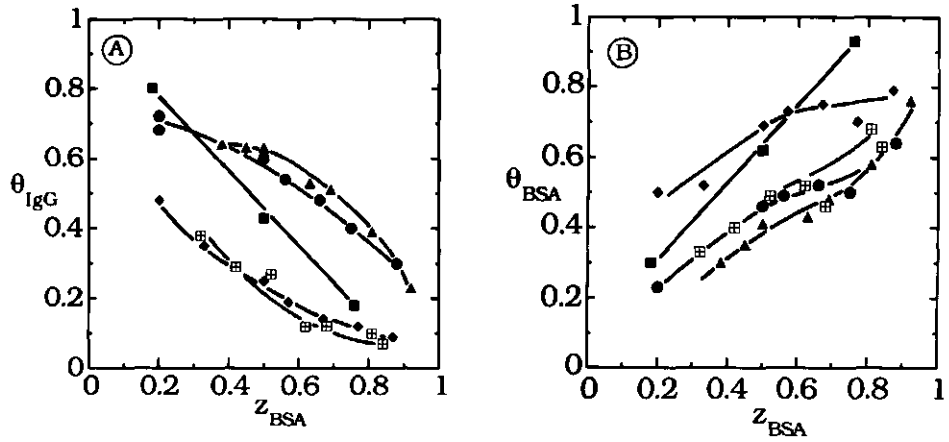

Figure 5.6 *Normalized adsorbed amounts under electrostatically attractive conditions as a function of Z_{BSA} (t_{ads}=2 min.), A) θ_{IgG} and B) θ_{BSA}. Symbols as in Table 5.4.*

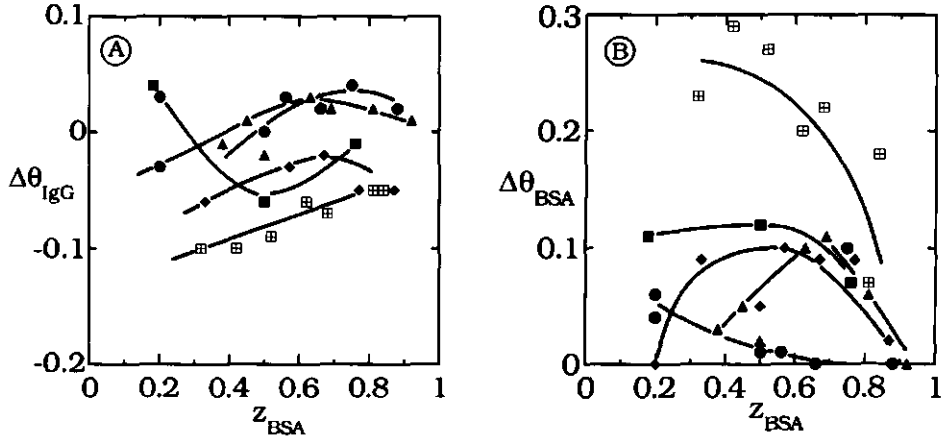

Figure 5.7 *Comparison of normalized amounts at two adsorption times, $\Delta\theta_i$=θ_i(24 hrs.)–θ_i(2 min.), as a function of Z_{BSA} under electrostatically attractive conditions for A) IgG and B) BSA. Symbols as in Table 5.4.*

Preferential adsorption of one of the proteins in the 'equilibrium' situation, as indicated by a deviation from Eq. [5.4], can be obtained by adding $\Delta\theta_i$-data from Figure 5.7 to θ_i-values in Figure 5.6. The adsorption data of both proteins are then more or less situated around $Z_i=\theta_i$, indicating that there is little preference under conditions of high affinity character for both proteins. This important finding is supported by the data for the adsorption of positively charged BSA and IgG-2B on the positively charged latex (Table 5.3, exp.#1). Also in that case where the single proteins develop high-affinity isotherms there is no preferential adsorption from the mixture, see Figures 5.4A and 5.5A.

• **overall adsorption**

In Figure 5.8 the fractions of the adsorbed proteins, as obtained after 24 hrs. adsorption time, are plotted against each other. Roughly, the plots show a correlation around the line for $\theta_{BSA}+\theta_{IgG}=1$. However, for electrostatically repulsive conditions the deviations from that line are somewhat larger, indicating that (non)-cooperative adsorption effects between the proteins on the surface occur. Since no 'simple' (electrostatic) trend is distinguished with respect to those deviations, it is concluded that other factors affecting the adsorption (e.g. hydrophobic interaction or structural stability of the proteins) overrule electrostatic interactions.

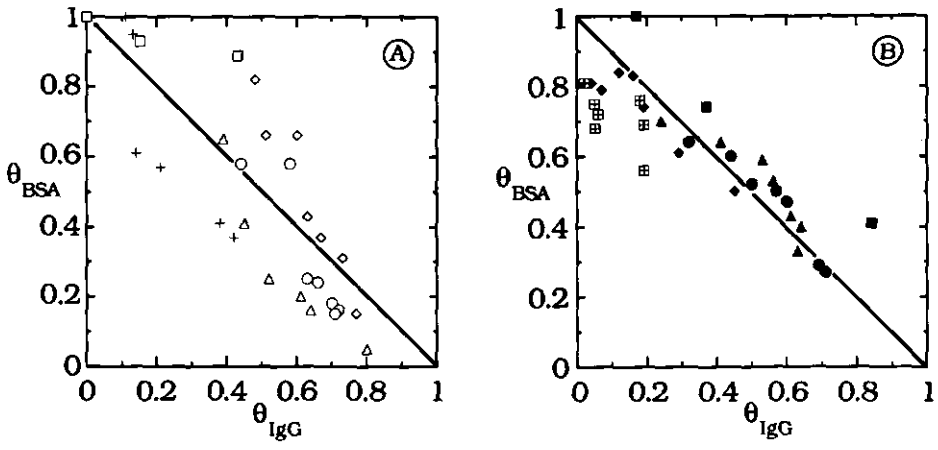

Figure 5.8 *Plot of θ_{BSA} against θ_{IgG} for A) electrostatically repulsive and B) attractive conditions. (t_{ads}=24 hrs.). Symbols as in Tables 5.3 and 5.4.*

5.3.2 ADSORPTION FROM MIXTURES OF DIFFERENT TYPES OF MONOCLONAL IgG's

Figure 5.2 shows that it is possible to discriminate analytically between IgG's having different isoelectric points in a mixture of monoclonal antibodies. Because the pH of the eluens and pH_{ads} are equal, the order of elutability of the IgG's from the (negatively charged) column is a qualitative measure of the charge density of the monoclonals in solution. With SDS-PAGE it was found that all monoclonals have two similar bands, indicating an equal molecular size of the IgG's (results not shown). When different IgG's have the same initial concentration their mass flux towards the surface must be equal, since the protein molecules have the same molecular size and shape. It follows that from a mixture of monclonal IgG's of different i.e.p. preferential adsorption is determined by electrostatic factors.

Table 5.5 *Competitive adsorption in [mg m^{-2}] from tertiary and binary mixtures of different types of monoclonals on two latices. In addition, the plateau values for single adsorption are given. For σ_o=+ 7.8 μC cm^{-2} c_o(IgG)=0.125 mg cm^{-3} and for σ_o=−11.6 μC cm^{-2} c_o(IgG)=0.25 mg cm^{-3}.*

	σ_o=+ 7.8 μC cm^{-2}		σ_o=−11.6 μC cm^{-2}	
	t_{ads}=2 min.	t_{ads}=20 hrs.	t_{ads}=2 min.	t_{ads}=20 hrs.
single:				
7B		2.2		5.6
4F		3.2		5.8
6A		1.1		5.5
binary:				
4F	0.76±0.03	0.75±0.16		
6A	0.19±0.06	0.39±0.17		
7B			2.37±0.17	1.75±0.17
4F			2.28±0.18	2.40±0.58
tertiary:				
7B	1.61±0.01	0.98±0.08	1.42±0.22	1.12±0.18
4F	0.32±0.12	0.09±0.09	1.33±0.17	0.88±0.05
6A	0.42±0.16	0.01±0.01	3.06±0.16	4.15±0.11

Experiments were performed at pH_{ads}=5.0 where electrostatically repulsive and attractive conditions between the IgG's and the adsorbent exists. Two differently charged latices at equal initial IgG concentrations were used. In Table 5.5 the adsorptions are given for binary and tertiary mixtures after two adsorption times.

