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Streptavidin - A Versatile Binding Protein for Solid-Phase Immunoassays

by

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To my family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications. They are referred to by Roman numerals in the text.

I Lasse Välimaa, Kim Pettersson, Markus Vehniäinen, Matti Karp, and Timo Lövgren (2003) A high-capacity streptavidin-coated microtitration plate. *Bioconjugate Chem.* **14:** 103 - 111.

II Lasse Välimaa, Kim Pettersson, Jaana Rosenberg, Matti Karp, and Timo Lövgren (2004) Quantification of streptavidin adsorption in microtitration wells. *Anal. Biochem.* **331:** 376 - 384.

III Lasse Välimaa, and Katja Laurikainen (2006) Comparison study of streptavidin-coated microtitration plates. *J. Immunol. Methods* **308:** 203 - 215.

IV Lasse Välimaa, Johanna Ylikotila, Hannu Kojola, Tero Soukka, Harri Takalo, and Kim Pettersson (2008) Streptavidin-coated spot surfaces for sensitive immunoassays using fluorescence surface readout. *Anal. Bioanal. Chem.* Published Online May 3, 2008. doi:10.1007/s00216-008-2120-y.

In addition, unpublished data are included mainly in sections 4.1, 4.4 and 4.5.

ABBREVIATIONS

AFM	atomic force microscopy
BCA	bicinchoninic acid
Bio-	refers to a biotinylated molecule
BSA	bovine serum albumin
CA125	cancer associated antigen 125
DDI	DNA-directed immobilization
DELFIA	dissociation-enhanced lanthanide fluorescence immunoassay
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
Eu	europium
Fab / Fab'	fragment antigen binding; refers to the antigen-binding fragment of immunoglobulin consisting of the variable and one constant domains
$F(ab')_2$	a fragment of immunoglobulin consisting of two linked Fab' fragments
Fc	fragment crystallizable; refers to the constant stem region of immunoglobulin
GA-SAv	refers to pretreated, polymerized high-capacity streptavidin
ΔG	Gibbs energy change
hCG	human chorionic gonadotropin
HRP	horseradish peroxidase
IgG	immunoglobulin G
IgM	immunoglobulin M
K_a	affinity constant
K_d	dissociation constant
k_{off}	dissociation rate constant
k_{on}	association rate constant
Mab	monoclonal antibody
NHS	N-hydroxysuccinimide
pI	isoelectric point
PSA	prostate specific antigen
QCM	quartz crystal microbalance
RCSB	Research Collaboratory for Structural Bioinformatics
RSA	random sequential adsorption
SAM	self-assembled monolayer
SATA	N-succinimidyl-S-acetylthioacetate
SAv	refers to unmodified, native streptavidin
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
SPM	scanning probe microscopy
SPR	surface plasmon resonance
STM	scanning tunneling microscopy
Tb	terbium
TR-IFMA	time-resolved immunofluorometric assay
TSH	thyroid-stimulating hormone

ABSTRACT

Streptavidin, a tetrameric protein secreted by *Streptomyces avidinii,* binds tightly to a small growth factor biotin. One of the numerous applications of this high-affinity system comprises the streptavidin-coated surfaces of bioanalytical assays which serve as universal binders for straightforward immobilization of any biotinylated molecule. Proteins can be immobilized with a lower risk of denaturation using streptavidin-biotin technology in contrast to direct passive adsorption. The purpose of this study was to characterize the properties and effects of streptavidin-coated binding surfaces on the performance of solid-phase immunoassays and to investigate the contributions of surface modifications.

Various characterization tools and methods established in the study enabled the convenient monitoring and binding capacity determination of streptavidin-coated surfaces. The schematic modeling of the monolayer surface and the quantification of adsorbed streptavidin disclosed the possibilities and the limits of passive adsorption. The defined yield of 250 ng/cm^2 represented approximately 65 % coverage compared with a modeled complete monolayer, which is consistent with theoretical surface models. Modifications such as polymerization and chemical activation of streptavidin resulted in a close to 10-fold increase in the biotin-binding densities of the surface compared with the regular streptavidin coating. In addition, the stability of the surface against leaching was improved by chemical modification. The increased binding densities and capacities enabled wider high-end dynamic ranges in the solid-phase immunoassays, especially when using the fragments of the capture antibodies instead of intact antibodies for the binding of the antigen. The binding capacity of the streptavidin surface was not, by definition, predictive of the low-end performance of the immunoassays nor the assay sensitivity. Other features such as non-specific binding, variation and leaching turned out to be more relevant.

The immunoassays that use a direct surface readout measurement of time-resolved fluorescence from a washed surface are dependent on the density of the labeled antibodies in a defined area on the surface. The binding surface was condensed into a spot by coating streptavidin in liquid droplets into special microtiter wells holding a small circular indentation at the bottom. The condensed binding area enabled a denser packing of the labeled antibodies on the surface. This resulted in a 5 - 6-fold increase in the signal-to-background ratios and an equivalent improvement in the detection limits of the solid-phase immunoassays.

This work proved that the properties of the streptavidin-coated surfaces can be modified and that the defined properties of the streptavidin-based immunocapture surfaces contribute to the performance of heterogeneous immunoassays.

1 REVIEW OF THE LITERATURE

1.1 Introduction

Streptavidin is a tetrameric protein of approximately 60 kD in size secreted by bacterium *Streptomyces avidinii*. Streptavidin was discovered in the early 1960s when the fermentation filtrates of *Streptomycetes* were searched for antibiotic activity (Chaiet *et al.*, 1963). The antibiotic activity of streptavidin is related to its ability to bind strongly a small growth factor, biotin, which is also known as vitamin H (György *et al.*, 1940). Each of the four identical streptavidin subunits can bind one molecule of biotin. The equilibrium constant of the streptavidin-biotin interaction (affinity constant, K_a) equals to 2.5×10^{13} M^{-1} and it is among the strongest non-covalent bonds in nature along with avidin-biotin interaction. Avidin, a biotin-binding protein from hen egg white, was discovered in the early 1940s (Eakin *et al.*, 1941) as a result of long studies involved in the biotin deficiencies and injuries found in animals fed with abundant egg white. Avidin and streptavidin both have similar tetrameric structures and biotin-binding features. Core streptavidin, consisting of 125 - 127 amino acids (subunit), shares an approximately 33 % sequence identity with full-length avidin, which consists of 128 amino acids (DeLange and Huang, 1971; Argaraña *et al.*, 1986; Green, 1990). Relevant differences between these proteins are related to their glycosylation and isoelectric point (pI). Avidin is a glycosylated and highly basic protein with a pI of 10.5 while streptavidin has no carbohydrates and is slightly acidic with a pI of 5 - 6. These proteins have provided scientists with an interesting paradigm to the molecular origins of strong binding. Furthermore, (strept)avidin-biotin technology has been widely used in several biotechnological applications. However, the positive charge and the presence of carbohydrates afford stronger non-specific binding features to avidin and therefore streptavidin is considered a more favorable alternative for several purposes. A number of principal reviews and entire volumes have been devoted either to the fundamental properties or the applications of avidin and streptavidin in recent decades (Green, 1975; Wilchek and Bayer, 1988; 1990; Diamandis and Christopoulos, 1991; Sano and Stayton, 1999).

One of the technological applications benefiting from the streptavidin-biotin system includes bioanalytical assays such as the immunoassays used in clinical diagnostics. Streptavidin can serve either as a solid-phase binding matrix or as a principal component in the signal collection and amplification system (Suonpää *et al.*, 1992; Schetters, 1999; Scorilas *et al.*, 2000; Qin *et al.*, 2001). Streptavidin-coated microtiter wells and sensor surfaces are used as universal surfaces for convenient binding of biotinylated molecules with well-retained activity. This thesis investigates the properties and applications of streptavidin and above all focuses on its use as a binding surface in solid-phase immunoassays.

1.2 Streptavidin and biotin

1.2.1 Streptavidin

1.2.1.1 Discovery and early characterization

Purification of the antibiotic activity in the fermentation filtrates of *Streptomycetes* led to the discovery of streptavidin in the early 1960s (Chaiet *et al*., 1963). The filtration broths contained two components of different sizes (termed 235L and 235S) which could be separated from each other by dialysis. None of the separated components alone showed antibiotic activity, except the smaller one when the bacteria were grown in a synthetic medium containing no biotin. This antibiotic effect was reversed by the addition of exogenous biotin, and thus it was postulated that the smaller component prevented the synthesis of intracellular biotin. Administration of either the larger component from the dialysis or alternatively avidin from the hen egg white compensated the effect of exogenous biotin and restored the antibiotic activity. Since this larger component showed biotin-binding properties similar to avidin it was named streptavidin. The characterization of the larger component showed that it was a tetrameric protein with a molecular weight of approximately 60 000 D, it could bind four molecules of biotin, it contained no carbohydrates and it was neutral or acidic in nature, in contrast to the basic protein avidin. (Chaiet *et al*., 1963; Chaiet and Wolf, 1964; Tausig and Wolf, 1964.)

1.2.1.2 Synthesis and post-translational modifications

The cloning and the sequencing of the streptavidin gene from the genomic libraries of *Streptomyces avidinii* (Argaraña *et al*., 1986) showed that the gene codes a polypeptide chain of 183 amino acids in length (Fig. 1). The first 24 residues constitute a signal sequence for secretion. The mature protein subunit consists of 159 amino acids and has a molecular weight of 16 500 D. The characterization of various streptavidin preparations had, however, shown smaller sizes of the protein as well as different amino acid sequences and compositions compared with the evidence from the gene sequence (Hofmann *et al.*, 1980; Argaraña *et al*., 1986; Pähler *et al.*, 1987). This indicated post-translational modifications of the mature protein into core streptavidin, the mechanisms of which were eventually characterized more comprehensively by Bayer and co-workers (Bayer *et al.*, 1989). They showed that mature streptavidin undergoes postsecretory proteolytic digestions both in the N- and C-termini by secreted extracellular proteases. Several studies, principally the above-mentioned, have characterized core streptavidin as follows. The core consists of 125 - 127 amino acids and the predominant truncated constituents start either at Ala-13 or Glu-14. The molecular weight of core streptavidin is about 13 200 D and it is stable against further proteolytic degradation. Core streptavidin retains full biotin-binding activity compared with the non-truncated mature form. The binding activity of biotinylated macromolecules to the core has been found to be even higher than to the non-truncated form. Core streptavidin is highly soluble in water in contrast to the full-length protein which tends to form aggregates.

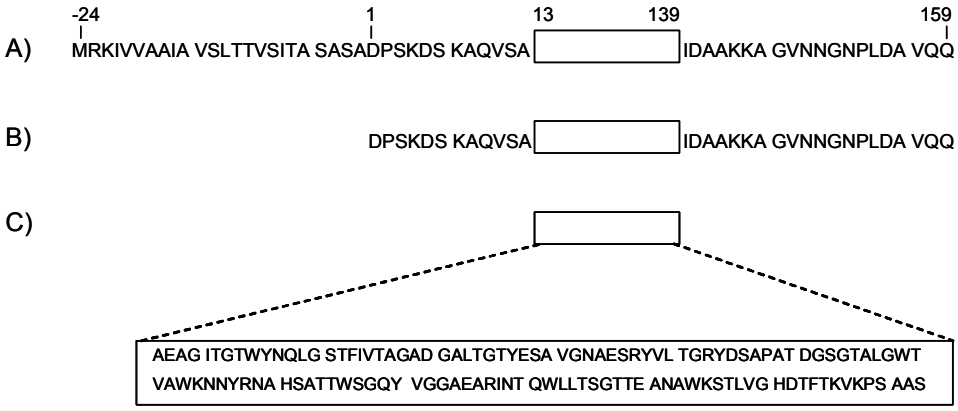

Figure 1. The amino acid sequence of streptavidin. A) The gene codes a polypeptide of 183 amino acids in length. The signal sequence is indicated by the negative numbering. B) The full-length mature protein consists of 159 residues. C) The mature protein goes through N- and C-terminal proteolytic digestions to core streptavidin consisting of 125 - 127 amino acids. One possible core including the residues 13 - 139 is shown as the white box. The amino acid sequence is from the Swiss-Prot, entry P22629.

The purification method used for isolation is supposed to affect the nature of a particular streptavidin preparation, whether it is predominantly the full-length protein or the truncated core form. Bayer *et al*. (1986) isolated a streptavidin preparation which exhibited the molecular weights of 18 000 D for the subunit and approximately 75 000 D for the tetramer in SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) analysis. The values implied a non-digested protein rather than core streptavidin. The authors used a modified iminobiotin-based matrix for the affinity purification of streptavidin. Iminobiotin, first synthesized by Hofmann and Axelrod (1950), is a biotin analog possessing a pH-dependent binding affinity to streptavidin. The modified purification method avoided a concentrating step in the process in contrast to the previous affinity purification and ammonium sulfate precipitation methods. It was suggested that the concentrating steps used in the previous purification processes simultaneously concentrated the proteases resulting in a more rapid degradation of mature streptavidin. In addition to the proteases of *S. avidinii*, several other proteases are able to convert mature streptavidin into the core molecule or close to it. These include Proteinase K, papain, subtilisin, thermolysin and elastase. Trypsin introduces partial digestion whereas pepsin and chymotrypsinogen were found unable to hydrolyze the mature protein into core streptavidin (Bayer *et al*., 1989). According to Bayer *et al*. (1990) commercial streptavidin preparations usually consist of core streptavidin.

1.2.1.3 Structure

The three-dimensional structure of streptavidin was resolved in the late 1980s (Pähler *et al*., 1987; Weber *et al*., 1987; Hendrickson *et al.*, 1989; Weber *et al.*, 1989). The subunit consists of eight adjacent antiparallel β-strands which are hydrogen-bonded to each other, forming a β-barrel structure. The β-strands are extended with hairpin struc-

tures at the ends of the strands and the biotin-binding site is located in one end of the barrel interior. Subunit pairs are hydrogen-bonded to each other forming a symmetric dimer and a pair of dimers interface each other, constituting the naturally occurring tetrameric structure which is sometimes described as a dimer of dimers. The dimeric nature of the subunit pairs is clearly recognized in the three-dimensional structures, where the interface of the dimers appears as a narrowing in the hour-glass resembling structure (Fig. 2).

Currently, the RCSB (Research Collaboratory for Structural Bioinformatics) Protein Data Bank (PDB) contains approximately 130 structures related to streptavidin, including the wild type protein and various mutated variants as apo-forms (without ligand) and with a number of bound ligands, ranging from biotin and its analogs to oligopeptides.