The main finding from Table 5.5 is that preferential adsorption from a mixture of different monoclonals is electrostatically determined. Adsorption from a tertiary mixture shows a preference for the most positively charged IgG (6A) on the negatively charged latex and a preference for the least positive one (7B) on the positively charged latex. The preference even increases with time. The two other proteins do no show preferential adsorption based on electrostatic interactions. It may therefore be suggested that the preferentially adsorbed IgG lowers the ζ-potential (cf. section 3.2) which, in turn, suppresses the electrostatic contribution to the adsorption of the two other monoclonals. The suggestion is in line with adsorption experiments from binary mixtures with these proteins, see Table 5.5. In these cases, electrostatically determined preferences are observed.

Competitive adsorption experiments with BSA never showed a decrease of the total adsorbed amount with time, see section 5.3.1. However, in some cases competitive adsorption between monoclonal IgG's leads to a decrease in the total adsorbed amount on prolonging the incubation time. A speculative explanation may be the following: at pH=5.0 IgG-7B is around its conformationally most stable situation, which leads to the highest initial adsorption rate (see chapter 6). However, at pH=5.0 the other proteins are conformationally less stable, possibly resulting in structural alterations upon adsorption. It could well be that the conformationally most stable protein molecules are displaced from the surface by those proteins that have a lower internal coherence and, hence, tend to optimize their contacts with the adsorbent surface. In this connection it is mentioned that for single albumin adsorption 'over-shoots' have been reported [12]. This phenomenon has been related to structural and/or conformational rearrangements of initially adsorbed proteins, leading to partial displacement of pre-adsorbed molecules. However, reflectometry and streaming potential measurements (cf. chapter 6) never point to such 'over-shoots' for single adsorption of IgG's.

5.4 CONCLUSIONS

Preferential adsorption of either one of the two proteins BSA and IgG is only observed when the proteins and adsorbent have the same charge sign. The protein with the higher affinity for the surface adsorbs preferentially, since in the initial stage of the process adsorption of the protein having the lower affinity for the surface is retarded. In later stages, additional adsorption of this protein does occur, but the preference is already determined in the early stage.

No preference is observed when both proteins show a high-affinity for the adsorbent surface. Under electrostatically attractive conditions the final composition of the adsorbed layer is already initially determined.

Adsorption competition between different monoclonal IgG's is governed by electrostatic interactions. Therefore, to study this competition the use of monoclonal antibodies having well defined isoelectric points has been a necessity. Contrary to adsorption from single protein solutions, adsorption 'over-shoots' are found for adsorption from mixtures of different IgG's.

5.5 REFERENCES

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CHAPTER 6

KINETICS OF PROTEIN ADSORPTION STUDIED BY REFLECTOMETRY AND STREAMING POTENTIAL MEASUREMENTS

6.1 INTRODUCTION

Generally, protein adsorption approaches its plateau value within seconds or minutes after exposing the solution to the adsorbent surface. After that, surface relaxation processes in the protein molecules may take place that proceed over more prolonged periods of time, i.e. hours or even days. To study the early stages of protein adsorption kinetics, detection techniques should be used that can monitor the adsorption process during its initial stages. Ellipsometry [1,2] and γ-photon spectroscopy using radiolabeled proteins [3] are well-established techniques. More recently, Total Internal Reflection (Intrinsic) Fluorescence [4,5,6] and (Attennuated Total Reflection)-Fourier Transformed Infra Red Spectroscopy [7,8] have become favourite techniques to monitor the time dependency of protein adsorption.

In this study two rather novel techniques are applied to acquire information on the kinetic aspects of protein adsorption *in situ*: (i) reflectometry, a spectroscopic technique that relates the change in intensity of polarized laser light reflected from a surface to the amount of adsorbate on that surface and (ii) a technique that monitors a change in the streaming potential of a (macroscopic) adsorbent surface due to adsorption of adsorbates. These two techniques, together with the earlier mentioned TIR(I)F and (ATR)-FTIR hold great promises in future research on protein adsorption, not in the least because proteins can be used without introducing an extrinsic label.

The study of the influence of the electric state of a protein on the initial adsorption rate is one of the main objectives of this chapter. As discussed before, the structural stability of proteins may be the dominating factor for its adsorption. Upon changing the charge of a protein molecule, besides the Coulomb interactions, its structural stability will also change. Hence, pH changes have a dual consequence and it is not always easy to discriminate between these two contributions.

Neither, reflectometry nor streaming potential measurements allow to discriminate between different proteins without standardising with single protein adsorption. Therefore, to interpret adsorption data in systems containing more than one protein (as in sequential and competitive adsorption), the results for single protein systems are indispensable.

6.2 EXPERIMENTAL

6.2.1 MATERIALS

Proteins

Monomeric Bovine Serum Albumin (BSA) and monoclonal Immuno gamma Globulins (IgG's) 2B, 4F and 6A (Organon Teknika, Boxtel, The Netherlands) were used in this study. Their isoelectric points (i.e.p.'s) are given in Table 6.1. In chapter 3 more characteristics of the proteins are presented. All adsorption experiments were performed in a buffer (NaH_2PO_4) of which the concentration was kept low (5 mM) to prevent suppression of the electrostatic component of the interaction.

Table 6.1 *Isoelectric points of the proteins used.*

Protein	BSA	IgG-2B	IgG-4F	IgG-6A
i.e.p.	4.7-5.0	4.9-5.2	6.8-7.0	7.9-8.1

Adsorbents

Protein adsorption on polystyrene (PS)-coated silica wafers (diameter=100 mm, thickness=500±20 μm, orientation=<111>, resistance≥100 Ω cm, Wacker Chemitronic GMBH, München, Germany) was followed by reflectometry and on PS-coated glass slides (length=76.0 mm, width=25.5 mm and thickness=1.0 mm, M.M.G., Goslar, GDR) by streaming potential measurements.

Before coating with PS, the glass plates were placed in concentrated chromic acid for two days. After washing with water and 30 min. soaking in 0.1 M NaOH, the plates were rinsed again with water and stored in absolute ethanol. Directly before the coating was applied, the glass plates were dried in a vacuum oven at 120 °C for 30 min.. Prior to coating, the silica plates were dried in a vacuum oven at 50 °C for at least 3 hrs. and subsequently rinsed with dichloromethane.

Coating procedure: PS latex having a surface charge density of +2.7 μC cm^{-2} (determined by conductometric titration [9]) was freeze-dried and subsequently dissolved in 1,2-dichloro-ethane to a concentration of 12 g dm^{-3} (for coating of the silica surface) and 15 g dm^{-3} (for coating of the glass plates). Half a cm^3 of a PS solution obtained in this way was spread over the adsorbent surface which was horizontally mounted on a disk. The PS coating was applied by spinning the disk at 2000 rpm. (rapidly attained) during 5 min. for the silica plates and 30 s. for the glass slides; with the glass surface the spin-coating was

repeated twice. The PS-coated adsorbents were placed in a vacuum oven; the PS-silica surfaces overnight at 50 °C and the PS-glass substrates for 1 hr. at 120 °C. The adsorbents were stored over $CaCl_2$ in a desiccator and they were used within one week. Prior to using for an adsorption experiment the PS-silica plates were cut into strips of about 1 cm width. The PS-layer thickness on silica was about 90 nm, as determined by reflectometry, see below.

Chemicals

Water was purified by reverse osmosis and subsequently passed through a Super-Q system (Millipore). Directly before use, water and buffer solutions were degassed to avoid the formation of small air bubbles during the adsorption experiment. All other chemicals (Merck) were of analytical grade and used without further purification.

6.2.2 METHODS

REFLECTOMETRY

Principle

In Figure 6.1 a schematic representation of the experimental set-up is given. The beam of the light source ①, a 10 mW He-Ne laser with λ=632.8 nm (Spectra Physics 105-1), has an angle of incidence at the sorbent surface ④ around the Brewster-angle, θ_B. The incident polarized beam, of which $I^o_{//}$ and $I^o_{\perp}$ are the intensities of the components that are polarized parallel and perpendicular with respect to the plane of incidence respectively, is reflected. After beam splitting ⑤, the reflected light beam has intensities $I_{//}(=R_{//}I^o_{//})$ and $I_{\perp}(=R_{\perp}I^o_{\perp})$ that are detected by photon detectors ⑥ (Diode S 1010E6 PL). At the Brewster angle, $R_{//}$=0. For λ=632.8 nm θ_B=70.6° for the silica-water interface. Any material adsorbed at the interface (having a refractive index different from that of the solution (n_{water}=1.33) and adsorbent material (n_{silica}=3.8, n_{PS}=1.59)) will change the reflectance. This change is most pronounced at θ_B. The reflection intensities are converted by an analogue signal processor ⑦ into a M-value, defined as

$$M = (R_{\perp} - aR_{//})/(R_{\perp} + aR_{//}) \qquad [6.1]$$

where $a=I^o_{//}/I^o_{\perp}$. For the silica-water system a=3, assuming equal sensitivity of the detectors for both polarization directions. M is recorded ⑧ (Philips PM 8202) as a function of time. From computer simulations, using theory for reflection in multilayer systems [10] it was found that an adsorbed amount of 1 mg m^{-2} corresponds to a change in M-value of 100 mV at the PS-silica surface with a PS-layer thickness of 90 nm. A more detailed discussion on the relation between the M-value and the adsorbed amount will be given by

Dijt *et al.* in a forthcoming paper [11]. Using a peristaltic pump ② (4-way valve 115VAC, Buchler Instruments), the protein solution could be continuously supplied into a cuvette ③ and by an 'impinging jet' directed perpendicularly to the adsorbent surface. The nozzle of the supplying device was close the point on the adsorbent surface where the light beam is reflected.