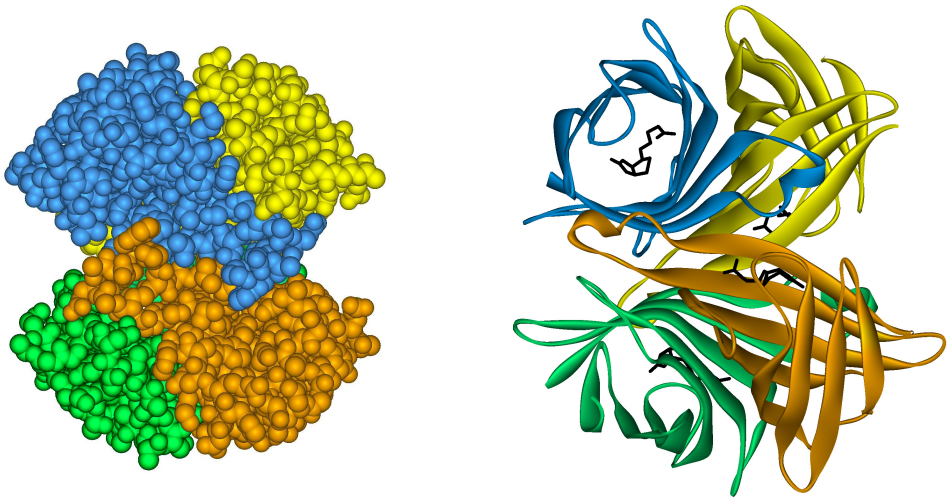

Figure 2. The three-dimensional structures of streptavidin. The space-filling model (left) shows the organization of the four identical subunits. The ribbon diagram (right) displays the predominant β-barrel structure and the intercalation of the biotin molecules in the binding pockets. The images were generated by the program Discovery Studio Visualizer (Accelrys, Inc.) using the RCSB Protein Data Bank entries 1SWC and 1SWE which are based on the work by Freitag *et al*. (1997).

1.2.2 Biotin

Biotin was first discovered as a growth factor in 1901 and afterwards it was purified from egg yolk and liver in the 1930s and early 1940s (reviewed by Streit and Entcheva, 2003). At this time the same molecule was studied in three contexts: vitamin H (a curative agent of "egg white injury"), biotin (a yeast growth factor isolated from egg yolk), and coenzyme R (a growth factor from rhizobia). In 1940 György and co-workers (György *et al*., 1940) suggested that all these were identical or closely related compounds. The history of biotin is closely related to the discovery and early phases of

avidin. Indications of the necessity of biotin for life was obtained when rats that were abundantly fed with egg white generated specific symptoms such as dermatitis and baldness (Boas, 1924; 1927). In 1940 Eakin and co-workers (Eakin *et al.*, 1940a) reported that the recognized condition, termed "egg white injury", was related to the deficiency of biotin in the tissues. The egg white which was included in the diet of the chicks was considered responsible for either destroying biotin or making it unavailable to the tissues. A constituent of the egg white was found to form a stable complex with biotin (Eakin *et al.*, 1940b) and this "avidalbumin" was considered responsible of the biotin deficiency (György *et al.*, 1941). This constituent was subsequently purified further and discovered as avidin (Eakin *et al*., 1941).

Biotin is an essential compound to all organisms. The natural function of biotin is to carry carboxyl groups and function as a cofactor for carboxylation reactions catalyzed by a group of enzymes, biotin-dependent carboxylases. Most prokaryotes and plants are able to synthesize biotin themselves while animals and humans need biotin in their diet. Comprehensive reviews of the biosynthesis and enzymatic mechanisms of biotin have been written for example by Knowles (1989) as well as Streit and Entcheva (2003).

The structure of biotin was presented for the first time as early as 1942 (du Vigneaud, 1942; Melville *et al.*, 1942) and the first chemical synthesis, starting from l-cystine, chloroacetic acid and glutaric acid was reported in 1944 (Harris *et al.*, 1944). Biotin consists of two fused rings and an aliphatic valeric acid side chain with a carboxyl group in the end (Fig. 3). The molecular weight of the compound is 244 D. In addition to the biotechnological purposes, biotin is used in bulk quantities as an additive in food, feed and cosmetology. Its annual production is estimated at approximately 10 - 30 tons. Commercially available biotin is mainly prepared by chemical synthesis. Microbial production is supposed to reduce the environmental burden caused by the chemical synthesis, but the yields of the biological production have not yet been economically profitable. (Streit and Entcheva, 2003.)

Numerous modifications of biotin have been synthesized and implemented for research purposes and biotechnological applications (Fig. 3). One group of the derivatives comprises those which have lower, environmentally sensitive binding affinity to avidin and streptavidin. These include, for example, 2-iminobiotin (Hofmann and Axelrod, 1950; Hofmann *et al*., 1980) and desthiobiotin (Hirsch *et al.*, 2002). These biotin derivatives exhibit reversible binding and they are useful in the purification processes which rely on streptavidin-biotin technology by enabling mild elution conditions. The molecular alterations have been typically targeted to the double-ring structure of biotin which is mainly responsible for the tight binding. Other main variants of biotin enable covalent coupling of biotin to target molecules (biotinylation) through chemically reactive groups introduced to the end of the side chain. These include, for example, the N-hydroxysuccinimide (NHS) ester derivatives (Becker *et al.*, 1971; Heitzmann and Richards, 1974; Bayer and Wilchek, 1990) and biotin-isothiocyanate (Mukkala *et al.*, 1993)

for coupling to primary amines as well as maleimide- biotin (Bayer *et al.*, 1985) for coupling to thiol-groups. Furthermore, some biotin derivatives possess specific functions in their side chain such as cleavable phenyl esters (Mouton *et al.*, 1982), disulfide bridges (Soukup *et al.*, 1995) and photochemical reactivity (Forster *et al.*, 1985; see section 1.4.3.2 for further use of photochemistry). In addition, various long-chain dendritic structures of biotin have been synthesized containing multiple branches and coupling sites (Wilbur *et al.*, 1999). These are mainly intended for therapeutic pretargeting purposes.

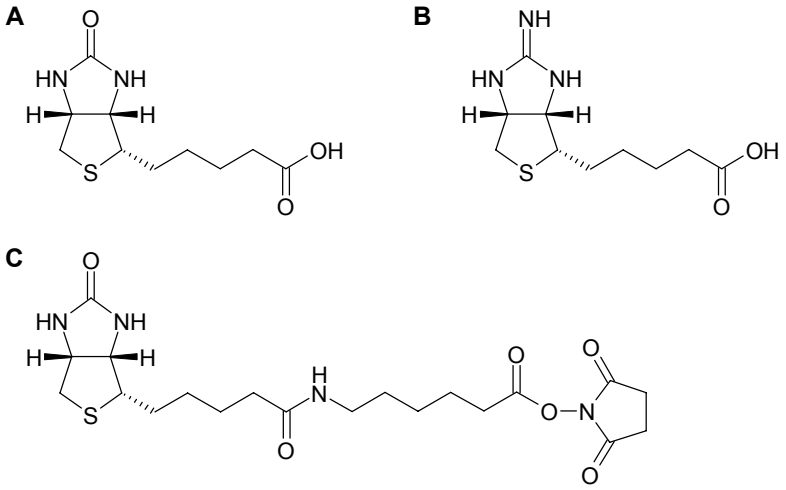

Figure 3. The structures of biotin and some derivatives. A) Intact biotin, drawn according to du Vigneaud *et al*. (1942). B) 2-iminobiotin has lower affinity than intact biotin to streptavidin and avidin. Drawn according to Hofmann and Axelrod (1950). C) Biotinamidohexanoic acid N-hydroxysuccinimide ester has an extended side chain with a reactive N-hydroxysuccinimide (NHS) ester functionality enabling the covalent coupling of biotin to other molecules through their amine groups. Drawn according to Costello *et al*. (1979).

1.2.3 Streptavidin-biotin interaction

1.2.3.1 Affinity

The binding affinity between biotin and streptavidin (and avidin) is obviously high, but there are few studies which actually report the equilibrium constants between intact biotin and wild type streptavidin. Two review publications by Green (1975; 1990) are the "golden standards" when referring to the strong interaction and high binding affinity. The latter publication reports a dissociation constant (K_d) of 4×10^{-14} M for the streptavidin-biotin interaction which equals to the widely accepted value of 2.5×10^{13} M^{-1} when expressed in terms of the affinity constant (K_a). The interaction between biotin and streptavidin is so strong that regular biochemical methods, for example, those which rely on an equilibrium binding accompanied by a separation of bound biotin from non-bound either by dialysis or ultrafiltration, do not evidently provide adequate separation efficiency and sensitivity for measuring the minute quantities

of unbound biotin. These assays are, therefore, conflicted by an upper limit in the measurable equilibrium constants. It has been stated that the equilibrium constant between (strept)avidin and biotin can only be estimated from the ratio of the rate constants for binding and dissociation (Green, 1963; 1990). Dissociation rate constants (k_{off}) have been determined in several studies. For example, Piran and Riordan (1990) reported a value of 2.4×10^{-6} s^{-1} measured at +25 °C whereas Chilkoti and Stayton (1995) reported a value of 5.4×10^{-6} s^{-1} at +25 °C and Klumb *et al*. (1998) defined a value of 4.3×10^{-5} s^{-1} at +37 °C for the dissociation of biotin from wild type streptavidin. The determination of the association rate constant (k_{on}) is obviously more complicated due to the rapid, high-affinity reaction. An association rate constant of 7.5×10^{7} $M^{-1}s^{-1}$ was recently measured by means of stopped-flow fluorescence technology relying on the decrease of the intrinsic fluorescence of streptavidin upon the binding of biotin (Hyre *et al.*, 2006). Based on the measured association rate constant, the study reported an affinity constant of 1.9×10^{13} M^{-1} for the streptavidin-biotin interaction.

Mutated streptavidin and biotin analogs frequently exhibit lower binding affinities which are measurable by regular chemical methods. The measurement methods used in this lower affinity region include, for example, equilibrium dialysis in a microdialyzer (Sano and Cantor, 1995), equilibrium binding followed by ultrafiltration (Chilkoti *et al.*, 1995b; Sano *et al.*, 1997), radiometric competition assay (Klumb *et al.*, 1998; Freitag *et al.*, 1999a; Howarth *et al.*, 2006) and a special modification of the common enzyme-linked immunosorbent assay (ELISA) (Chilkoti and Stayton, 1995). Furthermore, the energies of streptavidin-biotin interaction have been measured using isothermal titration calorimetry; these studies have reported Gibbs energy changes (ΔG) of approximately –18 kcal/mol (≈ –75 kJ/mol) for the binding of biotin to wild type streptavidin (Chilkoti and Stayton, 1995; Chu *et al.*, 1998). In addition, the binding forces between streptavidin and biotin have been measured using atomic force microscopy (AFM) and a special surface force measurement apparatus (Chilkoti *et al.*, 1995a; Allen *et al.*, 1996; Wong *et al.*, 1999). These studies have reported the binding forces in the range of 90 - 400 pN for the binding of biotin to various mutated and wild type streptavidin.

1.2.3.2 Structural origin and stability

The structural basis and the network of the bonds between streptavidin and biotin were defined in the structural studies published in 1989 (Hendrickson *et al*., 1989; Weber *et al*., 1989). The binding site is solvent-accessible when biotin is not bound. The binding of biotin displaces the water molecules and biotin becomes deeply buried in the binding site. The tight interaction is essentially enabled by three special binding motifs. One motif is the binding loop (residues 45 - 52) which is disordered when biotin is not bound but becomes ordered upon binding and closes the binding site (Freitag *et al*., 1997). The second motif comprises the hydrophobic interactions which are provided by Trp-79, Trp-92 and Trp-108 from the given subunit and Trp-120 from the adjacent subunit across the dimeric interface. The third binding motif is an extensive network of

the hydrogen bonds between the heteroatoms of biotin and the amino acid residues of streptavidin (eight bonds). In addition to the direct hydrogen bonds to biotin, the hydrogen bond network extends between several residues in the vicinity of the binding site, thus stabilizing the environment. A clear summary of the interactions between biotin and the amino acids of streptavidin has been written by Freitag and co-workers (Freitag *et al.*, 1999b).

Crystallographic studies have shown that the quaternary structure of streptavidin adopts a stabilized, more compact shape upon the binding of biotin so that the subunit barrels become slightly flattened and more tightly bound to each other (Weber *et al.*, 1989; Katz, 1997). These observations have suggested some co-operation or communication between the subunits. Evidence of the subunit communication was first obtained with avidin in a study by Green and Toms (1973) who reported lower affinity with an isolated subunit in comparison with the intact tetrameric molecule. The lower affinity of the monomeric form has also been confirmed with streptavidin (Qureshi and Wong, 2002; Wu and Wong, 2005). In streptavidin, the Trp-120 residue from the adjacent subunit plays a major role in the high biotin-binding affinity and binding-induced stability. The replacement of Trp-120 with phenylalanine reduces the binding affinity to the 10^8 M^{-1} level and weakens the tetrameric stability (Sano and Cantor, 1995). These two determinants are closely related to each other; tetrameric integrity is essential for complete biotin-binding activity and on the other hand, the binding of biotin increases the stability of the tetrameric composition. The streptavidin-biotin bond and the tetrameric integrity tolerates harsh conditions such as 6 M urea (Sano and Cantor, 1990; Kurzban *et al.*, 1991), a wide pH range and an excess of free biotin (Sano and Cantor, 1995). The tetrameric state of the protein with bound biotin is at least partially maintained upon heating in the presence of 8 M urea and 6 M guanidine hydrochloride (González *et al.*, 1997; 1999). In contrast, the same studies showed that streptavidin without bound biotin denatured at significantly lower temperatures. In another study (Sano *et al.*, 1995), streptavidin tolerated harsh conditions to some extent without bound biotin. It retained approximately 80 % of its biotin-binding activity after exposure to 6 M guanidine hydrochloride at pH 7.4. However, at pH 1.5, the remaining capacity decreased to 20 %.

The disruption of the streptavidin-biotin bond requires harsh conditions but some methods have been reported for the release of bound biotin or biotinylated molecules in somewhat milder conditions. For example, a mixture of formamide (95 %) and either ethylenediaminetetraacetic acid (EDTA) or sodium acetate combined with heating up to +90 °C has been used to elute biotinylated DNA from streptavidin-coated magnetic beads, resulting in yields of 95 - 98 % (Tong and Smith, 1992). Biotinylated oligonucleotides have been also recovered from streptavidin-based matrices in the presence of either 2.0 M 2-mercaptoethanol or 2.0 M dithiotreitol (Jenne and Famulok, 1999). At room temperature the release was partial but it was greatly increased upon heating to +95 °C. The release of biotinylated macromolecules appeared to be easier than the release of intact biotin, since biotin remained completely bound at room temperature.

Even upon heating, no more than 3 % was released. The conditions reported in the study enabled the recovery of the biotinylated target molecules in an active state as well as subsequent recycling of the streptavidin matrix. Another example of fairly mild conditions being able to release bound biotin from streptavidin was shown in a recent study where biotinylated DNA was reversibly recovered from streptavidin-coated magnetic beads only by brief exposure to elevated temperatures (+70 °C) in nonionic water solution (Holmberg *et al.*, 2005).

Owing to the conformational changes and the involvement of the tryptophan moieties in the binding interaction, the intrinsic fluorescence emission of streptavidin is decreased upon the binding of biotin (Kurzban *et al.*, 1990). The emission peak of streptavidin without bound biotin appears at 333 nm whereas the binding of biotin shifts the peak to 329 nm (blue-shift) and decreases the intensity. The total fluorescence intensity is reduced by 39 % and the peak intensity by 33 %. This feature, the quenching of the intrinsic fluorescence upon binding of biotin, has been used for quantitative determination of the biotin-binding capacities of streptavidin layers (Moll *et al.*, 2002; Huber *et al.*, 2006) and the determination of the association rates for binding using the stopped-flow measurement technology (Hyre *et al*., 2006).

1.2.3.3 Effect of the mutations

The streptavidin-biotin system has been an attractive research target when discerning the origins of the strong interaction between biomolecules. Analyses of mutated streptavidin and their interactions with biotin have provided further insight into the binding mechanisms and the importance of various amino acid residues for high binding affinity. As discussed above, Trp-120 from the adjacent subunit contributes significantly to the strong binding as well as to the stabilizing interaction between the subunits. A mutation of Trp-120 to phenylalanine reduced the affinity constant to the 10^8 M^{-1} level in one study (Sano and Cantor, 1995) and increased the dissociation rate constant (k_{off}) by 70-fold compared with wild type streptavidin in another study (Chilkoti and Stayton, 1995). Mutations of Trp-79-Phe and Trp-108-Phe increased the dissociation rate constant by 5.5 - 17-fold in comparison with wild type streptavidin (Chilkoti and Stayton, 1995). The effect of the tryptophan mutations in the other positions except 120 was less significant to the binding affinity which confirmed the overall importance of intersubunit contact in the strong interaction between streptavidin and biotin. Tryptophans in positions 79, 108 and 120 have also been replaced with alanine (Chilkoti *et al*., 1995b). The affinity constants of the Trp-79-Ala and Trp-120-Ala mutants were decreased down to the 10^7 M^{-1} level. The mutations to the binding site have not usually induced significant changes in the three-dimensional folding of tetrameric streptavidin. For example, X-ray crystallographic structure analysis of the mutants Trp-79-Phe, Trp-108-Phe, Trp-120-Phe and Trp-120-Ala showed only slight changes in the structure compared with the wild type protein, which implies conservation in the folding properties of streptavidin (Freitag *et al.*, 1998).

In addition to the binding motif of the hydrophobic interactions, the hydrogen bond network has been subjected to mutations. For example, elimination of one hydrogen bond by mutating Asp-128 to alanine resulted in a 1000-fold increase in the dissociation constant (Freitag *et al*., 1999a). The crystallographic data obtained in the study showed again no major changes in the overall folding, but instead, a 0.5 - 1.0 Å shift was observed in the position of the bound biotin which was regarded as a kind of snapshot from the biotin dissociation pathway consisting of successively breaking hydrogen bonds. The oxygen atom in the double-ring of biotin is coordinated by three hydrogen bonds, and the contribution of these principal bonds to the binding affinity was elucidated by mutating the residues Asn-23, Ser-27 and Tyr-43 one by one (Klumb *et al*., 1998). Furthermore, Ser-45 and Asp-128 were replaced with alanine individually or together in combination to produce a double mutant (Hyre *et al*., 2006). While the single mutants exhibited moderate decreases in the affinities, the biotin-binding affinity of the double mutant was decreased to 8.2×10^5 M^{-1}. The third binding motif, the loop structure which adopts a closed conformation upon the binding of biotin, also has an important role in the high binding affinity. Deletion of the loop, as studied by circularly permuted streptavidin (Chu *et al*., 1998), decreased the affinity constant down to approximately 2.3×10^7 M^{-1}. Though a sequence of four amino acids was removed from the structure, the X-ray analysis did not show remarkable alterations in the overall folding of this mutant either. A recent report introduced an interesting monovalent streptavidin exhibiting a biotin-binding affinity almost indistinguishable from wild type streptavidin (Howarth *et al*., 2006). This chimeric streptavidin was constructed by refolding subunits bearing three mutations with subunits of a wild type binding affinity at a 3:1 ratio. The mutated subunits had negligible biotin-binding affinity but they were capable of maintaining tetrameric integrity. This monovalent streptavidin enabled cellular imaging and the tracking of biotinylated cell-surface proteins by labeled streptavidin without adverse cross-linking, a property which was typically exhibited by tetravalent streptavidin.

In addition to the biotin-binding site, other mutations have been targeted to the subunit interfaces and to the overall structure of the protein. An interesting example is a mutant possessing increased stability of the tetrameric structure. Covalent bonds through the weaker dimeric interface were introduced by replacing His-127 either with cysteine or lysine, while the biotin-binding ability of streptavidin remained intact (Reznik *et al.*, 1996). An opposite objective has been to weaken the subunit interactions by mutations to produce monomeric streptavidin variants which have gained interest due to the reversible biotin-binding properties (Qureshi and Wong, 2002; Wu and Wong, 2005).

A recently published comprehensive review (Laitinen *et al.*, 2006) summarizes the numerous mutations made to streptavidin and avidin as well as describes their essential features. According to the review, none of the streptavidin mutants exhibit increased binding affinity compared with the wild type protein. A few mutants retain, or almost retain, wild type affinity. Another review from the same group surveys the possibilities

and applications of modified streptavidin and avidin in modern bioscience, medicine and nanotechnology (Laitinen *et al.*, 2007).

1.2.3.4 Other methods to control binding

In addition to the biotin derivatives of lower affinity, monomerization and the binding-site mutations as discussed above, some other strategies are available to control binding affinity. These are chemical modifications which result in environmentally sensitive binding ability. One approach is to use nitrated streptavidin (Morag *et al.*, 1996a; 1996b). Treatment of streptavidin (or avidin as well) with tetranitromethane results in the nitration of the tyrosine moieties. Some of them are involved in the binding of biotin and, therefore, the binding becomes dependent on the pH so that biotin is bound at low pH (4 - 5) and released either after increased pH or by the introduction of excess biotin. Another interesting technology to control biotin-binding activity is to use synthetic polymers attached close to the binding site (Ding *et al.*, 1999; Bulmus *et al.*, 2000; Ding *et al.*, 2001; Stayton *et al.*, 2004). Polymers of poly(N-isopropylacrylamide) and poly(N,N-diethylacrylamide) reversibly change their state between soluble linear and collapsed globular forms in response to changes in temperature, pH or light. These polymers can shield or expose the binding site upon mild environmental changes when coupled to the proximity of the binding site, as shown by site-specific coupling to a mutated streptavidin (Glu-116-Cys). Thus, these modifications were called "smart" streptavidin conjugates.

1.2.3.5 Streptavidin-binding peptides

In addition to biotin and its derivatives, various peptide motifs bind to streptavidin although with substantially lower affinity than biotin ($K_a \approx 10^3$ - 10^8 M^{-1}) (Weber *et al.*, 1992; Giebel *et al.*, 1995; Keefe *et al.*, 2001; Lamla and Erdmann, 2004). The well-defined peptides which have been applied in biotechnology are Strep-tag (Schmidt and Skerra, 1993) and Strep-tag II (Schmidt *et al.*, 1996), nine and eight amino acid peptides containing the common His-Pro-Gln sequence found in most streptavidin-binding peptides. Strep-tag II exhibits increased affinity to an engineered recombinant streptavidin ("Strep-Tactin") (Voss and Skerra, 1997), and this has further improved the versatility and utility of the Strep-tag affinity system in biotechnology. The Strep-tag system has been reviewed by Skerra and Schmidt (Skerra and Schmidt, 1999; Schmidt and Skerra, 2007). The main applications of the streptavidin-binding peptides are involved in affinity purification. The relatively low binding affinities of the peptides enable reversible release of the peptide-tagged fusion proteins from the streptavidin-based matrices by the addition of biotin or an appropriate analog. Furthermore, other reported uses of the peptides include peptide-tagged detection antibodies to serve the binding of an enzyme-labeled streptavidin for immuno-detection in membranes or in ELISA-type assays. In addition, a recent application used a nano-tag (a nine amino acid peptide) and a SBP-tag (a 38-amino acid peptide) to immobilize binding molecules to streptavidin-coated surface plasmon resonance (SPR) chips (Li *et al.*, 2006). Reversible binding enabled the release of the bound molecules and thereby convenient re-use of the expensive chips.

1.3 Solid-phase assays and the immobilization of antibodies

1.3.1 Solid-phase assays

1.3.1.1 Development of solid-phase assays

Immunoassays are widely used for recognizing and quantifying minute quantities of proteins and small molecules from various biological fluids. At present, the detection limits of some of the most sensitive assays reach the sub-femtomolar concentration range, for example a prostate specific antigen (PSA) assay using europium (Eu) -filled nanoparticle labels and time-resolved fluorescence detection (Soukka *et al.*, 2001). Active development in immunoassays originates in the 1950s. A recent review by Wide (2005) surveys the development of the immunoassays in those days. According to the author, one immunoanalysis method used at that time was Boyden's passive haemagglutination inhibition method which used antibodies and antigen-coated erythrocytes. Depending on the absence or presence of the antigen in the sample followed by the binding of the antibodies to the erythrocytes or inhibition of the binding by the antigen from the sample, the coated cells sedimented to the bottom of the test tubes in a specific manner. This technique was used in the first industrially manufactured diagnostic test kit launched in 1962 as a pregnancy test and was based on the detection of human chorionic gonadotropin (hCG) in urine.

Another important branch in the development of the immunoassays in the 1950s was involved in the studies of insulin metabolism which eventually led to the measurement of insulin by means of competitive binding between radioactively labeled and non-labeled insulin (Berson *et al.*, 1956; Berson and Yalow, 1958). This technique was first used to assay animal insulin (beef, pork) employing antiserums from human subjects. As expected, it was not applicable for the measurement of human insulin due to weak binding until Yalow and Berson (1959) discovered that antiserum from guinea-pigs immunized with beef insulin cross-reacted strongly with human insulin. This provided the basis for the quantitative measurement of human insulin by means of competitive binding between endogenous insulin and ^{131}I-labeled insulin (Yalow and Berson, 1960). Independently of the above work, Ekins (1960) determined the concentrations of thyroxine (T_4) in human plasma by measuring the distribution of ^{131}I-labeled thyroxine between albumin and a thyroxine-binding protein in a saturation assay. Though not using antiserum or antibodies, and thus not literally an immunoassay, the work by Ekins is also regarded as a milestone publication in the context of the immunoassays, or in wider sense, in measuring the concentrations of substances by means of a competitive ligand binding accompanied by a labeled component.

Heterogeneous immunoassays require separation of the bound analyte from the non-bound before measuring the signal from a labeled component. In the first assays, separation was enabled by chromatographic or electrophoretic means. In subsequent assays, solid materials were used to facilitate separation. For example, charcoal was capable of binding free insulin but not antibody-bound insulin and, therefore, it was used for separation in the immunoassays of insulin (Herbert *et al.*, 1965; Keane *et al.*, 1968). Solid

phases with immobilized antibodies were likewise reported at that time. According to Wide (2005), competitive binding assays of several hormones were evaluated using antibody-coated formalinized sheep erythrocytes and ^{125}I-labeled hormones in 1965 at the University Hospital in Uppsala. Subsequently, solid phases made from polymeric materials were introduced. For example, Catt *et al*. (1966) reported the use of an antibody-coated graft polymer of styrene and polytetrafluoroethylene in an assay of human growth hormone. In another work, Wide and Porath (1966) used antibodies coupled to Sephadex-isothiocyanate for the assays of hCG and luteinizing hormone. The solid materials in the first solid-phase assays were provided in the format of particles or powder in suspension, and separation by centrifugation constituted an advantage over the earlier chromatographic or electrophoretic methods. A shift to a more solid format was introduced by Catt and co-workers, first with solid discs prepared from synthetic polymers (Catt *et al.*, 1967) and then with tubes made from polystyrene or polypropylene (Catt and Tregear, 1967). In the latter work, the antibodies were immobilized to the inner surfaces of the plastic tubes by passive adsorption and the tubes were successfully used for the assays of human placental lactogen and human growth hormone. The tube format enabled convenient separation and washing under tap water. Earlier studies had essentially used reactive chemistries to couple antibodies to the solid phases, whereas the current study relied on unsubstituted polymers where the antibodies adsorbed spontaneously. The spontaneous adsorption of the proteins to the solid phases had already been recognized and reviewed earlier; for example, according to Neurath and Bull (1938) some findings of protein adsorption to solid phases had been made in 1905. Afterwards, Bull (1956) reported and modeled the adsorption of albumin to glass. Furthermore, the search for blood-compatible, non-thrombogenic, plastics had revealed the adsorption of blood proteins to unmodified plastic surfaces (Leininger *et al.*, 1966). The work by Catt and Tregear (1967), however, introduced the adsorption of antibodies to be utilized among an emerging solid-phase assay application.

Further significant advances in immunoassays were introduced by the end of the 1960s. One was the principle of an immunoradiometric reagent-excess assay using ^{125}I-labeled antibodies (Miles and Hales, 1968) which yielded an ascending linear relationship between the amount of the added antigen and the measured radioactivity, instead of the descending relation in the previous competitive binding assays. Another development was the introduction of a non-competitive two-site assay by Wide and coworkers (Wide *et al.*, 1967). This was first employed to detect allergen antibodies using a set-up where a solid-phase coupled allergen bound specific antibodies from serum followed by detection with a ^{125}I-labeled antibody against the allergen antibody. Soon after, the principle of using a solid-phase coupled catching antibody in the two-site assay was presented and the term "sandwich assay" was launched (Wide, 1969); the assay format that currently is probably the most common non-competitive immunoassay type.

1.3.1.2 Introduction of avidin- and streptavidin-coated surfaces

Immobilized avidin and streptavidin were first used in separation and purification matrices before their entry into solid-phase assays. For example, sepharose and agarose matrices with covalently coupled streptavidin were used to immobilize biotinylated capture molecules such as growth hormones, monoclonal antibodies or lectins and their soluble targets to facilitate separation and purification (Haeuptle *et al.*, 1983; Updyke and Nicolson, 1984; Buckie and Cook, 1986). Avidin-biotin technology was introduced in the context of the enzyme immunoassays in the late 1970s. In the work by Guesdon *et al.* (1979) avidin was used to mediate the binding of both biotinylated and covalently coupled reporter enzymes to the immunocomplex in bridging sandwich assays. Avidin-coated solid phases of the immunoassays were introduced in the mid 1980s. Sutton and co-workers (Sutton *et al.*, 1985) used avidin-coated microtiter plates to immobilize biotinylated bacterial polysaccharides in an ELISA-assay. Odell and colleagues (Odell *et al.*, 1986) used avidin-coated polystyrene beads in a rapid radioimmunoassay of human thyrotropin (hTSH). The beads were first coated with biotinylated bovine serum albumin (BSA) to immobilize avidin, which further bound the biotinylated capture antibody through the remaining unoccupied biotin-binding sites. The same principle was subsequently used in the assays of hCG (Griffin and Odell, 1987), lutropin (Odell and Griffin, 1987) and corticotropin (Zahradnik *et al.*, 1989).