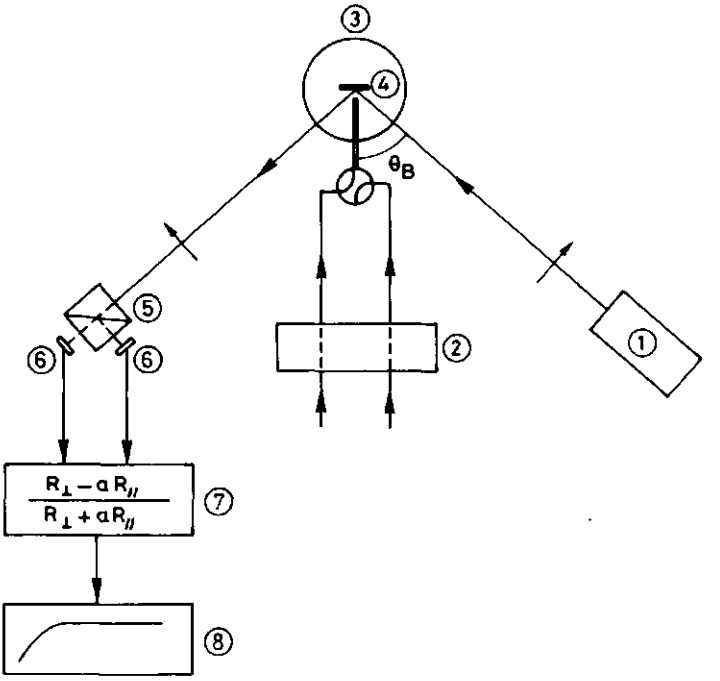

Figure 6.1 *Schematic representation of the reflectometry system. The numbers in the figure are explained in the text. Taken from reference [19].*

For such an impinging jet supply, Dabrós and van de Ven [12] derived a theoretical equation of the maximum protein flux (J_{pr}) onto the adsorbent

$$J_{pr} = 0.287\, \nu^{-1/3}\, \phi^{2/3}\, R^{-5/3}\, D^{2/3}\, c_b \qquad [6.2]$$

where ν is de kinematic viscosity of the solution, ϕ the volume flux, R the radius of the nozzle of the supplying device, D the diffusion coefficient of the protein in solution and c_b the concentration of the protein in the supplied solution. Eq. [6.2] is only valid under the following conditions: (i) the nozzle of the supplying device is cylindrically shaped, (ii) the distance (h) of the nozzle to the adsorbent is much larger than R, (iii) the particles in the

supplied solution are uncharged and spherical, (iv) the effect of gravity on the particles can be neglected, (v) the systems are very dilute and (vi) all particles arriving at the surface must actually adsorb.

Protein adsorption

Starting an adsorption experiment, the cuvette was filled with buffer and the angle between the plane of polarization of the incident beam and the plane of incidence was chosen such that the resulting M-value is zero. Due to protein adsorption the M-value changes.

Single protein adsorption was followed until a plateau value was reached (ca. 30 min.). Then, by switching the 4-way valve, first buffer was supplied for about 5 min. followed by a solution of another protein. Competitive adsorption experiments from a mixture of two different proteins were performed in the same way as with single adsorption. All experiments were performed at 20±1 °C in degassed buffer solution of 5 mM NaH_2PO_4 in which the total protein concentration ranged between 1-100 mg m^{-3}.

In this chapter initial adsorption rates as a function of protein concentration and pH are described and compared with J_{pr}. The following experimental conditions and literature data were used: $\nu=10^{-6}$ m^2 s^{-1}, $\phi=1.4$ 10^{-8} m^3 s^{-1}, $h=2$ 10^{-3} m, $R=(1.5\text{-}2.0)$ 10^{-4} m, $D_{IgG}=4.0$ 10^{-11} m^2 s^{-1} [13] and $D_{BSA}=7.4$ 10^{-11} m^2 s^{-1} [3,4].

STREAMING POTENTIAL MEASUREMENTS

Principle

When, as a result of a mechanical pressure difference, Δp, a solution moves relative to a charged solid phase, counterions in solution, i.e. beyond the hydrodynamic slipping layer of the solid surface, are transported by the flow of the solution. The resulting charge separation creates an electric potential difference which, in turn, causes a conductive ion flux in the direction opposite to the solvent flow. This electric potential is constant under stationary conditions and referred to as the streaming potential, V_s. It can be measured using a high-impedance Volt meter [14]. The streaming potential over a flow cell is related to the zeta-potential ζ, that is the potential at the slipping plane (i.e. the plane separating the flowing and stagnant layer) adjacent to the (adsorbent) cell surface through [15]

$$V_s/\Delta p=\varepsilon\zeta/\eta C\kappa \qquad [6.3]$$

where ε is the dielectric permittivity , η the viscosity of the solvent , C a cell constant and κ the conductance of the cell filled with the solvent. The cell constant C has been determined by measuring the conductance of the cell filled with an electrolyte solution of relatively high salt concentration (0.1 M KBr). At high ionic strength it is allowed to neglect the contribution of surface conductance along the cell wall to the overall conductance.

Due to protein adsorption the ζ-potential of the adsorbent surface changes (cf. chapter 3). According to Eq. [6.3] V_s varies proportionally with the ζ-potential. Then, to convert changes in V_s to the amount of protein adsorbed, Γ, a relation must be obtained between Γ and the ζ-potential. For a number of proteins a linear relation between ζ and the surface occupancy has been established [16]. Figures 3.5 and 3.12 also indicate approximate linearity between ζ and the occupancy of PS surface with BSA and IgG.

In the case of laminar flow, an analytical formula for combined convective-diffusive mass transport towards an adsorbent surface has been derived by Lévêque [17]. For a planar surface it reads

$$dn_i(y)/dt = 0.81\,(\gamma/yD_i)^{1/3}\,D_i\,c_b \qquad [6.4]$$

where $dn_i(y)/dt$ is the mass flux of protein i at a distance y from the entrance of the flow cell, i refers to BSA or IgG, γ is the shear rate at the cell wall; D and c_b were introduced before (cf. Eq. [6.2]). Equation [6.4] is valid under steady-state conditions with respect to the concentration boundary layer, implying that the adsorption rate must be controlled by diffusion across this layer. The steady-state conditions are expected to be satisfied at low protein concentration and at the initial stages of adsorption ('virginal' surface). Furthermore, if all protein molecules arriving at the surface do adsorb, n_i may be read as Γ_i. Since V_s (and, hence, ζ) is measured over the total length (L) of the cell, the adsorption rate must be averaged over L to yield the adsorption rate that corresponds to the change in V_s:

$$d\Gamma_i/dt = 1.22\,D^{2/3}\,c_b\,\gamma^{1/3}\,L^{-1/3} \qquad [6.5]$$

The validy of Eq [6.5] has been proven for the adsorption of various proteins on glass [16], i.e. the initial adsorption rate has been found to vary linearly with $\gamma^{1/3}$ for $3.7<\log\gamma<4.2$ and with c_b up to values of $2.0\ 10^{-3}$ mg cm^{-3}.

Protein adsorption

The flow cell used is a modification of the one described by Van Wagenen and Andrade [18]. A photograph of the cell is shown in Figure 6.2. The two adsorbent plates are placed parallel to each other in the cell and separated by a 0.1 mm Teflon gasket. The

platinum electrodes (rectangular plates of 5.0 x 25.0 mm^2) are placed in the solution on both ends of the plates.

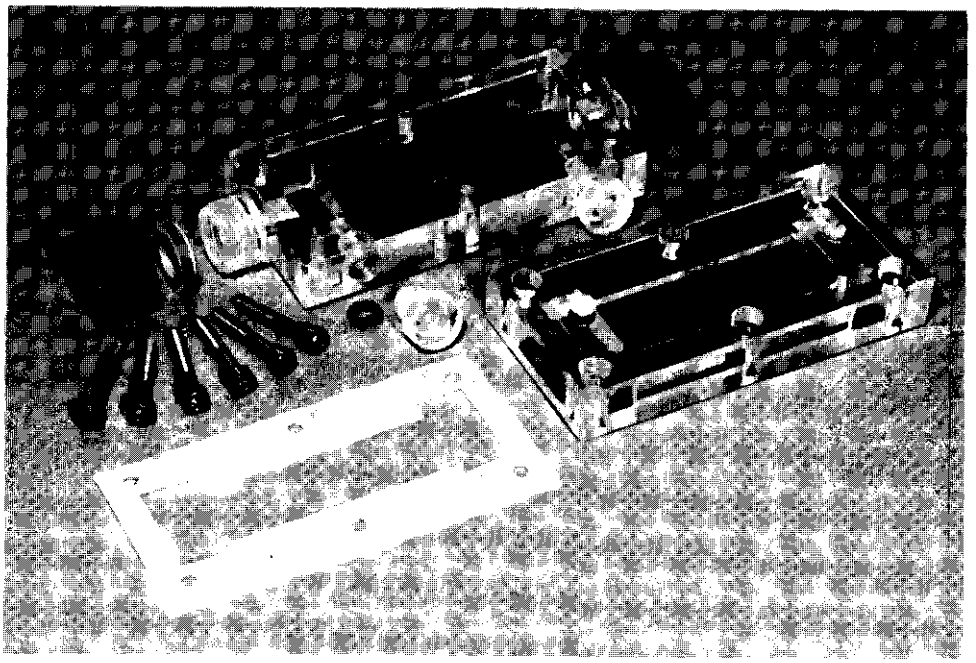

Figure 6.2 *Cell used for streaming potential measurements between two parallel plates. A Teflon gasket, used as a plate spacer, is separately shown. On both ends of the cell bottles are placed, one filled with a (protein) solution that is pressed between the plates. Taken from reference [19].*

After establishing a linear relation between V_s and Δp, independent of the flow direction of the solution through the cell, all ensuing streaming potential measurements have been performed at a pressure difference of 1.0 10^4 N m^{-2}. First, a buffer solution (5 mM NaH_2PO_4) is forced through the cell until a constant value for V_s is reached. Subsequently, a protein solution of known concentration is supplied, which enters the the cell at a time that is defined as t=0. The change of V_s upon protein adsorption is recorded. When the flask with the protein solution is nearly empty, the flow direction is quickly reversed and the adsorption continued.

Single protein adsorptions are monitored until a constant value of V_s is reached (ca. 20 min.). For sequential protein adsorption, after completion of the adsorption of the first

protein the cell is flushed with buffer solution and V_s measured (to verify whether desorption of adsorbed proteins occurs) and then exposed to another protein solution for ca. 10 min. Competitive adsorption experiments from a mixture of two different proteins were performed in the same way as for single adsorption, except that now a protein mixture is supplied. The concentration of the proteins were chosen between 0.2 and 2.0 mg m^{-3}. All experiments were performed at 25±1 °C.

Special attention is paid to the initial protein adsorption rate as a function of pH. First, V_s-data are converted to ζ-potentials (using Eq. [6.3] with ε=6.99 10^{-10} [C V^{-1} m^{-1}] and η=0.89 10^{-3} [N s m^{-2}]), followed by applying Eq. [6.6], that expresses a linear dependence of ζ with Γ:

$$(d\Gamma/dt)_{t\to 0}=(d\zeta/dt)_{t\to 0}.(\Gamma_{pl}/\Delta\zeta_{pl}) \qquad [6.6]$$

where $\Delta\zeta_{pl}$ is the difference between the ζ-potentials of the bare (ζ_o) and the protein-covered adsorbent surface under plateau conditions (ζ_{pl}), see also Figure 6.4B; standardization is possible by using for Γ_{pl} values taken from reflectometry. The adsorption rate thus derived is compared with the (maximal) initial adsorption rate as predicted from Eq. [6.5].

6.3 RESULTS AND DISCUSSION

6.3.1 ADSORBENT SURFACE PROPERTIES

Figure 6.3 shows the ζ-potential of bare PS-coated glass plates as a function of pH.

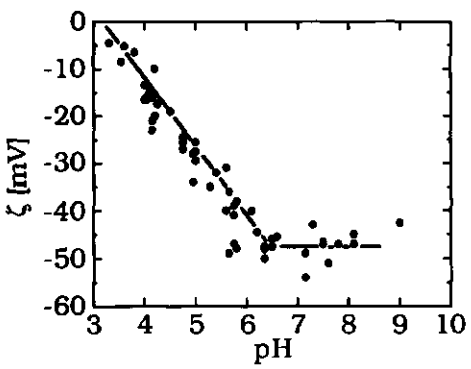

Figure 6.3 *The ζ-potential of bare polystyrene-coated glass plates in 5 mM NaH_2PO_4.*

Negative values for ζ are found for pH>3.0, indicating that the surface charge density of the positively charged groups of the polystyrene molecules is less than the negative charge density of the underlying glass. Above pH≈6.5 the variation of ζ with pH levels off, which

may be ascribed to an increase in ionic strength, i.e. with increasing pH of the buffered medium $H_2PO_4^-$ ions are titrated into HPO_4^{2-}, leading to higher ionic strengths. The ζ-potentials of the negatively charged glass surfaces coated with positively charged PS are less negative than those for the negatively charged PS-latices (cf. section 3.1).

For bare PS-coated silica plates a similar trend of (the negative) V_s with pH was observed. In this case, the V_s-values were not converted into ζ-potentials, because of the relatively high surface conductance of the silica plates.

6.3.2 SINGLE PROTEIN ADSORPTION

Qualitative patterns for a reflectometry and streaming potential measurement are presented in Figure 6.4. V_s-data are converted into ζ-potentials applying Eq. [6.3] and Γ is obtained from M-values, see section 6.2.2.

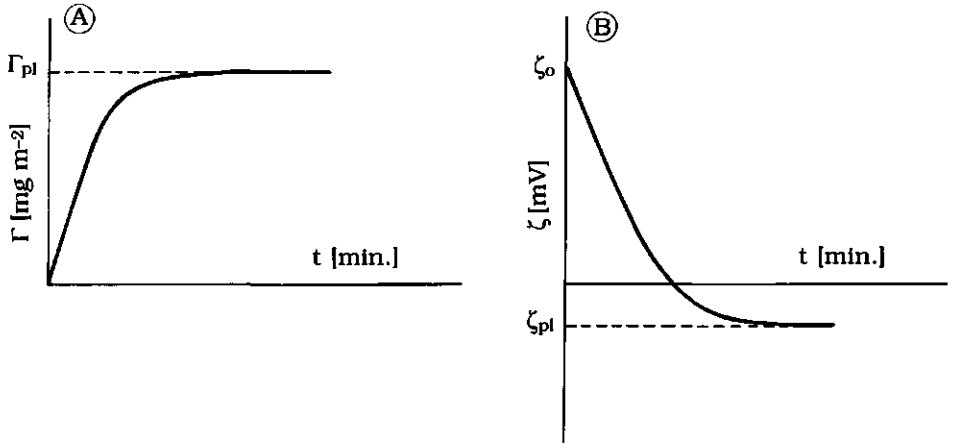

Figure 6.4 *Typical protein adsorption pattern for A) reflectometry and B) a streaming potential measurement.*

After an initial change a plateau value is reached. In both figures, the initial slopes represent the initial adsorption rate. In the cases of equal protein concentrations in both types of experiments, $\Delta\zeta_{pl}$ can be related directly to Γ_{pl}; in the other situations this relation was obtained by extrapolation. Under electrostatically attractive conditions between the protein and the adsorbent surface, the sign of V_s reverses, which is in line with the variation of ζ with the amount of protein adsorbed on PS particles (see chapter 3), and which is indicative of specific adsorption (i.e. adsorption under the influence of electrostatic and non-electrostatic interactions).

Plateau adsorption

In Figure 6.5 plateau adsorption as derived from reflectometry is plotted as a function of pH. The Γ_{pl}-values are lower than those obtained for adsorption on latices, (cf. Figures 3.3 and 3.11). The difference may be ascribed to the more negative ζ-potentials of the latices as compared to the macroscopic surfaces. Furthermore, $\Gamma_{pl}(t)$ decreases with lower protein concentration, which may point to larger structural changes in protein molecules that adsorb from a solution of a lower concentration. Streaming potential measurements with BSA of 0.2 and 1.0 mg m^{-3} gave identical ζ_{pl}-values, whereas with IgG-4F at 1.0 mg m^{-3} ζ_{pl} is less negative (by about 20 mV) than at 0.2 mg m^{-3}.