Streptavidin-coated solid phases of the immunoassays were likewise reported in the mid 1980s by Suter and Butler (1986). In fact, rather than an actual immunoassay, the authors essentially studied the amounts and the activities of the monoclonal antibodies which were either directly adsorbed to a plastic surface or immobilized by a novel "protein avidin biotin capture" (PABC) system. In this concept, streptavidin was immobilized via biotinylated interface polymers (proteins or gelatin) to the surface of the immunoassay well and the remaining biotin-binding sites of streptavidin served for the binding of a biotinylated monoclonal antibody. Though the direct adsorption resulted in larger amounts of the immobilized antibodies, the PABC system was superior in terms of the antigen binding activity of the immobilized antibody. Subsequently, the system was comprehensively studied and optimized in stoichiometric terms (Suter *et al.*, 1989). A covalent coupling of streptavidin to immunoassay surfaces was soon reported using bromoacetyl-activated polystyrene beads (Peterman *et al.*, 1988). The antigen-binding activities of the antibodies immobilized to the streptavidin surface were again excellent in comparison with the activities of the passively adsorbed or directly coupled antibodies. Covalent chemistry was, furthermore, used to couple streptavidin to the surfaces of magnetic beads to facilitate the separation of biotinylated human chromosomes from human-hamster hybrid cell lines (Dudin *et al.*, 1988) and to enable magnetic bead based solid-phase DNA-sequencing (Hultman *et al.*, 1989). These studies thus extended the use of the streptavidin-coated solid phases to the area of nucleic acid research.

1.3.2 Immobilization of antibodies

1.3.2.1 Adsorption and streptavidin-based immobilization

Immobilized proteins continuously constitute an important part of solid-phase immunoassays and it is reasonable to review some particular aspects involved in the immobilization of the antibodies. The biologically active binding layer and its quality are essential for the optimal performance of the assay and it may become a challenge to introduce a binding surface of correct and adequate functionality. Owing to its simplicity and cost-efficiency, direct coating of the capture antibodies by means of passive adsorption (also called physisorption) has been widely used to functionalize solid phases. Despite the well-characterized disruptive effects of the adsorption on protein conformation and activity, some scientists have until recently recommended it as the first choice before proceeding to more complicated methods (see a review by Butler, 2000). In addition to providing a universal surface for binding of any biotinylated molecule, the non-adsorptive immobilization of the antibodies via streptavidin constitutes an advantage with respect to the activity of the immobilized antibody, as will be reviewed below.

Adsorbed proteins tend to occur in clusters of active proteins instead of forming an evenly distributed layer. The cluster formation has been demonstrated with monoclonal antibodies on polystyrene using various imaging methods such as scanning electron microscopy (SEM), scanning tunneling microscopy (STM) and atomic force microscopy (AFM) (Butler *et al.*, 1992; Davies *et al.*, 1994a; 1994b). Furthermore, clustering has been observed with the adsorption of ferritin on various substrates (polycarbonate, carbon, quartz) as visualized by electron microscopic studies (Feder and Giaever, 1980; Nygren, 1988). Biotinylated antibodies immobilized to the streptavidin-coated surfaces have appeared in uniform distribution instead of clustering when visualized by STM and AFM (Davies *et al*., 1994a; 1994b).

Studies have suggested that the interactions between the binding molecules are stronger on the solid-liquid interface than in the solution (Lehtonen, 1981; Nygren *et al.*, 1985). These observations are usually explained by the formation of the binding clusters where the increase in local densities results in secondary bonds which increase overall binding avidity. This model originates from the well-known lateral diffusion of the receptor molecules in the lipid layer of the cell membranes when binding their ligands. Likewise, lateral diffusion of the adsorbed proteins has been shown (Michaeli *et al.*, 1980). The clustering of the antibodies on the surface may be, nevertheless, an advantage in immunoassays since possible cross-linking accompanied by slower dissociation rates should guarantee better preservation of the bound analyte on the surface during the repeated washing and incubation steps.

Adsorbed antibodies (and proteins in general) typically exhibit decreased activity and antigenicity. According to Butler (2000), conformational changes of the adsorbed proteins have been recognized at least since the 1950s. More comprehensive and quantitative studies of the adsorption-induced changes to the activity of immunoassay antibod-

ies were commenced by Butler and co-workers in the 1980s. A common finding has been the better survival of the polyclonal antibodies compared with the corresponding monoclonal antibodies in adsorption (Butler *et al.*, 1986; Suter *et al*., 1989; Butler *et al*., 1992; Joshi *et al.*, 1992; Butler *et al.*, 1993). One explanation for the better survival of the polyclonal antibodies is that among the range of the antibodies of multiple specificities there are always some variants which tolerate adsorption better than others. Another suggestion is that the multiple specificities of the antibody preparations result in a more efficient cross-linking of the bound antigen followed by a pronounced avidity effect. Some quantitative values and estimates of the magnitude of the lost or remaining activities have also been reported. For example, Joshi *et al*. (1992) reported that 5 - 7 % of polyclonal antibodies while less than 1 % of the monoclonal antibodies retained their activity when adsorbed on polystyrene. Fluorescein-specific polyclonal antibodies were reported to retain the activities of 5 - 10 % and monoclonal antibodies less than 3 % (Butler *et al*., 1992). In another similar study the respective values were 22 % and 2 - 6 % (Butler *et al*., 1993). In addition, Davies *et al*. (1994a; 1994b) reported that 5 % of the adsorbed monoclonal antibodies were functional for binding ferritin, which was used as an antigen when studying the surfaces by means of atomic force microscopy and scanning tunneling microscopy.

The preservation of binding activity is perhaps one of the strongest arguments in favor of streptavidin surfaces. For example, Suter and co-workers found 5 - 400-fold (Suter and Butler, 1986) and 5 - 6-fold (Suter *et al*., 1989) higher antigen-binding activities with antibodies immobilized to the streptavidin-coated surface in comparison with the antibodies adsorbed directly to polystyrene. Davies and co-workers (Davies *et al*., 1994a; 1994b) reported that 60 % of the antibodies remained functional on the streptavidin-coated surface compared with 5 % for the adsorbed antibodies. A panel of fluorescein-specific monoclonal antibodies showed a functionality of less than 3 % when adsorbed, whereas immobilization by a streptavidin-coated surface preserved the functionality at around the 20 % level (Butler *et al*., 1992). Another study from the same group reported an approximately 5-fold improvement in the activity of the capture antibodies when immobilized via streptavidin instead of direct adsorption (Butler *et al*., 1993). Monoclonal antibodies have benefited more from streptavidin-based immobilization than polyclonal antibodies (Suter *et al*., 1989; Butler *et al*., 1993). This is obvious considering that typically a lower fraction of the monoclonal antibodies survive passive adsorption and, thereby, the non-destructive immobilization by streptavidin certainly has a greater effect. In addition to the antibodies, immobilization by streptavidin has also been shown to be beneficial for other proteins, as exemplified by a bridging ELISA-assay of the antibodies against recombinant erythropoietin (Gross *et al.*, 2006). The immobilization of the antigen (erythropoietin) to the streptavidin-coated surface resulted in a 100 - 300-fold more sensitive assay than an identical assay using adsorbed erythropoietin. Again, the lower activity of the adsorbed antigen was explained by conformational changes and the loss of epitopes induced by adsorption.

Spitznagel and co-workers (Spitznagel and Clark, 1993; Spitznagel *et al.*, 1993) reported decreased specific activities of antibodies (meaning active binding sites per antibody) on surfaces of high antibody densities; an especially dramatic decrease was observed above certain surface densities. Furthermore, Xu *et al*. (2006; 2007) studied the adsorption and the binding capacities of a monoclonal antibody against hCG on silicon oxide substrate by means of neutron reflection and ellipsometry. Specific activities of 0.1 - 0.3 were found with the antibodies that were adsorbed at pH 4 - 7, whereas the ratio was 0.8 at pH 8.0. In the higher pH, the amount of the adsorbed antibody was also found to be lower. The reduced specific activities of the antibodies on the dense surfaces can largely be explained by steric hindrances and conformational issues rather than by the denaturation of the antibodies. After increased packing, the accessibility of the binding sites evidently becomes hindered at least for large antigens. In the extreme, the high antibody density on the surface may result in the reduction of the total binding capacity of the surface, as shown for example by Bae *et al*. (2005).

1.3.2.2 Antigenic specificity and heterogeneity

Adsorbed proteins may exhibit altered antigenic specificities. This is potentially a more severe problem than sole activity loss in immunoassays, since the lost activity can be partially compensated by relatively large quantities of the adsorbed antibodies. The new antigenic determinants introduced by adsorption are usually the regions of the proteins which are buried in the native conformation but become exposed upon adsorption-induced changes. Evidence of the altered antigenicity of the adsorbed proteins was found already in the 1960s (Kochwa *et al.*, 1967) when altered specificities were reported in rabbit antisera which were raised by immunizing the animals with particles bearing adsorbed antigens. In a later study (Djavadi-Ohaniance *et al.*, 1984), monoclonal antibodies of eight epitope specificities against *Escherichia coli* tryptophan synthase were monitored with respect to their antigenic specificities. Five of the eight antibodies reacted very slowly with a native antigen in solution, while they reacted rapidly with antigens adsorbed to a microtiter plate. In a subsequent study (Friguet *et al.*, 1984), the same antibodies were tested in solution both against the native antigen and against the antigen that was first subjected to denaturation by N-ethylmaleimide. Those five antibodies that reacted rapidly with the adsorbed antigen in the previous study recognized the denatured antigen in the solution reasonably well. This suggested adsorption-induced changes which resemble those that are introduced by chemical denaturation of protein structure. Butler *et al*. (1997b) showed that intradomain allotypes (supposedly) of immunoglobulin G2a (IgG2a) were more readily recognized by respective monoclonal antibodies when the antibody was passively adsorbed instead of being immobilized on the streptavidin-coated surface (although streptavidin-based immobilization almost invariably constituted an advantage in terms of activity). This was explained by the exposure of the buried domains of the antibody upon adsorption. In addition, the study showed that the antibodies which were adsorbed after denaturation by 6 M guanidine hydrochloride did not show remarkable differences in binding activities compared with the antibodies that were adsorbed without prior denaturation. On

the streptavidin surface, however, the difference in activity between the chemically denatured antibody and the one not denatured was remarkable.

Adsorbed antibodies present heterogeneity in their antigen binding affinities (Rabbany *et al.*, 1997; Vijayendran and Leckband, 2001). The unique feature of the monoclonal antibodies, the single homogeneous antigen binding affinity value in solution, is lost upon adsorption and the antibody surface exhibits multiple heterogeneous affinities. This probably originates both from the different survival of the individual antibody molecules upon adsorption and from the varying orientation and the accessibility of the binding sites. Streptavidin surfaces may alleviate the problem of non-optimal orientation to some extent and recover the homogeneity of the affinities as shown, for example, by Vijayendran and Leckband (2001). The study found that the antibodies that were biotinylated at their carbohydrate moieties and immobilized to an oriented streptavidin surface showed the highest and most homogeneous affinities in comparison with alternative methods. The orientation of the streptavidin surface was enabled by a biotinylated interface consisting of biotin that was covalently bound to the solid substrate (more about this type of surface will be reviewed in section 1.4.4). The alternative surfaces in the study consisted of either covalently bound antibody, covalently bound streptavidin or protein G surfaces. The covalent coupling of the antibody and streptavidin was mediated through amines. This is a somewhat random process and, therefore, the surfaces were unable to render the homogeneity of the bound proteins.

1.3.2.3 Other non-adsorptive immobilization techniques

Typically, the antibodies that are immobilized to a superficial second layer preserve their activity significantly better compared with antibodies that are in direct contact with solid substrate (Butler *et al*., 1997b). In addition to streptavidin surfaces, an alternative method to separate the antibodies from the inorganic substrate is provided by antibody-binding antibodies which are employed as the underlying first layer. This approach retains the activity of the immobilized antibodies fairly well, even better than immobilization via streptavidin. At best, complete preservation of the activity is retained (Butler *et al*., 1993). However, as the underlying antibody layer is vulnerable to adsorption-induced losses in binding activity, the total amount of the immobilized antibody may become quite low. This may eventually decrease the actual number of functional binding sites to a lower level than obtained by direct adsorption (Butler *et al*., 1992).

Other means of immobilizing the antibodies to the second layer is to use Fc-binding proteins such as protein A and protein G. Forsgren and Sjöquist (1966) found that protein A from the cell wall of *Staphylococcus aureus* binds to the Fc part (fragment crystallizable, the constant region of the antibodies) of the antibody and this principle was afterwards exploited in the immunoaffinity columns using the protein A-Sepharose matrix (Moseley *et al.*, 1977; Gersten and Marchalonis, 1978). Protein G, a streptococcal cell wall protein, constitutes a more versatile binder since it has a wider specificity range to different IgG isotypes in comparison with somewhat limited specificities of

protein A (Langone, 1978; Björck and Kronvall, 1984; Reis *et al.*, 1984). A more universal binder, employing the specificities of both of these, was provided by a recombinant chimeric fusion of protein A and protein G (Eliasson *et al.*, 1988; 1989). Examples of the use of protein A and protein G in the bioassays include the capturing of antibodies on magnetic beads (Widjojoatmodjo *et al.*, 1993), the capturing of pre-equilibrated antigen-antibody complexes to a microtiter plate (Protein A antibody capture ELISA, PACE) (Ngai *et al.*, 1993), the immobilization of capture antibodies to fiber optic biosensors (Anderson *et al.*, 1997) and the capturing of antigen-antibody complexes to a protein G column in an enzyme flow immunoassay of herbicides (Bjarnason *et al.*, 2001).

1.3.2.4 Orientation

Fc-binding proteins enable the immobilization of antibodies in a correctly oriented manner. In addition to protein A and protein G, Fc-specific antibodies can be used to facilitate correct orientation, as shown by immobilizing enzymes on the surface via an oriented antibody bridge (Gunaratna and Wilson, 1990; 1992). The streptavidin surfaces can facilitate correct orientation which is accomplished by combining the streptavidin-based immobilization with site-specifically biotinylated antibodies. Another feasible means of orientation relies on the carbohydrate moiety present in the Fc domain of the antibodies. The reactive aldehyde groups which emerge through oxidation with mild sodium periodate (O'Shannessy and Quarles, 1985) can be biotinylated with the hydrazide derivatives of biotin (O'Shannessy *et al.*, 1984) or they can be covalently coupled to hydrazide-activated surfaces (Hoffman and O'Shannessy, 1988). Furthermore, carbohydrate moieties can be used for oriented immobilization by lectins. Lectins comprise a diverse group of carbohydrate-binding proteins (Lis and Sharon, 1986) which have been successfully used to immobilize antibodies, for example, to sensor surfaces (Starodub *et al.*, 2005). Furthermore, one popular method of orientation relies on the disulphide bridges present in the hinge region of the antibody. The thiols generated upon mild reduction can be used either for covalent coupling or for targets of biotinylation by thiol-reactive biotin derivatives (Domen *et al.*, 1990; Cho *et al.*, 2007). This thiol-based modification and immobilization has proven applicable to intact antibodies, but above all it is useful for the immobilization of the Fab, Fab' and $F(ab')_2$ fragments as was demonstrated by Prisyazhnoy *et al*. (1988) and subsequently characterized in other studies (Lu *et al.*, 1995; Bonroy *et al.*, 2006). The oriented, site-specific immobilization of the antibodies has typically resulted in favorable effects with respect to surface homogeneity, binding affinities and capacities (Domen *et al*., 1990; Shmanai *et al.*, 2001; Vijayendran and Leckband, 2001; Peluso *et al.*, 2003; Bonroy *et al*., 2006; Kang *et al.*, 2007). Comprehensive reviews on the methods and effects of the oriented protein immobilization have been written, for example, by Lu *et al*. (1996), Rao *et al*. (1998) and Turková (1999).