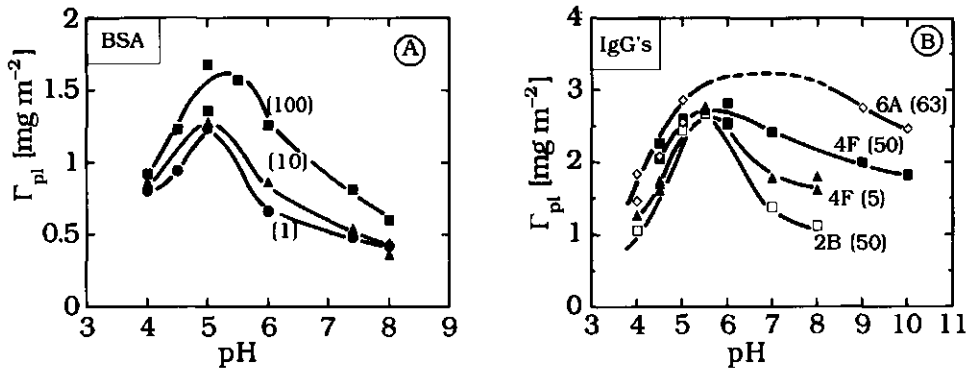

Figure 6.5 *Plateau adsorption of A) BSA after 45min. and B) IgG's after 30 min. using reflectometry. Protein concentrations (mg m^{-3}) are indicated in brackets. Because of instability of IgG-6A in the pH region between 5.5 and 7.5 the corresponding part of the curve is interpolated (dashed in Figure 6.5B).*

For all the proteins Γ_{pl} as a function of pH passes through a maximum ($\Gamma_{pl,max}$). In Table 6.2 pH-values at which $\Gamma_{pl,max}$ occurs are tabulated together with those for adsorption on PS latices. This table shows that, with respect to the Γ_{pl}(pH) pattern, the adsorption on the negatively charged PS-coated silica surface is similar to that on a negatively charged latex. Apparently, the positive charge of the polystyrene coating, which is less than the underlying negative charge on the silica surface, does not affect the protein adsorption behaviour. In chapter 3 for protein-PS latex systems, the existence of a maximum plateau value has been ascribed to maximum structure stability of the adsorbed protein molecules around the isoelectric state of the protein-adsorbent complex. Similarly, with macroscopic surfaces it is found that the pH values where reflectometry attains maximum plateau

adsorption corresponds rather well with those where streaming potential data indicate isoelectricity, i.e. 4.9 for BSA and 5.3 for IgG-4F.

Table 6.2 *The pH where $\Gamma_{pl}(pH)$ attains a maximum value.*

protein	PS-coated silica (ζ<0)	PS latices (ζ<0)	(ζ>0)
BSA	5.0	4.5	6.1
IgG-2B	5.5	5.0	5.6
IgG-4F	6.0	5.6	7.0
IgG-6A	6–7	~7	9.0

Initial rate of protein adsorption

Initial adsorption rates as a function of pH are given in Figure 6.6. For the calculation of the initial adsorption rate from the streaming potential measurements with 0.2 mg m^{-3} IgG-4F solution, Γ_{pl}-values from reflectometry for a solution of 1.0 mg m^{-3} are used. Therefore, the value of $(d\Gamma/dt)_{t\rightarrow 0}$ for IgG-4F adsorbing from a 0.2 mg m^{-3} solution may be somewhat overestimated. The Figures 6.6A and 6.6B show good agreement between the adsorption rates determined by the two different techniques. For BSA and IgG-4F an increase of the initial adsorption rate with increasing concentration is found, as expected. BSA, IgG-2B and, to a lesser extent, IgG-6A show maximum initial adsorption rates around their isoelectric points.

Around their i.e.p. the different monoclonals have about the same initial adsorption rates. The decrease for BSA and IgG-2B with increasing pH can be attributed to an energy barrier caused by overlapping electric fields of the protein molecule and the adsorbent, that are both negatively charged. The adsorption is then retarded by a factor $e^{-A/kT}$ [20], where A is the activation energy required to overcome the energy barrier and kT is one unit of thermal energy. For a BSA solution of 1.0 mg m^{-3} the experimentally determined initial adsorption rates are compared with the rates of supply calculated using Eq. [6.2] for reflectometry and Eq. [6.5] for streaming potential measurements. Generally, the experimentally determined initial adsorption rates were lower than the predicted ones from Eqs. [6.2] and [6.5], indicating that not all molecules supplied really adsorb. These lower values for the adsorption rates can be explained by assuming activation energies that at pH=5.0 amount to 0.2 kT (streaming potential measurement) and 1.6 kT (reflectometry)

and at pH=7.5 to 2.3 kT (streaming potential measurement) and 6.5 kT (reflectometry). Because the A-values derived from reflectometry are larger, the fraction of protein molecules that really attach to the surface is smaller. The transport of the protein towards the adsorbent surface might be quicker for streaming potential experiments, implying a less thick Nernst diffusion layer.

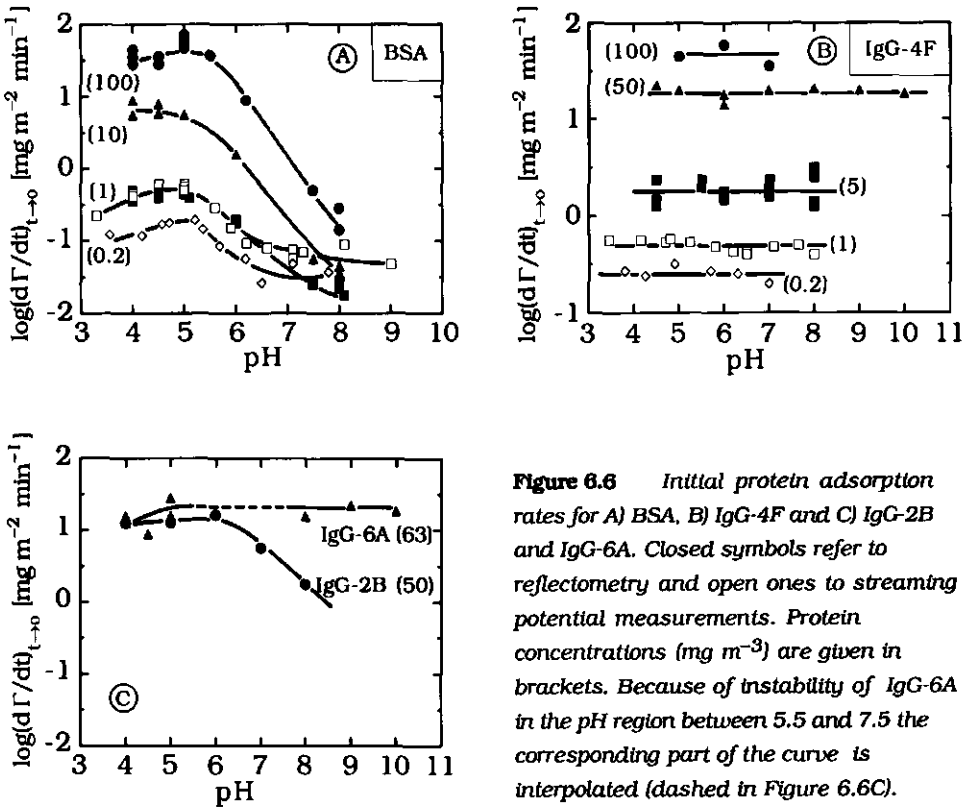

Figure 6.6 *Initial protein adsorption rates for A) BSA, B) IgG-4F and C) IgG-2B and IgG-6A. Closed symbols refer to reflectometry and open ones to streaming potential measurements. Protein concentrations (mg m^{-3}) are given in brackets. Because of instability of IgG-6A in the pH region between 5.5 and 7.5 the corresponding part of the curve is interpolated (dashed in Figure 6.6C).*

The initial adsorption rate of IgG-4F is independent of the pH, indicating that other than electrostatic factors dominate the adsorption behaviour. Since it is believed that the F_c-fragment of an IgG-molecule is more hydrophobic than the two F_{ab}-parts [21], it may be speculated that IgG-4F adsorbs in an 'end-on' fashion onto the surface. The A-values for IgG-4F are somewhat smaller as compared to those for BSA, i.e. 1.3 kT (reflectometry) and about 0 kT (streaming potential). This finding supports the idea that electrostatic interactions are of minor importance for the adsorption of IgG-4F.

6.3.3 SEQUENTIAL PROTEIN ADSORPTION

Sequential adsorption experiments were performed with BSA (i.e.p.=4.7-5.0) and IgG-4F (i.e.p.=6.8-7.0) at various pH-values. After pre-adsorption no (or negligible) desorption was observed upon flushing with 5mM NaH_2PO_4 buffer for about 10 min. using either technique. As mentioned before, the two techniques do not allow to discriminate between different proteins. Therefore, any change in the total adsorbed amount, after supplying the second protein to the pre-adsorbed one, may be caused by either or both additional adsorption and displacement.

In Table 6.3 the protein concentrations used are tabulated for both sequences.

Table 6.3 *Protein concentrations in sequential adsorption experiments.*

method	c_{IgG-4F} [mg m^{-3}]	c_{BSA} [mg m^{-3}]	sequence
str. pot.	0.2	1.0	1st IgG-4F, 2nd BSA
str. pot.	1.0	2.0	1st IgG-4F, 2nd BSA
str. pot.	0.2	0.2	1st BSA, 2nd IgG-4F
reflec.	5.0	2.6	1st IgG-4F, 2nd BSA
reflec.	5.0	2.6	1st BSA, 2nd IgG-4F

Competition between BSA and pre-adsorbed IgG

The change in adsorbed amount ($\Delta\Gamma$) determined by reflectometry are given in Figure 6.7A. For comparison purposes, $\Delta\Gamma$ for the sequential adsorption on PS latices is also given (calculated from the data in Table 4.3). The data for two negatively charged latices are averaged. According to both techniques in the pH range studied $\Delta\Gamma$ is positive over the entire pH range, which includes conditions of electrostatic attraction between the two proteins (cf. Figure 4.2). Below, where the opposite adsorption sequence will be discussed, more attention is paid to this feature. Displacement of IgG is observed from negatively charged latices for 4<pH<7 and from the positively charged latex for pH>5.5, see also Table 4.3. Reflectometry data point to minimum amounts of additionally adsorbed BSA, but displacement of IgG cannot be excluded.

Initial adsorption rates of the additionally supplied BSA, derived from streaming potential measurements, are given in Figure 6.7B. In addition, the values for single BSA adsorption at 1.0 mg m^{-3} are given. A maximum in the initial adsorption rate is found in the pH range 4-5. More precisely, the pH where this maximum occurs shifts to lower values

with increasing amount of pre-adsorbed IgG. The initial adsorption rates for 1.0 mg m^{-3} BSA are unaffected by the presence of pre-adsorbed IgG, indicating that intrinsic properties of the BSA molecules are of greater importance than adsorbent characteristics.

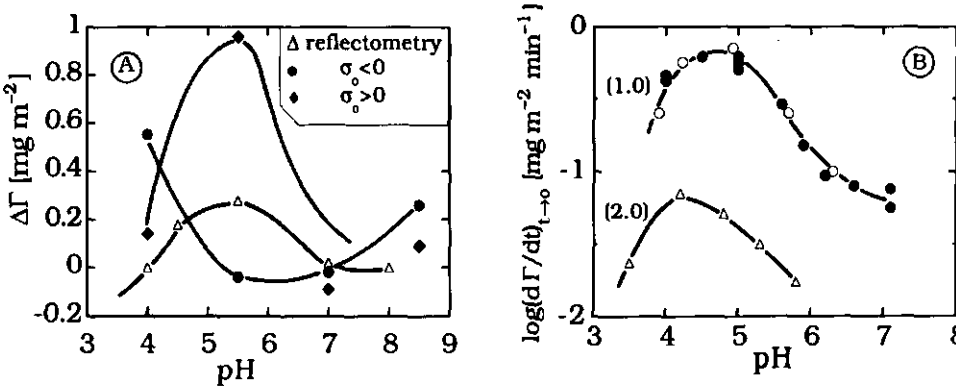

Figure 6.7 *Competition of solute BSA with pre-adsorbed IgG-4F A) change of amount adsorbed and B) initial adsorption rate: open symbols indicate sequential adsorption, closed circles single adsorption. The BSA concentration (mg m^{-3}) is indicated.*

The finding that the initial adsorption rate of BSA, is not affected by the presence of pre-adsorbed IgG indicates that in the sequential adsorption experiment displacement of IgG is not a prerequisite for additional adsorption. Furthermore, from Figure 4.6 it was concluded that displacement of IgG occurs only for amounts of adsorbed IgG >3 mg m^{-2}, a value that is not achieved under the present conditions, i.e. low protein concentrations.

Competition between IgG and pre-adsorbed BSA

From data obtained with PS latices it was found that BSA is hardly displaced by different monoclonal IgG's (cf. section 4.3.2). Therefore, the increase of the total amount of protein adsorbed, as found with the two techniques, is completely ascribed to additional adsorption of IgG.

In Figure 6.8A the amount of additionally adsorbed IgG ($\Delta\Gamma_{IgG}$) determined by reflectometry is given, together with data for the PS latices, taken from Table 4.4. The increase of additionally adsorbed IgG with increasing pH may (partly) be attributed to the decrease in the amount of pre-adsorbed BSA.

Using streaming potential measurements, the effect of electrostatic interaction between the pre-adsorbed and solute protein can be inferred from the initial adsorption rate. In

Figure 6.8B the initial adsorption rate of IgG is given as a function of pH together with that for single IgG adsorption from a solution of 0.2 mg m^{-3}. In sequential adsorption the initial adsorption rate decreases with increasing pH, whereas for single IgG a constant initial adsorption rate was found. The difference illustrates the influence of the pre-adsorbed BSA on the subsequent IgG adsorption.

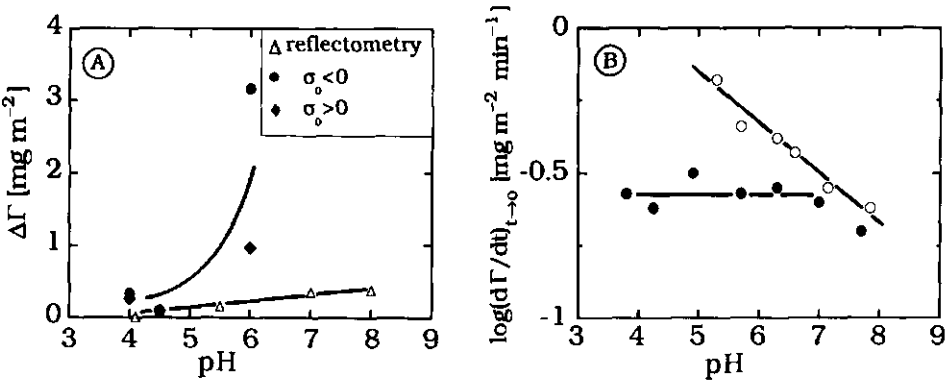

Figure 6.8 *Competition of solute IgG-4F with pre-adsorbed BSA A) change of amount adsorbed and B) initial adsorption rate: open circles indicate sequential adsorption, closed circles single adsorption.*

In accordance with the finding for PS latices, under electrostatically attractive conditions (in the pH range 5.7-6.8) between the secondly supplied protein and the pre-adsorbed one, additionally adsorbed amounts are measured. To verify whether this additional adsorption is caused by electrostatic attraction between the two proteins at the surface after adsorption of the second protein, the cell was flushed with an electrolyte solution of higher ionic strength (0.02 M NaH_2PO_4) for 5 min. Then, the cell was refilled with 5 mM NaH_2PO_4 and after achieving a constant streaming potential, this final value was compared with that obtained directly after sequential adsorption. Assuming that due to the increase of ionic strength only the additionally adsorbed protein molecules are prone to desorb, the results can be summarized as follows: (i) around pH=5.5 100% of the additionally adsorbed IgG desorbs, (ii) with increasing pH the desorbed fraction, which is inversely related to the additionally adsorbed amount, becomes smaller and (iii) the less unambiguous results for the opposite sequence (pre-adsorption of IgG) are probably due to the small amounts of BSA additionally adsorbed.

6.3.4 SIMULTANEOUS ADSORPTION OF BSA AND IgG-4F

Initial adsorption rate

Streaming potential measurements show that from solutions of low BSA and IgG-4F concentrations (i.e. both 0.2 mg m^{-3}) and in the pH range 4-8, the initial adsorption rate equals the sum of the initial single adsorption rates. This observation is indicative of the absence of interaction between the two adsorbing proteins and hence it supports the premises underlying the application of Eq. [6.5]. Increasing the concentration of one of the proteins to 1.0 mg m^{-3}, the initial adsorption rates at 5.5<pH<7 become larger than the sum of the individual initial adsorption rates. This cooperative effect takes place in the pH range where, in solution (cf. Figure 4.2), the two proteins attract each other electrostatically and it may therefore be attributed to complex-formation on the adsorbent surface.

Reflectometry, using solutions of pH=4.5 and 8.0 with BSA and IgG concentrations of 1 and 5 mg m^{-3}, and 50 and 5 mg m^{-3}, respectively, also points to the absence of interaction between the adsorbing proteins. In contrast, from a solution of 1 mg m^{-3} BSA and 5 mg m^{-3} IgG (pH=6.5) the initial adsorption rate is again larger than the sum of the single initial rates. An adsorption 'over-shoot' was found within 4 min., followed by a further slow decrease in adsorbed amount (ca. 0.2 mg m^{-2} in 50 min.). The last mentioned phenomenon might indicate that the adsorbed protein-protein complex is disrupted, possibly due to re-orientation and/or structural rearrangements of protein(s) in the adsorbed state.