1.3.3 Aspects of protein adsorption

1.3.3.1 Mechanisms and conditions

Adsorption is in many situations the prevailing mechanism in the adhesion of proteins to solid substrates, even though covalent chemistries or some other advanced methods are employed. The presence of passive adsorption must always be taken into account when a protein and a solid substrate interact, unless adsorption is prevented by using detergents or very hydrophilic surfaces which confer little adsorption. The multitude of protein features with respect to the size, shape, isoelectric point, hydrophobicity and rigidity combined with a range of the solid substrates of various surface hydrophobicity, charge and format constitute a range of conditions whereby it has proved difficult to find useful, well-accepted models of physisorption (Dent and Aslam, 1998). Some rules, however, prevail which will be briefly discussed.

In order to occur spontaneously, adsorption must be an exothermic reaction, meaning that Gibbs energy (ΔG) must be less than zero. Gibbs energy is defined by the following equation

$$\Delta G = \Delta H - T\Delta S$$

where H refers to the enthalpy of the system, T is the absolute temperature and S is the entropy. While the change in enthalpy during the adsorption process is small, the positive gain in entropy dominates, resulting in overall negative Gibbs energy. Therefore, adsorption is said to be an entropically driven process. The positive gain in entropy is supposed to originate from the dehydration of the substrate and the protein surfaces. Typically, the constituents are first surrounded by well-organized water molecules (solvated). The hydrophobic regions of the substrate and the protein tend to interact minimally with water. This is achieved upon contacting the hydrophobic regions of the protein and the substrate while dislodging water from these sites. The released water molecules then increase the entropy of the system. This explains why the proteins adsorb preferentially to hydrophobic surfaces contributed by hydrophobic interactions. The driving force of dehydration is not so strong on hydrophilic surfaces and passive adsorption is weaker. The theory of entropically driven adsorption was postulated by Norde and Lyklema (1979) and, thereafter, it has been reviewed in several studies and reports (Lyklema, 1984; Andrade and Hlady, 1986; Norde, 1986; Dent and Aslam, 1998).

Adsorption is preferred to hydrophobic surfaces, but extreme hydrophobicity is not favorable either (Lee and Ruckenstein, 1988). Rather, surfaces with both non-polar and polar properties have been optimal for passive adsorption. This is because of the other interactions such as hydrogen bonds, electrostatic effects and van der Waals forces (transient polarities) contributing to adsorption (Esser, 1997b). The electrostatic interactions are, however, considered less dominant, though not negligible, on the surfaces where the hydrophobic interactions predominantly take place. For example, IgG adsorbs to negatively charged surfaces in the pH values above its isoelectric point (pI)

where its net charge is negative and thus repulsive forces are expected (Bagchi and Birnbaum, 1981; Buijs *et al.*, 1995). Several studies have confirmed that the optimal pH for adsorption is usually close to the pI of the protein (see Dent and Aslam, 1998). At the isoelectric point, where the net charge of the protein equals zero, the protein adopts the most compact structure and exhibits the least repulsion between the neighboring protein molecules. Outside the isoelectric point the repulsion between the adjacent molecules and the more extended conformation obstructs denser packing of the molecules into a layer. The adsorption peak at the pI has also been observed on hydrophilic surfaces, where the electrostatic interactions should be more favorable. For example, the decrease in the pH of the solution and consequent increase in the positive charge of the protein should facilitate more binding to a negatively charged hydrophilic surface due to the pronounced opposite charges. This is not always true, probably because of the increased repelling forces emerging between the adjacent molecules (Buijs *et al*., 1995; Xu *et al*., 2006). Increased ionic strength of the coating solution decreases the electrostatic interactions and the repulsion between the adjacent molecules, and it indeed facilitates adsorption (Lee and Ruckenstein, 1988). The reduced electrostatic effects by the increased ionic strength should also apply to the interactions between the protein and the substrate, so that under repulsive electrostatic conditions adsorption is enhanced while under attractive conditions binding is decreased. Therefore, the effect of the ionic strength is quite strongly linked to the pH of the solution. Studies have shown, however, that the relations between the particular protein and its pI, solution pH and the ionic strength of the solution are not that straightforward (Buijs *et al*., 1995; Butler *et al.*, 1997a).

Adsorption is generally considered an irreversible, non-equilibrium reaction (for reviews see Haynes and Norde (1994) and Nakanishi *et al*. (2001)). Dilution of the solution usually results in a hysteresis in the adsorption isotherms, meaning unequal ascending and descending branches of the isotherm. This is indicative of an irreversible process (more about isotherms will be reviewed below). On the other hand, some desorption and replacement of the adsorbed protein is well documented, especially under rinsing or in the presence of replacing proteins (Vroman and Adams, 1969; 1986; Wahlgren *et al.*, 1993; Butler *et al*., 1997a; Xu *et al*., 2006). Desorption is obvious from the surfaces which have been coated using saturating protein quantities (Lee and Ruckenstein, 1988), since beyond the monolayer-forming concentrations the emergence of the loosely bound secondary layers is evident. When the desorption of the adsorbed protein is monitored, it is essential to recognize whether the question is about the breakage of the protein-solid substrate or the weaker protein-protein interactions before making further conclusions on the stability of the adsorbed layer and the interactions of the particular protein and the solid phase.

1.3.3.2 Adsorption isotherms

Adsorption isotherms can be used to link the amount of the adsorbed protein to the amount of the non-adsorbed protein at the equilibrium state (Fig. 4). The simplest physically plausible isotherm is the Langmuir isotherm which was originally developed

to model the adsorption of gas molecules to solid phases (Langmuir, 1916) and it has been further applied to the adsorption of proteins. It defines the surface coverage, or the fraction of the occupied binding sites as follows:

$$\theta = \frac{b[P]}{1 + b[P]}$$

In the equation, θ refers to the surface coverage, [P] to the protein concentration in the solution and b is a constant, analogous to the equilibrium constant of a ligand-receptor binding equilibrium, here indicating the affinity (or preferably avidity) of the protein to the surface. Due to the several prerequisites of the Langmuir isotherm such as reversible binding, monolayer requirement (no secondary upper layers), homogeneous binding sites and solutes as well as the absence of lateral interaction between the molecules, the isotherm is frequently criticized for its unsuitability to protein adsorption. Indeed, scientists have found their adsorption data to fit variably in the Langmuir isotherm (Wilkins Stevens and Kelso, 1995; Li *et al.*, 2005; Zhang *et al.*, 2005). It nevertheless provides a simple model for an empirical description of adsorption behavior.

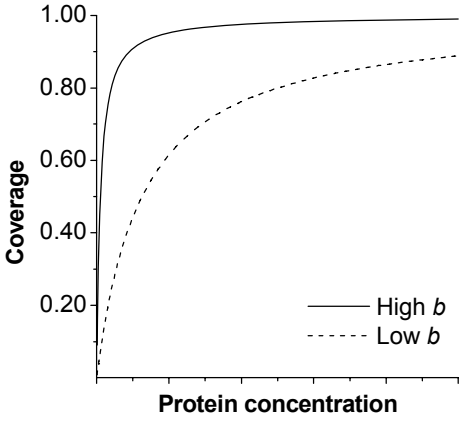

Figure 4. Langmuir adsorption isotherms with low (broken line) and high (solid line) values of the constant b. The coverage on the y-axis refers to θ in the Langmuir equation.

Another well-established adsorption isotherm is an empirical Freundlich isotherm which defines surface coverage as follows:

$$\theta = a[P]^{1/n}$$

In the equation a and n are constants and [P] refers to protein concentration. The Freundlich isotherm remains convex through the whole range, in contrast to the Langmuir isotherm which tends to linearity near the origin and approaches saturation in the high concentrations. The Freundlich isotherm is supposed to consider the issues of

lateral interactions between molecules and surface heterogeneity better than the Langmuir isotherm. (Dent and Aslam, 1998; Yang, 1998.)

Another useful means to describe adsorption behavior is the percentage bound plot (Fig. 5) which is defined as the ratio of the adsorbed protein to the added amount of the protein. In several adsorption situations these plots have shown a constant value in the dilute region (linear binding region) followed by a clear decrease in the percentage bound value (Cantarero *et al.*, 1980; Butler *et al*., 1992; Joshi *et al*., 1992; Butler *et al*., 1993; 1997a). The percentage value of the linear binding region (or the region of independence) describes the protein's "avidity" to the surface and the end of the linear region corresponds to the formation of a protein monolayer. Cantarero *et al*. (1980) showed that the percentage value of the linear region corresponded mainly to the size of the protein. The values ranged from less than 20 % for α-lactalbumin (14 kD) to more than 80 % for immunoglobulin M (IgM, molecular weight close to 1000 kD) which showed that the larger proteins had higher overall adsorption capability. The fact that the larger size enhances adsorption is explained by a larger number of individual binding sites. Proteins are supposed to rearrange on the surface after initial adsorption contacts and under this process small units may lose all their footholds while larger polymers more probably have some sites attached all the time (Dent and Aslam, 1998).

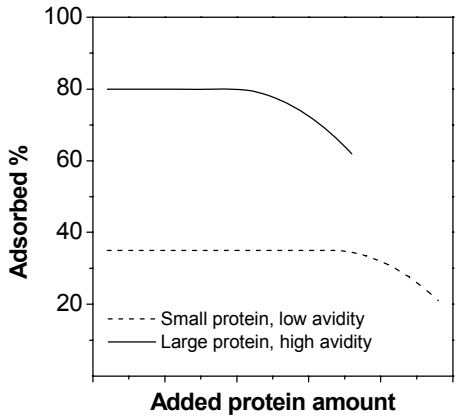

Figure 5. A schematic presentation of the percentage bound plots, showing the behavior of the proteins with "high avidity" (solid line) and "low avidity" (broken line) to the surface.

Several studies have reported the total quantities of proteins adsorbed to various surfaces and some of these values have been collected by Dent and Aslam (1998) and Nakanishi *et al*. (2001). The actual maximum quantities of the adsorbed proteins range from a few dozen to several hundred nanograms per square centimeter depending on the type of protein and surface.

A substantial number of adsorption studies have been carried out using immunoglobulins as the model proteins, which is understandable considering their wide use in the solid-phase assays (Underwood and Steele, 1991; Buijs *et al*., 1995; Wilkins Stevens *et al.*, 1995; Wilkins Stevens and Kelso, 1995; Butler *et al*., 1997a; Vermeer *et al.*, 2001). In addition, the use and applications of streptavidin in a variety of binding surfaces have been widely reported. However, the fundamental adsorption characteristics of intact streptavidin on different substrates in various physical and chemical conditions have not been comprehensively reported.

The structural and quantitative features of adsorbed streptavidin layers have been studied using, for example, scanning probe microscopy (SPM) techniques and quartz crystal microbalance (QCM, brief descriptions of the various techniques are reported in section 5). Cooper *et al.* (1994) imaged individual streptavidin molecules adsorbed on a graphite surface using scanning tunneling microscopy (STM). The images revealed some denaturation of the protein and the organization of four adsorbed subunits on the surface in a somewhat separated manner. Atomic force microscopy (AFM) images have been acquired from streptavidin layers covalently bound to mica and gold substrates (Kim J. *et al.*, 2004) and to biotin-functionalized Langmuir-Schaefer layers chemisorbed on gold (Ihalainen and Peltonen, 2004). In the latter study, the tip-sample convolution typically recognized with the AFM (smoothing of the lateral resolution due to a non-optimal curvature of the tip head) was corrected by means of software which enabled the acquisition of sharp, high-resolution images of the streptavidin layers. The study revealed pronounced clustering and high local densities of adsorbed streptavidin after increasing protein bulk concentration and the amount of adsorbed protein. A surface coverage of 49 % was measured at the highest level. In the same study, comparative measurements using QCM revealed a maximum quantity of adsorbed streptavidin of 250 ng/cm^2 on a dried surface. This represented a surface coverage of 63 %. A typical property, the clustering of the adsorbed protein was thus recognized with streptavidin, although elsewhere streptavidin surfaces have enabled a smooth surface distribution of biotinylated capture antibodies compared with passively adsorbed antibodies (see section 1.3.2.1).

1.4 Advanced coating technologies

1.4.1 Description of techniques

Adhesion mechanisms frequently make use of several simultaneous physical and chemical phenomena. Therefore, it may be inappropriate to strictly categorize which particular mechanism is employed in a given application. However, some suggestive classification of the techniques is useful in order to elucidate the principles of various mechanisms. One clear definition is whether the particular protein is coated either directly to the solid substrate or through an interface layer consisting of a kind of "carrier" molecule. Another determinant is whether adhesion is based on either plain physisorption or whether other interactions such as covalent chemistry are used to facilitate binding. Furthermore, the target protein and the carrier molecule can be introduced either in monomeric or polymerized forms. An additional, unique feature involved in

the immobilization of streptavidin evolves from its multiple biotin-binding properties. A biotinylated interface layer can be used to immobilize streptavidin while still retaining multiple unoccupied binding sites to serve the immobilization of the desired biotinylated molecules.

1.4.2 The features of streptavidin relevant to adsorption

As shown in several studies (I, II, Davies *et al*., 1994b; Huang *et al.*, 1994; Allen *et al*., 1996) streptavidin adsorbs spontaneously to the polystyrene surfaces in optimized conditions. The adsorption property of streptavidin is frequently explained by its hydrophobic nature. Some chromatographic data supports the hydrophobicity of streptavidin (Reh *et al.*, 1986; Schwidop *et al.*, 1990; Tischer *et al.*, 1991) but on the other hand, hydrophilic features can also be recognized for streptavidin. For example, the grand average of hydropathicity (GRAVY) -value computed at the ExPASy Proteomics Server (http://au.expasy.org, based on the algorithm by Kyte and Doolittle (1982), using entry P22629) returns the GRAVY-value of –0.331 for core streptavidin, the negative value thus classifying the protein as hydrophilic rather than hydrophobic. In addition, core streptavidin is readily dissolved in water in concentrations as high as 30 - 50 mg/ml (our experimental results) and it remains soluble without tendency to precipitate. The hydrophobicity plot (computed at the ExPASy) shows two distinct regions of greater hydrophobicity in the subunit and these hydrophobic patches are likely to be responsible for the hydrophobic character recognized for streptavidin. Though directly adsorbed streptavidin provides functional biotin-binding surfaces, there is some ongoing discussion on the adequacy of the surface density, the binding capacity and the stability of the adsorbed protein layer. Surface-bound streptavidin has also shown decreased binding affinities for biotinylated macromolecules. Fujita and Silver (1993) reported an affinity constant (K_a) in the order of $10^{12}\ M^{-1}$ for the binding of biotinylated DNA to the streptavidin-coated magnetic beads whereas Huang *et al*. (1996) reported affinity constants in the range of 0.6×10^8 - $1.1 \times 10^{10}\ M^{-1}$ for the binding of biotinylated DNA to the streptavidin-coated polystyrene particles.

The large number of the publications and reports generated in the context of the protein coatings may reflect the fact that in the absence of well-defined adsorption models scientists have been obliged to find the appropriate conditions for their application experimentally. Another explanation for the active research and development in the field relates to the prominent commercial share of the applications using immobilized proteins, of which streptavidin-coated microtiter wells and sensor surfaces are representative examples. Due to commercial interests, manufacturers do not by definition disclose the coating techniques in the scientific literature, but informative technical details can be found in the patent files instead. Some advanced protein coating and immobilization technologies will be reviewed below using interesting examples of streptavidin and avidin coatings reported in the literature.

1.4.3 Covalent coupling

1.4.3.1 Regular covalent chemistries

Covalent coupling is a useful alternative to improve the immobilization efficiency of proteins since a variety of chemistries enabling straightforward coupling are available. Perhaps the most common coupling approach is based on the reactivity of the ε-amines of the lysine residues and the N-terminal amines of the polypeptide chains. Widely used amine-reactive chemical groups include N-hydroxysuccinimide (NHS) esters, iso(thio)cyanates and aldehydes. Though easy to perform, amine-mediated coupling may result in quite random modification owing to the abundance and diverse distribution of lysines in proteins. Other modification targets of the proteins include the carboxyl groups of aspartate and glutamate. These react with amine-containing modification reagents in carbodiimide-mediated reactions. More controlled and site-specific modification relies on the carbohydrate residues of proteins which are reactive with amines and hydrazides after oxidation by mild sodium periodate and consequent formation of the aldehyde groups. Furthermore, one of the most important protein modification methods utilizes reactive sulfhydryl- (or thiol) groups of cysteines which represent efficient modification targets at exact locations. Thiol-groups can be generated by a mild reduction of the cystine disulfide bridges to two cysteines. Common sulfhydryl-reactive chemistries include maleimides, haloacetyls and alkyl halides which form stable thioether bonds with the sulfhydryls. Furthermore, the sulfhydryls can be used to create disulfide bridges in the oxidation reactions and thiol-disulfide exchange reactions. Examples of the latter include the reactions of the thiols with pyridyl disulfides and with the reagents containing the 5-thio-2-nitrobenzoic acid (TNB) structure. The disulfide bonds can be cleaved quite easily by mild reduction enabling specific breakage of the linker which is a useful property in certain applications. (Hermanson, 1996a; 1996c.) When inherent cysteines are absent or scarce, additional introduction of sulfhydryls can be used to render reactivity. Appropriate thiolating agents include, for example, 2-iminothiolane (Traut's reagent, Traut *et al.*, 1973) and heterobifunctional agents such as N-succinimidyl-S-acetylthioacetate (SATA, Duncan *et al.*, 1983). Supplemental thiolation renders, however, a more random distribution since thiols are usually introduced through amines.

One of two main strategies can be used when coupling proteins to other proteins and to the solid substrates. First, homobifunctional cross-linkers such as dialdehydes and bifunctional NHS-esters enable simultaneous coupling of proteins in a single reaction. Homobifunctional coupling is easy to perform and it is best suited for a homogeneous "self-conjugation" of proteins. The polymerization degree is hard to control and the result is a mixture of polymers containing the initial proteins in various ratios. In contrast, heterobifunctional cross-linkers allow more controlled reactions in two- or three-step procedures. A representative example of their use is a conjugation process where maleimide functionality is applied in one partner and sulfhydryl functionality (inherently present or chemically added) in the other partner to serve the covalent conjugation of the molecules. Some special heterobifunctional reagents (for example SATA)

provide chemically protected groups which are non-reactive until exposed by certain de-protective agents. (Hermanson, 1996b.)

A feasible strategy for the covalent coupling of the proteins to the solid phases is to activate the substrate first with reactive chemical functionalities and then introduce the protein of interest containing a specific reactivity to the activated support (Niveleau *et al.*, 1993; Shriver-Lake *et al.*, 1997; Suzuki *et al.*, 1997; Page *et al.*, 1998; Wang and Jin, 2004; Pyun *et al.*, 2005). When compared with the passive coating methods in parallel, the covalent approaches have regularly shown higher amounts of bound protein. Covalent modification and immobilization techniques have also been applied for streptavidin, though due to the absence of cysteines and carbohydrates only amine- or carboxyl-reactive chemistries work. The covalent binding of streptavidin to solid phases was reported already in the early studies quite soon after the first reports of using streptavidin-based solid phases for the assays. Peterman *et al*. (1988) bound streptavidin to the bromoacetyl-activated polystyrene spheres through nucleophilic groups such as amines and found the covalent binding to increase binding capacity and decrease dissociation compared with passively adsorbed protein. Dudin *et al*. (1988) coupled streptavidin to magnetic beads using sulfonyl chloride chemistry (amine-reactive) to be used in chromosome separation. Amine surfaces and isothiocyanate-based coupling chemistries were also used in a recent magnetic particle application (Zhang *et al.*, 2007). Carbodiimide chemistry has been used to coat streptavidin to carboxyl-terminated Eu-nanoparticles to be used as reporters in highly sensitive immunoassays (Härmä *et al.*, 2001). Further, Schiestel *et al*. (2004) used carbodiimide chemistry for the covalent coupling of streptavidin to amine- and carboxyl-terminated silica nanoparticles. The coupling of streptavidin to the amine surface through the protein's carboxyls proved ineffective. This was explained by the higher reactivity of lysines of streptavidin compared with the amines on the surface. In contrast, the binding of streptavidin to carboxyl nanoparticles through the protein amines was successful resulting in (according to the authors) the highest binding densities reported on the nanoparticle surfaces by that time (1.1 streptavidin molecules per 100 nm^2). Furthermore, Douglas and Monteith (1994) reported a 96-well plate that bound proteins covalently through amines when introduced in a buffer with the pH above the pI of the protein. These plates were used for the covalent binding of streptavidin and the resulting surfaces showed significantly higher uniformity and increased biotin-binding capacities compared with the passively coated reference plates.

1.4.3.2 Photochemistry

A particular technique in covalent binding is photochemistry, which makes use of the chemical reactivities that are induced by the exposure of special chemical structures to light (usually ultraviolet light). The most popular photosensitive groups are aryl azides which form reactive nitrene intermediates upon photolysis. These intermediates go through either an addition reaction to the double bonds or an insertion reaction into the C–H and N–H bonds. They can also react with nucleophiles such as primary amines. Other photosensitive functionalities include benzophenones, diazo compounds and

diazirine derivatives. Upon photoactivation, the principal binding targets of all these are the C–H and N–H bonds, hence the site-specificity of the coupling is rather weak. However, photochemistry enables the binding of ligands to the targets where the regular chemical functionalities are lacking or scarce. Various heterobifunctional cross-linkers have been synthesized, which contain both a photoreactive end and a regular thermochemical end. This may be amine-reactive, sulfhydryl-reactive or carboxyl-reactive. The photoreactive groups are typically inert until exposed by a light pulse and, therefore, these reagents enable controlled and timed initiation of the cross-linking. (Hermanson, 1996d; 1996e.)

In addition to the regular photosensitive groups mentioned above, common fluorescent reporter dyes such as fluorescein and Alexa 594 are capable of forming bonds when extensively photobleached by prolonged exposure to light (Holden and Cremer, 2003). These are activated by longer wavelengths (470 nm and 560 nm) and thereby some of the potential drawbacks of ultraviolet light are avoided.

One approach to immobilize proteins is to couple a photoreactive substance to an inert solid support by photoactivation while maintaining the other, thermochemical functionality of the reagent available for the covalent coupling of the protein. For example, an aryl azide derivative, 1-fluoro-2-nitro-4-azidobenzene (FNAB) has been used to activate polystyrene and polycarbonate surfaces (Nahar *et al.*, 2001; Bora *et al.*, 2006) and these surfaces have been shown to rapidly bind high quantities of proteins. Furthermore, they have introduced higher sensitivities and heat-stability in ELISA-assays in comparison with non-treated surfaces (Bora *et al.*, 2002; 2004). In another study, the thermoreactive end of FNAB was first reacted with a carrier polymer while photoreactive functionality was used for protein coupling (Naqvi and Nahar, 2004). Alternatively, quinones can be used to activate surfaces for photochemical coupling (Jacobsen and Koch, 2000). An alternative to the activation of the solid phase is to activate the protein of interest with a photoreactive ligand. Nakajima and co-workers (Nakajima *et al.*, 2006) bound a photoreactive ligand to glucose oxidase and horseradish peroxidase, after which the activated enzymes were patterned inside microfluidic channels by means of a laser beam. Regioselective patterning is conveniently enabled by photochemistry since the target proteins can be bound from a bulk solution to exact locations by means of a laser beam or exposure of light through special photomasks.

Streptavidin and avidin are typically linked to photochemistry through photoactivatable derivatives of biotin (Fig. 6). A common strategy has been to couple an aryl azide derivative of biotin (photobiotin, Forster *et al*., 1985) to the solid substrate by a photochemical reaction and consequently use the biotinylated surfaces for the immobilization of either streptavidin or avidin (Hengsakul and Cass, 1996; Dontha *et al.*, 1997; Sabanayagam *et al.*, 2000; Wilde *et al.*, 2001; Choi *et al.*, 2005). Some of these studies were confined to visualizing the patterning of biotin by streptavidin-conjugated enzymes and fluorophores, whereas some studies further proved the availability of the unoccupied biotin-binding sites by immobilizing biotinylated antibodies and oligonu-

cleotides to the surface. The concept is also applicable in reverse order. Pritchard *et al*. (1995) immobilized photobiotin to an avidin-coated surface through the biotin-end and the remaining photochemical functionality was used for the coupling of an antibody. Another photosensitive derivative of biotin, biotin-4-fluorescein, has also been used to immobilize streptavidin-conjugated enzymes inside microchannels (Holden *et al.*, 2004). Photochemical biotinylation, as photochemical reactions in general, can be focused to confined locations enabling subsequent introduction of streptavidin-enzyme conjugates and biotin-binding surfaces to defined regions.

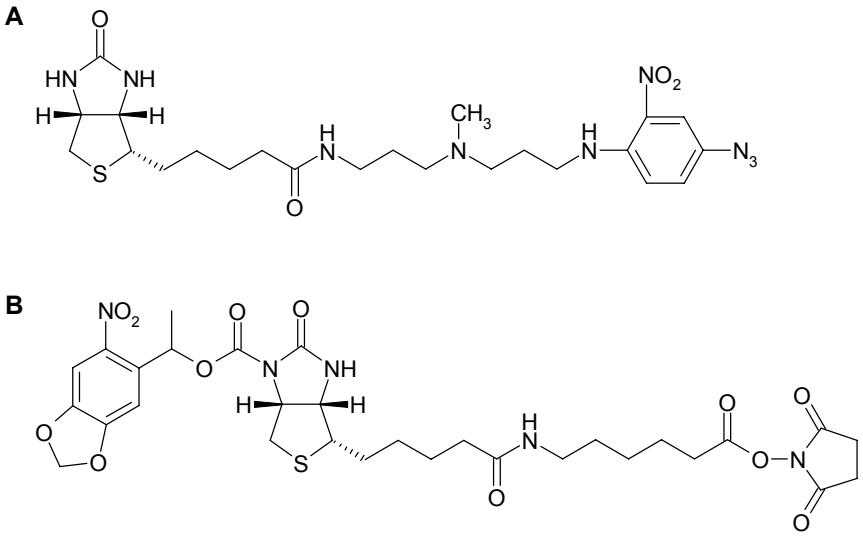

Figure 6. A) Photobiotin, N-(4-azido-2-nitrophenyl)-N'-(N-d-biotinyl-3-aminopropyl)-N'-methyl-1,3-propanediamine, contains a photoactivatable terminal group to enable a photochemical coupling of biotin to the solid substrates. Drawn according to Forster *et al*. (1985). B) Methyl α-nitropiperonyl-oxycarbonyl biotin - aminocaproic-NHS ester (MeNPOC-biotin-AC–NHS), or "caged" biotin, contains a photolabile protective group which obstructs the binding of biotin to avidin and streptavidin. Drawn according to Pirrung and Huang (1996).

In (strept)avidin-biotin technology, the use of photochemistry is not only limited to the photochemically mediated coupling of biotin through the reactive group of the aliphatic side chain. In addition, the double ring structure of biotin can be protected with a photolabile group which can be removed by light exposure and thus recover the binding activity of biotin to avidin and streptavidin (Sundberg *et al.*, 1995). The chemistry of this "caged" biotin relies on a photoremovable nitrobenzyl group which is bound to one nitrogen in the double ring of biotin and this biotin derivative is named as methyl α-nitropiperonyl-oxycarbonyl biotin (MeNPOC-biotin). These biotin derivatives have been coupled to solid substrates either by direct covalent coupling (Sundberg *et al*., 1995), using BSA as an interface protein (Pirrung and Huang, 1996; Blawas *et al.*, 1998) and by self-assembled alkanethiol monolayers on gold (Yang *et al.*, 2000). The immobilization of streptavidin by this approach is probably not very efficient; for ex-

ample, Blawas *et al*. (1998) yielded densities of 9×10^9 streptavidin molecules per square millimeter (≈ 0.015 pmol) and an efficiency of 20 % for the immobilization of streptavidin to BSA-biotin surfaces. In addition to the photochemically activatable biotin derivatives, an electrochemically activatable variant, a hydroquinone-caged biotin has been reported (Kim K. *et al.*, 2004). This biotin derivative was likewise used for the patterning of streptavidin to the solid phase using an electrode array instead of a photomask. The examples discussed above concerned the use of photochemistry for creating biotinylated surfaces, but an opposite approach was presented by Koyano *et al*. (1996). Surface-bound biotins were ruptured by ultraviolet light exposed through a mask while the unexposed sites retained their ability to bind streptavidin.

1.4.4 Biotinylated interface

Biotin-functionalized surfaces can also be introduced either by regular covalent chemistries or by self-assembled monolayers on gold surfaces in addition to photochemistry (more about the self-assembly in section 1.4.6). Covalent methods include, for example, the coupling of NHS-biotin to amine-terminated microtiter well surfaces (Bugari *et al.*, 1990; Rasmussen, 1990; Bicknell *et al.*, 1996), to aminosilanized TiO_2 surfaces (Ye *et al.*, 2007) and to hydrazide functionalized surfaces (Wang *et al.*, 2005). Other functionalities are also feasible, such as the binding of maleimide-biotin to a thiolated surface (Vijayendran and Leckband, 2001) and the binding of unmodified biotin to a titania layer through the inherent carboxyl group of biotin (Huang *et al.*, 2006). The strong binding of sulfur to gold is exploited in the self-assembly of the thiol-terminated biotin derivatives into ordered monolayers (Pérez-Luna *et al.*, 1999; Pradier *et al.*, 2002a; Xia *et al.*, 2004; Jung *et al.*, 2006). Another way to prepare the biotinylated monolayer is to use a thiolated, self-assembling interface molecule containing a reactive terminal group to serve subsequent covalent binding of biotin (Yam *et al.*, 2001; Pradier *et al.*, 2002b; Liu and Amiridis, 2005).