Plateau adsorption

For all situations studied (conditions mentioned above), constant adsorbed amounts are reached within 30 min. with values that are between those obtained from single protein adsorption. This is observed both with reflectometry and streaming potential measurements. It seems plausible that both proteins are present on the surface. The plateau value for adsorption of the mixture tends to approach the plateau value for the protein component that has the highest concentration in the mixture. This feature agrees with the results obtained for competitive adsorption on PS latices (cf. Figures 5.4-5.7) and proves once more that the initial situation is virtually frozen in.

Reflectometry with protein solutions at various conditions showed that even after a prolonged incubation time (>60 min.) the adsorbed amount suddenly slightly increases. As has been stated in chapter 5 the effect may be caused by the retarded adsorption of the protein having the lowest affinity for the bare adsorbent surface. Streaming potential measurements were not continued over a sufficiently long period of time to verify the validity of this hypothesis.

6.4 CONCLUSIONS

Reflectometry and streaming potential measurements gave comparable results for the initial adsorption rate of the proteins BSA and monoclonal IgG-4F on PS-coated macroscopic surfaces.

For BSA, the initial adsorption rate as a function of pH shows a maximum around its isoelectric point. Because a similar variation with pH has been observed for the plateau value, it is concluded that the ultimate surface composition of the adsorbed BSA layer is largely determined by the initial stage of the adsorption process. For monoclonal IgG-2B a similar observation was made. The pattern of the initial adsorption rate of BSA as a function of pH is not affected by pre-adsorption of IgG-4F at the PS surface. It is therefore concluded that the adsorption rate of BSA is primarily determined by intrinsic properties of the BSA molecule rather than by the surface characteristics of the adsorbent.

For monoclonal IgG-4F, the initial adsorption rate was independent of pH, indicating that factors other than electrostatic interaction between the protein and the adsorbent are dominating. The variation with pH of the plateau adsorption at the macroscopic PS-coated surface is similar to that observed with negatively charged latices: the occurence of a plateau value reaches a maximum at the isoelectric state of the IgG-adsorbent complex. These observations lead to the conclusion that the initial adsorption behaviour of IgG-4F does not dominate the final surface composition. In this respect BSA and IgG-4F behave quite differently.

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SUMMARY

The subject of this thesis is proteins at interfaces. The main purpose of the work was to acquire more insight into the mechanism of adsorption of Bovine Serum Albumin (BSA) and monoclonal Immuno gamma Globulins (IgG's), both individually and in competition. Another aim was to achieve optimum conditions for the colloidal stability of polystyrene (PS) latices coated with these proteins, a feature that is important in the development of diagnostic test systems.

First, in **chapter 2**, a literature overview is given with special emphasis on (i) the adsorption of protein mixtures and (ii) the dynamic aspects of protein adsorption. The reason for studying the latter is that there are many indications that protein adsorption is to a certain extent irreversible. Hence, the ultimate properties of a protein adsorbate may partly be determined by its history. The structure stability of a protein molecule is considered to be one of the most important parameters influencing its (competitive) adsorption behaviour. The heterogeneity of an adsorbed protein layer can also be related to this intrinsic property of the protein. The 'reversibility' aspects are reflected in the extents of desorption, exchange and/or displacement. The molecules that arrive the latest on the surface will be more easily removed compared to those adsorbing in a more initial stage, having more time to adapt themselves optimally.

In **chapter 3** single adsorption of BSA and monoclonal IgG's on differently charged PS latices is described. Monoclonal IgG's having different isoelectric points were used, allowing us to systematically investigate the electrostatic aspects of interaction. Much attention is paid to the proper purification and characterization of the materials used. The main finding in this chapter is the occurrence of maximum protein adsorption as a function of pH around the isoelectricity of the protein-latex complex, rather than that of the solute protein. An important aspect in the overall protein adsorption process is the involvement of low molecular weight ions.

The competition between BSA and monoclonal IgG's has been studied by sequential (**chapter 4**) and simultaneous (**chapter 5**) adsorption.

IgG molecules are hardly able to displace pre-adsorbed BSA from PS latices, whereas BSA molecules are capable to partially displace pre-adorbed IgG from these surfaces. More precisely, partial displacement of IgG occurs from a positively charged PS latex at pH≈7 and from negatively charged latices at lower pH. The lower structural stability of BSA molecules

compared to that of the (larger) IgG molecules may account for this effect. In addition, the extent of displacement depends on the electrostatic interaction between the respective proteins and the surface. Additional adsorption of different IgG's on BSA-coated latices follows an electrostatically determined sequence, whereas no such trend is found for the reverse case of additional adsorption of BSA on different IgG pre-coated latices. Under electrostatically attractive conditions between the secondly supplied and pre-adsorbed protein, large additionally adsorbed amounts are found, a phenomenon that may partly be attributed to the formation of a protein-protein complex on the surface.

Simultaneous adsorption of BSA and IgG only leads to preferential adsorption of one of the two proteins, when the proteins and the adsorbent have the same charge sign. Initially, protein molecules having the lower electrostatical affinity for the surface are retarded. Additional adsorption of this protein does occur with time, but the preference is already determined in the early stage. Fixation of the initial surface composition is also found under electrostatically attractive conditions between proteins and adsorbent. Furthermore, competitive adsorption between different IgG's is governed by electrostatic interactions. It is concluded that electrostatic interactions are important in competitive protein adsorption. The finding is helpful to predict the ultimate preference in the adsorbate.

In **chapter 6** kinetic aspects of the adsorption on macroscopic PS surfaces is studied by reflectometry and streaming potential measurements. The initial adsorption rate and plateau adsorption of BSA both show a maximum around its isoelectric point, leading to the conclusion that the final surface composition is already frozen in in the initial stages. For one monoclonal (isoelectric point ≈7.0), the initial adsorption rate was found independent of the pH. Apparently, other than electrostatic interactions are dominating. Finally, single and competitive adsorption of these proteins are comparable with those observed on the latices.

SAMENVATTING

Dit proefschrift behandelt de adsorptie van eiwitten aan vaste oppervlakken. Met name wordt aandacht besteed aan de concurrentie die kan optreden als verschillende eiwitten tegelijk of na elkaar aangeboden worden. Er zijn veel biologische processen te noemen waarin deze problematiek een rol speelt. Eiwitten komen voor in diverse biologische vloeistoffen zoals bloed, urine, traanvocht, melk en ook in vele vruchtesappen en zeewater. Elk eiwitmolecuul is opgebouwd uit een lineaire reeks van vele (tot enkele honderden) aminozuur-eenheden. In een waterig milieu nemen de moleculen van de meeste eiwitten een globulaire (= opgevouwen) structuur aan. Deze komt tot stand door een subtiele balans van interacties binnen het molecuul zelf en die tussen het molecuul en zijn omgeving. Wanneer een eiwitmolecuul adsorbeert vanuit een waterige oplossing aan een vast oppervlak treedt er een 'omgevings'-verandering op, waardoor deze balans wordt beïnvloed. Een gevolg kan zijn dat eiwitten aan oppervlakken van conformatie (= structuur) veranderen. Uit de literatuur (**hoofdstuk 2**) blijkt dat voor sommige eiwitten het optreden van deze conformatieveranderingen een van de oorzaken kan zijn dat inderdaad adsorptie plaatsvindt.

De conformatie van een eiwit molecuul bepaalt mede de biologische functie. Een eiwit dat is gebruikt in dit onderzoek is runder serum albumine (BSA), een relatief klein 'sigaar'-vormig eiwit dat de pH en osmotische druk reguleert en daarnaast het transport van vetzuren, hormonen en (metaal)ionen verzorgt. Tevens zijn monoklonale immuno γ-globulinen (IgG's) gebruikt, dit zijn grotere Y-vormige moleculen die zorgen voor de afweer van lichaamsvreemde stoffen (= antigenen). Monoklonale IgG's zijn moleculen met een zeer grote uniformiteit, verkregen met behulp van een speciale 'hybridoma' techniek. Daarentegen bevat conventioneel (natuurlijk) IgG moleculen met verschillende fysische eigenschappen. Van de 'afweer'-eigenschap van IgG moleculen wordt dankbaar gebruik gemaakt in diagnostische testsystemen. Het principe van zo'n testsysteem is de agglutinatie (= samenklontering) van met IgG bedekte bolvormige deeltjes ten gevolge van een (immunologische) reactie met het complementaire antigeen. De bolvormige 'drager'-deeltjes zijn noodzakelijk om de reactie te visualiseren. Tijdens dit onderzoek zijn bolvormige deeltjes gebruikt (polystyreen (PS) latices genaamd) van enkele honderden nanometers klein met verschillende elektrische ladingen aan het oppervlak. Aangezien de meeste eiwitten ook lading bevatten, is het duidelijk dat elektrostatische factoren het adsorptie gedrag mede zullen beïnvloeden. In dit onderzoek is ondermeer gekeken naar de effecten

van elektrostatische- en conformatie factoren op de adsorptie van BSA en IgG aan PS latices, wanneer de eiwitten afzonderlijk en concurrerend worden aangeboden.