A biotinylated polymer in the interface between the solid substrate and the streptavidin layer frequently constitutes a further advantage compared with the surfaces of directly coupled biotin. The interface layer better prevents direct contact of streptavidin with inorganic solid material and introduces a three-dimensional structure enabling higher quantities of immobilized streptavidin. Furthermore, it can facilitate the orientation of streptavidin to the uppermost layer (Fig. 7) when a more readily adsorbing polymer is used, or when the surface is constructed successively in a layer by layer manner. The principle of a biotinylated interface polymer was already used in early reports of streptavidin-coated surfaces. Suter and Butler (1986) used biotinylated keyhole limpet hemocyanin (KLH), gelatine and rabbit gamma globulin as the primary layer for the immobilization of streptavidin. In their study, the antibody-binding capacity of the adsorbed streptavidin surface was only 1 - 10 % compared with the streptavidin surfaces immobilized via the biotinylated interface. Berger *et al*. (1993) described the use of heat-treated, denatured and polymerized BSA (thermo-BSA, see the next section) as the biotinylated interface polymer. This, in combination with chemically polymerized streptavidin, exhibited high biotin-binding capacity and good desorption stability

against detergents as well as providing greater response in an assay of thyroid stimulating hormone (TSH) compared with control surfaces. Furthermore, the same patent issue described the use of biotinylated aminodextran and biotinylated polyaminoacid (a copolymer of lysine and phenylalanine) as the interface polymers for the immobilization of streptavidin. Biotinylated BSA has also been used in a co-coating approach to create a solid phase to serve the simultaneous detection of TSH and thyroxin (T_4) (Wu *et al.*, 2003). In the first step, a monoclonal antibody against TSH and extensively biotinylated BSA were adsorbed to the surface. The latter provided subsequent binding of streptavidin and the immobilization of a biotinylated monoclonal antibody against T_4. Passive adsorption of both antibodies had resulted in the reduction of activities and inadequate assay performance, but in contrast, this co-coating approach proved useful in this assay combining the principles of the sandwich-assay and the competitive assay in one vessel. Other interface proteins are also useful, for example, Strohner and coworkers (Strohner, 2000; Strohner and Immer, 2001) yielded surfaces of high biotin-binding capacity when using biotinylated, cross-linked IgG as the interface layer for the immobilization of a mixture of avidin and streptavidin to the second layer.

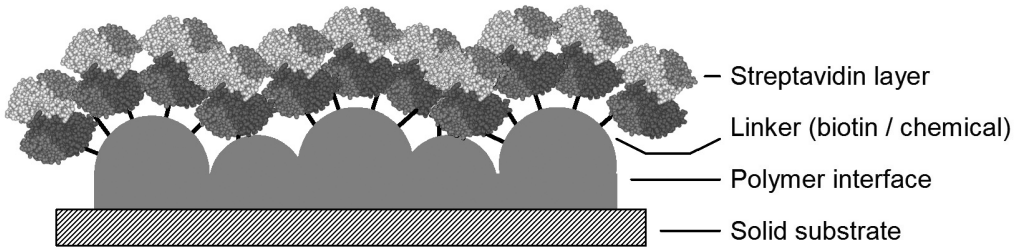

Figure 7. A schematic presentation of the immobilization of streptavidin using an interface layer.

1.4.5 Various interface polymers and homogeneous conjugation

Instead of a biotin link, streptavidin can be coupled to appropriate polymers by means of chemical conjugation. For example, Tischer *et al*. (1991) coupled streptavidin either to BSA or β-galactosidase and obtained significantly steeper calibration curve slopes in a TSH-assay compared with those obtained using surfaces coated with unmodified native streptavidin. An even more substantial effect was observed when using thermo-BSA as the conjugation partner (Tischer *et al*., 1991; Berger *et al*., 1993; Schmitt *et al.*, 1994). In these experiments, BSA was denatured and polymerized by heating to a polymer of the molecular weight above 700 kD. This polymer was activated chemically to provide reactive thiols and streptavidin was modified to offer maleimide moieties. Upon combination, these were efficiently conjugated into a heteropolymer and the polymer was coated into the microtiter wells. In addition to excellent assay performance, the thermo-BSA-streptavidin surfaces showed minimal desorption of the adsorbed polymer by detergents.

In addition to proteins, carbohydrates can be used as carrier polymers in chemical conjugation and in biotin-mediated immobilization. A patent publication by Dapron *et al*. (2005) described a method where benzophenone and thiol moieties were first coupled to a dextran polymer. Subsequently, photochemical irradiation was used to couple the polymer to the solid substrate through the benzophenone groups while the thiols enabled the coupling of maleimide-activated streptavidin to the polymer. Streptavidin quantities of 3.3 - 6.67 μg/cm^2 were bound in the wells and the immobilization capacities for biotinylated albumin were 1.13 - 1.76 μg/cm^2. In another dextran-based approach, functional streptavidin surfaces were constructed on gold-layered piezoelectric crystals (Chen and Lin, 2007). The first layer was fabricated with amine-terminated alkanethiols to which periodate-oxidized dextran was coupled through a reaction between amines and dextran aldehyde moieties. Streptavidin was immobilized either covalently by reductive amination to the dextran aldehyde groups or through biotin linkers coupled to dextran. Both these hydrogel-based streptavidin-coatings showed further ability to bind biotinylated BSA.

Polyamidoamine (PAMAM) dendrimers are synthetic copolymers of repeated monomeric units such as ethylenediamine and methylacrylate. These structures have a defined number of either reactive amine-, hydroxyl- or carboxyl-groups on their surface (Singh *et al.*, 1994; Singh, 1998; Nourse *et al.*, 2000). The well-characterized structure and functionality of the dendrimers favor their use as the building blocks in biological structures such as the interface layers for the immobilization of proteins. Amine-terminated dendrimers have been used as the carrier polymers for the immobilization of streptavidin and avidin to aminosilylated glass chips (Benters *et al.*, 2001), to magnetic particles (Gao *et al.*, 2005) and to gold substrates for the analysis of the surface by various means (Hong *et al.*, 2003; Liu and Amiridis, 2004; Mark *et al.*, 2004; Hong *et al.*, 2005; Kim *et al.*, 2006). Both covalent coupling and biotinylated dendrimers have been used for the immobilization of streptavidin. Various surface research methods in all these studies have shown either increased binding capacities or improved homogeneity of the surfaces with dendrimer-based coatings in comparison with respective control surfaces without the dendrimer interface.

Polylysine, a synthetic polymer of amino acid lysine, is widely used to render negatively charged solid substrates, for example glass and metal oxides, to positively charged surfaces and, furthermore, to provide primary amines for covalent coupling (Eisen and Brown, 1999; Haab *et al.*, 2001; Kusnezow *et al.*, 2003). Polyethylene glycol (PEG) and polyethylene oxide (PEO, referring to longer polymers) are known for their ability to resist non-specific binding of proteins when layered on surfaces (reviewed by Kingshott and Griesser, 1999). A co-polymer of polylysine and PEG, poly(L-lysine)-grafted poly(ethylene glycol) (PLL-g-PEG), has been used as an interface layer for the immobilization of streptavidin (Ruiz-Taylor *et al.*, 2001a; 2001b). Defined ratios of polylysine, PEG and biotin-terminated PEG strands reacted to produce a desired co-polymer. When layered on the titanium oxide surface, the primary amines of polylysine provided binding of the polymer to the negatively charged sub-

strate whereas the PEG-strands, some of them terminated with biotin, protruded out of the surface. The grafting and the biotinylation degrees and thus the properties of the surface could be modified by adjusting the stoichiometric ratios of the reagents. Low non-specific binding of proteins to this surface was observed and streptavidin was efficiently immobilized by the biotinylated polymer up to monolayer densities of 6.3 pmol/cm^2. This type of streptavidin surface was subsequently used by Peluso *et al*. (2003) in a study which compared the performance of random and oriented immobilization of biotinylated antibodies and Fab' fragments.

The interface polymer is not a prerequisite for enhanced coating performance. Homogeneous polymerization of streptavidin without other macromolecules may also be utilized, as shown, for example, in one of the study (I). In another example, Desai *et al*. (2003) used maleimide- and thiol-chemistry to polymerize streptavidin, resulting in a mixture of products consisting of monomeric to tetrameric conjugates. When coated on polystyrene, this surface showed a 25-fold greater biotin-binding capacity and a 70 % higher binding capacity for biotinylated peptide compared with the surface manufactured using non-polymerized streptavidin. Other studies have also reported the enhanced performance of surfaces made from homogeneous streptavidin polymers (Tischer *et al*., 1991; Berger *et al*., 1993).

1.4.6 Recombinant approach and self-assembly

Some articles have reported that recombinant and fusion protein technologies improve the immobilization properties of streptavidin, albeit most of the numerous mutations of streptavidin have been targeted at the biotin-binding site and at the subunit interfaces mainly for scientific interest (see section 1.2.3.3). One genetically modified variant (Reznik *et al.*, 2001) possessed a six-residue immobilization sequence (Gly-Gly-Ser-Gly-Cys-Pro) fused to the C-terminus of the streptavidin subunit. This recombinant streptavidin was covalently coupled to a maleimide-functionalized microtiter plate through the unpaired thiol groups of the cysteine residues. The biotin-binding properties of this variant were minimally affected when bound on the surface and the accessibility of the binding sites for biotinylated macromolecules was improved in comparison with natural core streptavidin due to the linkers that separated the protein from the surface.

The fusion protein derivatives and the genetic variants of streptavidin have been frequently used in combination with self-assembled monolayers. The self-assembled monolayers are highly ordered single-molecular layers which are generated spontaneously by the inherent properties of the organizing molecules. A classical example of this is the organization of alkanethiols on gold surfaces (Nuzzo and Allara, 1983; Porter *et al.*, 1987). In these structures, aliphatic alkyl molecules with a thiol-group in one end bind tightly to the gold surface due to the strong interaction between gold and sulfur. Meanwhile the protruding alkyl chains are organized in highly ordered manner. Self-assembling properties have also been recognized with some proteins. A well-characterized protein which self-assembles *in vitro* and has potential in nanotechnology

is the S-layer protein from the surface of gram-positive bacteria (Jaenicke *et al.*, 1985; Pum and Sleytr, 1999; Sleytr and Beveridge, 1999; Sleytr *et al.*, 1999; 2007). In one application, Moll *et al*. (2002) fused an S-layer protein SbsB from *Geobacillus stearothermophilus* to a streptavidin subunit and constructed four N-terminal and two C-terminal fusions. The fusion proteins were produced in *E. coli* and then refolded in a ratio of 1:3 with native subunits of core streptavidin in order to construct a hetero-tetrameric streptavidin containing one subunit with the S-layer protein extension. The transmission electron microscopy images showed that the tetramers with the N-terminal, but not the C-terminal, streptavidin fusions formed highly ordered layers. When layered on liposomes, the tetramers retained their capability to bind biotinylated proteins. Huber *et al*. (2006) introduced a principally similar construct; streptavidin was fused to the C-terminus of an S-layer protein SbpA from *Bacillus sphaericus*. The self-assembling property of this S-layer protein is dependent on the calcium ions which enables a controllable assembly *in vitro*.

Other self-assembling proteins, applicable in biotechnology, are hydrophobins from filamentous fungi (Wösten, 2001; Hektor and Scholtmeijer, 2005). Hydrophobins are involved in the growth and extension of the aerial hyphae of fungi and they self-assemble into amphipathic membranes at interfaces. Hydrophobins HFBI and HFBII from the mold *Trichoderma reesei* have been extensively studied with respect to their surface organization and feasibility for the immobilization of useful proteins in biotechnology (Linder *et al.*, 2001; 2002; Torkkeli *et al.*, 2002). Szilvay and co-workers (Szilvay *et al.*, 2006; 2007) constructed genetically engineered variants of HFBI containing either N- or C-terminal short amino-acid linkers with an unpaired cysteine moiety serving for biotinylation with maleimide-biotin. The biotinylated HFBI-variants were used to immobilize avidin to the surface in a highly ordered manner yielding amounts of 8.1 - 9.8 pmol/cm^2 which represented 140 - 170 % surface coverage compared with a complete monolayer density.

1.4.7 Oligonucleotide-mediated immobilization

The principle of oligonucleotide-mediated, or DNA-directed immobilization (DDI), is to immobilize a protein-oligonucleotide conjugate by hybridization to a complementary oligonucleotide on the solid surface (Fig. 8). The streptavidin-oligonucleotide conjugates have been constructed either through an affinity-based binding of biotinylated oligonucleotides to streptavidin or by the covalent coupling of chemically activated streptavidin and DNA. Scouten and Konecny (1992) constructed a complex consisting of biotinylated single-stranded polyadenylic acid (poly[dA]), streptavidin and biotinylated antibody for immobilization and separation on thymidine-coated (poly[dT]) magnetic beads. The reversible binding of the complex to the particles was shown since 80 % of the bound radioactivity was eluted in mild conditions. In another application, Niemeyer *et al*. (1994) covalently coupled a 5'-thiolated oligonucleotide to streptavidin which was functionalized with maleimide groups by means of a heterobispecific cross-linker. A 1:1 complex of streptavidin and DNA was observed after the purification and characterization. The covalent DNA-streptavidin conjugates were

combined with biotinylated capture antibodies and subsequently the complexes were hybridized to complementary oligonucleotide-surfaces in microtiter wells. The immunoassays performed in the wells demonstrated the transformation of an oligonucleotide surface into a functional protein surface.

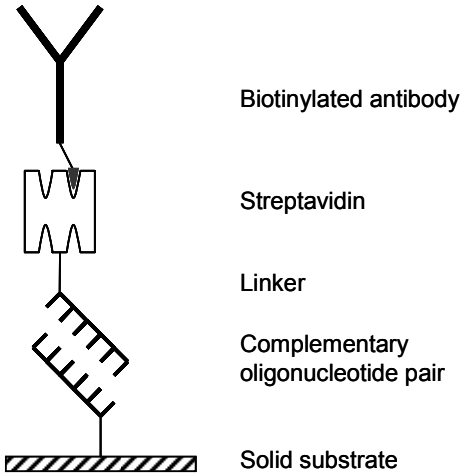

Figure 8. The principle of oligonucleotide-mediated, or DNA-directed, immobilization of streptavidin and a biotinylated antibody. The linking between the oligonucleotide and streptavidin can be either biotin-mediated binding or based on covalent coupling of the protein to DNA.