In **hoofdstuk 3** zijn voor zowel BSA als vier verschillende monoklonale IgG's enkelvoudige (met één soort eiwit) adsorpties aan verschillend geladen PS latices beschreven. De gebruikte monoklonalen verschillen onderling in isoelektrisch punt (= de pH waar de electrokinetische lading nul is), zodat de elektrostatische bijdrage tot de eiwit adsorptie systematisch kan worden bestudeerd. Het blijkt dat de hoeveelheid geadsorbeerd eiwit maximaal is wanneer het eiwit-latex complex geen elektrokinetische lading bevat. Dit is een precisering van de eerdere opvatting dat maximale eiwitadsorptie plaatsvindt rond het isoelektrisch punt van het eiwit in oplossing. Tevens duidt deze trend op het belang van kleine ionen tijdens het adsorptieproces, waarbij vooral aan ioninbouw tussen eiwit en oppervlak wordt gedacht.

De concurrentie tussen BSA en monoklonale IgG's aan verschillend geladen PS latices is bestudeerd voor opeenvolgende- (**hoofdstuk 4**) en gelijktijdige adsorptie (**hoofdstuk 5**) van deze eiwitten.

Wanneer BSA als eerste eiwit wordt geadsorbeerd dan blijkt het niet of nauwelijks door later toegevoegd IgG van de PS deeltjes verdrongen te worden. In de omgekeerde situatie blijkt BSA wel in staat om een deel (maximaal 30%) van gepreadsorbeerd IgG te verdringen. Mogelijk draagt de lagere conformatie-stabiliteit van BSA moleculen ten opzichte van de IgG moleculen bij tot dit resultaat. Verder blijkt de mate van verdringing afhankelijk van de elektrostatische wisselwerking tussen de eiwitten en het kale oppervlak. De additionele adsorptie van de verschillende IgG's aan met BSA bedekte latices volgt een elektrostatisch bepaalde volgorde. In de omgekeerde situatie wordt deze trend niet gevonden. Als opeenvolgende adsorptie wordt uitgevoerd onder elektrostatische aantrekking tussen het tweede- en het gepreadsorbeerde eiwit worden grote hoeveelheden extra geadsorbeerd. Dit verschijnsel kan deels worden toegeschreven aan de vorming van een eiwit-eiwit complex op het oppervlak.

Wanneer een mengsel van BSA en IgG gelijktijdig aan PS latices wordt aangeboden wordt alleen voorkeur voor één van beide eiwitten waargenomen wanneer het oppervlak en de eiwitten eenzelfde ladingsteken (dus onder elektrostatische afstoting) hebben. Tijdens het begin van het proces wordt de adsorptie van het eiwit met de laagste elektrostatische affiniteit voor het oppervlak vertraagd. Hoewel er nog wel extra adsorptie van dit eiwit plaatvindt, is de voorkeur voor het andere eiwit hierdoor reeds vastgelegd. Bij elektrostatische aantrekking tussen het oppervlak en de eiwitten wordt de samenstelling van de geadsorbeerde eiwitlaag reeds in de beginfase van het adsorptieproces gefixeerd.

Gelijktijdige adsorptie van een mengsel van verschillende IgG's aan PS latices blijkt (dan) ook elektrostatisch bepaald. Elektrostatische wisselwerkingen zijn dus van belang in de concurrentie tussen eiwitten om te adsorberen. Het blijkt mogelijk om een voorspelling te doen over het eventueel verrijken van de geadsorbeerde laag ten aanzien van één van de eiwitten.

In **hoofdstuk 6** zijn kinetische aspecten van eiwit adsorptie aan makroscopische PS oppervlakken bestudeerd. Er zijn twee verschillende methoden gebruikt, te weten reflectometrie en een stromingspotentiaal techniek. De adsorptiesnelheid in de beginfase en de uiteindelijk geadsorbeerde hoeveelheid BSA als functie van de pH blijken een identiek verloop te hebben; beide vertonen een maximum waarde rond het isoelektrisch punt van het BSA. Hieruit kan worden geconcludeerd dat de uiteindelijke situatie op het oppervlak reeds in de beginfase wordt vastgelegd. Voor één monoklonaal IgG, met een isoelektrisch punt van 7.0, blijkt de beginsnelheid van de adsorptie onafhankelijk van de pH te zijn, terwijl de uiteindelijk geadsorbeerde hoeveelheid IgG wel pH-afhankelijk is. Blijkbaar bepalen in dit geval andere dan elektrostatische wisselwerkingen de beginsnelheid van de adsorptie.
De resultaten van enkelvoudige-, opeenvolgende- en gelijktijdige adsorptie experimenten van deze eiwitten aan macroscopische PS oppervlakken komen goed overeen met die van PS latices.

CURRICULUM VITAE

Alfred Victor Elgersma werd geboren op 13 februari 1962 te Weststellingwerf. In 1980 behaalde hij het Atheneum-B diploma aan de Rijksscholengemeenschap te Harderwijk. Aansluitend begon hij de studie Chemische Technologie aan de Technische Hogeschool Twente, tegenwoordig Universiteit Twente, te Enschede. De doctoraalstudie werd verricht binnen de vakgroep Macromoleculaire Chemie en Materiaalkunde, onderzoekgroep Biomedische Materiaaltechniek onder leiding van Prof. dr. A. Bantjes. Het doctoraalexamen werd afgelegd in augustus 1986.

Van oktober 1986 tot april 1990 verrichtte hij het in dit proefschrift beschreven onderzoek onder leiding van Prof. dr. J. Lyklema en Dr. W. Norde bij de vakgroep Fysische- en Kolloïdchemie aan de Landbouwuniversiteit te Wageningen. Het onderzoek werd uitgevoerd in samenwerking met AKZO Corporate Research te Arnhem.

Vanaf augustus 1990 zal hij voor de periode van één jaar werkzaam zijn bij het 'Research Institute for Polymers and Textiles' te Tsukuba (Japan).

DANKWOORD

Dit boekje is het resultaat van enkele jaren onderzoek bij de vakgroep Fysische- en Kolloïdchemie. Ik wil alle (ex)-medewerkers danken voor de getoonde interesse in mijn onderzoek en de bijzonder prettige sfeer op en rond de werkplek. Het onderzoek werd financieel mogelijk gemaakt door AKZO Corporate Research (Arnhem), waarvoor mijn dank.

Graag wil ik enkele personen in het bijzonder vernoemen:
Allereerst mijn twee leer(groot)meesters Hans Lyklema en Willem Norde.
Beste Willem, mijn dank aan jou is groot. In vele opzichten heb je mij een onvergetelijke, leerzame tijd bezorgd, waarbij ik echt altijd bij je terecht kon. Mijn gevoelens laten zich het best verwoorden door te zeggen dat ik het zal missen!
Beste Hans, ik wil je hartelijk danken voor de grote mate van vrijheid waarin ik de onderzoeker kon spelen onder jou hoede. Van je kritische commentaren bij de verschillende versies van de hoofdstukken heb ik veel geleerd. Ik wens je veel succes met de voltooiing van je boek(werk).
Hans en Willem, ik beschouw het als een groot voorrecht om met jullie te hebben samengewerkt.
Rob Zsom van AKZO-CR wil ik hartelijk dank zeggen voor zijn enthousiaste en stimulerende betrokkenheid bij het onderzoek. Tevens wil ik een ieder van AKZO-CR en Organon Teknika (Boxtel) die heeft bijgedragen aan de constructieve werkbesprekingen danken.
Stefan Blankenborg, Lucie van de Steeg en Coen van Delden hebben in het kader van hun doctoraalopdracht significant bijgedragen aan mijn onderzoek, hiervoor mijn oprechte dank. Ik vond het zeer plezierig en leerzaam om met jullie te bomen over vele (niet) wetenschappelijke onderwerpen.
Erna Rouwendal wil ik danken voor het uitvoeren van de adsorptie experimenten die op het laatste ogenblik noodzakelijk bleken.
Voor de vormgeving van dit proefschrift ben ik Gert Buurman en Bert Bouman mijn dank verschuldigd.

Fred