A comparative immobilization of biotinylated reporter enzymes (horseradish peroxidase, β-galactosidase and alkaline phosphatase) either by the DDI-process or by direct binding to the streptavidin-coated microtiter plates showed 1.7 - 2.7-fold higher intensities for the DDI-method (Niemeyer *et al.*, 1999). Similar improvements, compared either with direct physisorption or with immobilization to an adsorbed streptavidin surface, were observed for the immobilization of antibodies in the same study. The results showed the general efficiency and gentleness of the DDI-process. Niemeyer *et al*. (2003) have, furthermore, reported a combination of the DDI of the capture antibody with immuno-PCR detection technology (Sano *et al.*, 1992), yielding an extremely sensitive assay concept. The DDI of the capture antibody has been further used in an array-based microscale fluorescence immunoassay (DDI-μFIA) for simultaneous detection of four protein antigens using a Cy5 label (Wacker and Niemeyer, 2004), resulting in reasonable sensitivities and reproducibility. Ladd *et al*. (2004) described the functionalization of the biosensor surfaces by combining the self-assembled monolayers with DDI. The surface consisted of successive layers of biotin monolayer, streptavidin, biotinylated oligonucleotide and an oligonucleotide-coupled antibody to provide the capture of hCG and subsequent detection by surface plasmon resonance. Wacker *et al*. (2004) reported a comparison of three different capture concepts in an array-based antibody-assay. A capture antibody was immobilized either by DDI, direct adsorption or by streptavidin-coated surface for the binding and determination of rabbit IgG. Equal detection limits (150 pg/ml) were observed with each surface, but the DDI-method provided the most homogeneous spots when visualized by imaging and exhib-

ited the least variation between the repeated tests. Furthermore, DDI enabled equal performance with the use of 100 times less antibody compared with direct spotting.

1.4.8 Peptide tags

Various peptide tags fused to desired target proteins have recently become popular in biotechnology, essentially enabling reversible immobilization of recombinant fusion proteins to affinity purification matrices (reviewed by Terpe, 2003). In addition to purification purposes, peptide tags have been used to some extent to immobilize target proteins to surfaces in a specified orientation through the tag. For example, a positively charged arginine-tag has been used to facilitate the immobilization of proteins to negatively charged mica (Nock *et al.*, 1997) and the FLAG-tag (a well-defined common octapeptide) has been used to enable an oriented immobilization of subtilisin to a surface coated with anti-FLAG monoclonal antibodies (Wang *et al.*, 2001). Perhaps the most common peptide-tag mediated immobilization is based on the polyhistidine tag which makes use of the interaction of histidine with certain metal ions such as nickel (Ni^{2+}). This technology has been used, for example, in surface plasmon resonance sensor chips (Gershon and Khilko, 1995; Nieba *et al.*, 1997), for the immobilization of enzymes (Carlsson *et al.*, 1996) and for the oriented immobilization of Fab fragments (Vallina-García *et al.*, 2007).

Immobilization using peptide tags requires a functional primary layer such as antibodies against the tags or a metal-activated surface such as a nitrilotriacetic acid (NTA) matrix or other nickel chelators (Johnson and Martin, 2005). The additional layers may be sources of instability and they can introduce some inconvenience to the preparation of the surface. Thus, peptides exhibiting direct affinity to a solid substrate, for example to polystyrene, would be of interest for direct immobilization. Loomans and coworkers (Loomans *et al.*, 1997; 1998a; 1998b) showed that certain N-terminal extensions (oligo-lysine moieties) enhanced the adsorption of desired epitope peptides on polystyrene which enhanced their immunoreactivity in ELISA-assays. Sakiyama *et al.* (2004) and Kumada *et al*. (2006) have screened dodecapeptides exhibiting affinity to polystyrene from an *E. coli* random peptide display library using the enzymatic activity of glutathione-S-transferase as a reporter of the adsorption efficiency. One of their screened peptides was chemically linked to either streptavidin or monoclonal antibody (Kumada *et al.*, 2007). These proteins showed higher binding to the hydrophilic polystyrene surface and also enabled improved performance over conventional technology in ELISA-assays. In addition to the polystyrene-specific peptides, polypeptides which have direct affinity to various inorganic surfaces have been designed and synthesized, facilitating the assembly of nanostructures to sensor surfaces (reviewed by Sarikaya *et al*., 2003).

2 AIMS OF THE STUDY

The aim of the study was to discover the effects of specified streptavidin surface properties on the performance of solid-phase immunoassays and examine the potential to improve assay performance by modifications of the binding surface. The specific aims were to:

1. Model the surface and investigate the adsorption capabilities of streptavidin.

2. Prepare various reporter molecules and implement straightforward assays for the characterization and monitoring of the surfaces.

3. Develop surface performance by selected, straightforward modifications to the protein and coating process.

4. Investigate various streptavidin-coating chemistries in use and determine the influence of the defined surface properties on the efficiency of various immunoassay types through a comprehensive study of the surfaces exhibiting distinct features.

5. Investigate the potential of preparing condensed, high density capture surfaces and study their influence on immunoassays which are based on the surface readout measurement of time-resolved fluorescence.

3 SUMMARY OF THE MATERIALS AND METHODS

An overview of the materials and methods is given below. Detailed descriptions are presented in the original publications.

3.1 Streptavidin and coating (I - IV)

3.1.1 Streptavidin

Streptavidin was mainly from Biospa (Milan, Italy). It was delivered as lyophilized powder, then dissolved in pure water (usually 10 mg/ml) and stored in aliquots at −20 °C. The product was characterized at the Department of Biotechnology using N-terminal sequencing and SDS-PAGE analysis.

3.1.2 Solid substrates

Microtiter wells either in the C12 strip format, in separate single wells or as special spot wells were used as the solid substrates for the coatings. The wells were fabricated from low-fluorescent polystyrene and were irradiated to enable maximal protein adsorption capability. The spot wells (IV) had an indentation of approximately 0.2 mm in depth and either 2.5 mm, 3.5 mm or 4.5 mm in diameter at the bottom of the single-well format microtiter well. The spot was introduced in the well within the injection molding. All wells and strips were from Nunc A/S (Roskilde, Denmark).

3.1.3 Coating

Native, unmodified streptavidin or some of the modified variants were diluted in an appropriate coating buffer which typically consisted of 100 mM Na_2HPO_4 and 50 mM citric acid adjusted to pH 5.0. The coating volume was 200 µl per well. The plates were incubated at +35 ºC and the evaporation of the solution was minimized by closing the plates in a humid container. The plates were washed after coating using a wash solution containing Tween 20 (0.05 % v/v) as a detergent to provide efficient washing. The streptavidin-coated surfaces were subsequently incubated with protective and blocking agents (BSA, D-sorbitol). The blocking solution was aspirated after overnight incubation and the plates were dried, packed with desiccant and stored at +4 ºC. Typically, the coated plates retained their initial biotin-binding activities for several years.

3.1.4 Spot surfaces (IV)

The purpose of the spot approach was to introduce the binding surface to coincide with the excitation beam and condense the capturing of the labeled antibodies to increase the signal-to-background ratios of the assays that utilize the direct surface readout measurement. Modified single microtiter wells holding a circular indentation at the bottom were used as templates for the droplets of the coating solution. The droplet volume ranged from 4 to 15 µl depending on the spot diameter. Excluding the volumes and concentrations used, the stages of the coating process were principally similar to the regular protocol including the overnight incubation and the blocking steps. Avoid-

ance of evaporation was considered especially critical while incubating the droplets at the elevated temperature.

3.1.5 Modified streptavidin (I, III, IV)

Streptavidin was subjected to amine reactive glutaraldehyde prior to coating (I, IV) in a reaction containing approximately 100 mM (1 % v/v) glutaraldehyde and 35 µM streptavidin. The reaction mixture was purified by gel filtration or successive desalting columns. The pretreatment resulted in a modified, polymerized protein (referred to as GA-SAv) with enhanced adsorption capability. The reaction conditions in terms of the temperature, time and concentrations were studied in order to obtain an optimal polymerization degree and adsorption capability.

Alternatively, streptavidin was modified using thiol-chemistry (III). Streptavidin was first reacted with a 40-fold molar excess (2500 µM) of amine reactive N-succinimidyl S-acetylthioacetate (SATA, Pierce Biotechnology, originally described by Duncan *et al*., 1983). The incorporated heterobifunctional linkers were deacetylated using hydroxylamine to reveal reactive thiols which served for polymerization and efficient binding to the surface. The number of the reactive thiols incorporated to streptavidin was determined by Ellman's reaction using cysteine as a calibrator of the sulfhydryl quantity.

3.1.6 Fractionation and size-exclusion chromatography (I)

Streptavidin and the polymerized variant (GA-SAv) were analyzed and fractionated by size-exclusion chromatography on Superose 12 HR10/30 column, run by the ÄKTA explorer system (both from GE Healthcare, Uppsala, Sweden). Alkaline buffers, for example, Tris-Cl or borate buffers at pH 8.4 - 8.8, without NaCl, were found suitable for the elution of streptavidin and GA-SAv.

3.2 Biotinylation and labeling of the antibodies and reporter molecules

3.2.1 Biotinylation and labeling with lanthanide chelates

Modified biotin containing a reactive isothiocyanate group in the end of the extended side chain (Mukkala *et al*., 1993) was used for the biotinylation of proteins (I, III, IV). The pH of the biotinylation reaction was adjusted to 9.8 to provide optimal conditions for the reaction between the amines and the isothiocyanate group. Typically, an 80-fold molar excess of biotin over the protein was used and the protein concentrations in the reactions were approximately 6 - 8 µM for the antibodies and 70 - 80 µM for myoglobin. The biotinylated proteins were separated from non-reacted biotin on NAP- or PD-10 desalting columns (GE Healthcare). The biotinylated antibodies were characterized using an in-house test where a sample of the antibody was immobilized to a streptavidin-coated well and subsequently an aliquot of the liquid was transferred to an anti-mouse IgG-coated plate to assess the fraction not bound to the streptavidin surface. The test indicated the proportion of the antibody molecules bearing functional biotin moieties and the result was typically 90 - 100 %.

The principles of the lanthanide chelate labels and their measurement will be described below in section 3.3. Proteins were labeled with inherently fluorescent seven-dentate (Takalo *et al.*, 1994) or nine-dentate (von Lode *et al.*, 2003) Eu-chelates (I, III, IV). Alternatively, a terbium- (Tb) chelate was used (Mukkala *et al.*, 1989) for the labeling of streptavidin (II). Amine reactive isothiocyanate chemistry was used for the coupling. The protein concentrations in the labeling reactions were approximately 4 - 10 µM for the antibodies, 40 µM for myoglobin and 40 µM for streptavidin. The chelates were used in 5 - 60-fold molar excess over the protein depending on the desired purpose; the biotinylated reporter molecules were labeled with a lower amount whereas the detection antibodies for the immunoassays were labeled with larger excess to guarantee a higher degree of labeling. The labeled proteins were purified by size-exclusion chromatography on a Superdex 200 HR10/30 column (GE Healthcare).

The concentrations of the proteins, after labeling or any other process, were typically assayed by measuring absorbance at 280 nm or by a Bradford protein assay (Bradford, 1976). Quantification by absorbance was based on the protein-specific absorbance values which were 1.34 AU (absorbance units) for the antibodies and 2.8 AU for streptavidin when the concentration of the given protein is 1 mg/ml. The value for streptavidin deviated a little from that reported in the literature (3.1 - 3.4 AU), but the value used was experimentally confirmed with several dissolved batches. The Bradford protein assay frequently resulted in a weak response with streptavidin. An enhanced method based on heating (Sharma and Tihon, 1988) increased the absorbance values but complicated the assay. Therefore, the Bradford assay was not routinely used for assaying the concentrations of streptavidin. An alternative color-forming assay, the Micro BCA assay (Pierce Biotechnology) was used instead.

The labeled proteins were further characterized with respect to their lanthanide ion contents. The concentrations of the ions were measured against respective calibrators using DELFIA® measurement (Dissociation-Enhanced Lanthanide Fluorescent Immunoassay, PerkinElmer Life Sciences and Analytical Sciences - Wallac Oy, Turku, Finland). The lanthanide concentration defined was divided by the protein concentration to calculate the degree of labeling (lanthanide chelates/protein).

3.2.2 Reporter molecules

Biotinylated and labeled reporter molecules provided a straightforward, direct means for studying streptavidin-coated surfaces (I - IV). Biotinylation was first introduced to a part of the amines and subsequently a part of the remaining amines was used for the coupling of the chelates. Monoclonal antibody H117 (Lövgren *et al.*, 1995; Piironen *et al.*, 1998) was used (I, III) for the determination of the binding capacities for a large molecule (MW ≈ 160 000 D) whereas myoglobin (I) represented a smaller macromolecule (MW ≈ 17 000 D). The labeling degrees of the reporter molecules were adjusted to be quite low (approximately 1 - 2) to avoid too high signal intensities in the assays where abundantly bound reporters were measured.

The smallest reporter molecule, Eu-biotin, was synthesized from biotin and the seven-dentate Eu-chelate in one of the studies of this thesis (II). The product was purified using reverse phase high performance liquid chromatography (HPLC) and its molecular weight was assayed 1036 D by means of mass spectroscopy. Eu-biotin was intended to simulate the binding of intact biotin and report the total binding capacity of the surface without steric constraints. The degree of labeling for Eu-biotin was exactly 1.0.

3.3 Fluorescence measurement (I - IV)

The fluorescence detection technology utilized in this study was based on lanthanide chelate labels. Lanthanide chelate labels consist of lanthanide ions (inner transition metals) encompassed by strong chelators (ligands). Typically, trivalent europium (Eu) and terbium (Tb) ions are used, but samarium (Sm) and dysprosium (Dy) are also useful. While bare ions are poor absorbers, the ligand absorbs the excitation energy and transfers the energy from the excited S_1 state to the triplet state T_1 (intersystem crossing) and further through an intermolecular energy transfer to the bound lanthanide ion which then emits specific fluorescence. The lanthanide labels exhibit narrow emission peaks and large Stokes' shift (the excitation and the emission wavelengths are separated by hundreds of nanometers) which prevents the scattering of the excitation light into the measurement window. The main advantage, however, is the long-lasting fluorescence enabling time-resolved recording after the decay of prompt background fluorescence typically generated by biological material. The characteristics of lanthanide measurement are high sensitivity and wide dynamic range. For example, the dissociation-enhanced measurement (see below) of the Eu ions exhibits a detection capability of approximately 10^{-14} - 10^{-13} M concentrations and a linearity range of six decades. (Siitari *et al.*, 1983; Hemmilä *et al.*, 1984; Soini and Lövgren, 1987.)

Time-resolved fluorescence of the lanthanide labels was measured using Victor 1420 Multilabel counters (Wallac Oy). A 340 nm excitation filter was used and the emission filters were 615 nm and 545 nm for Eu and Tb, respectively. The Eu measurement used 1000 µs cycles (time between successive flashes) with 400 µs delay and 400 µs recording times. The respective values for the Tb measurement were 2000 µs (cycle), 500 µs (delay) and 1400 µs (recording window). Two principally different measuring methods were employed (Fig. 9).

In dissociation-enhanced measurement, the lanthanide ion is dissociated from a coupling chelate (a chelate where the ion is coupled to an other molecule, for example to a protein) into a solution by lowering the pH. In the solution, the ion is chelated by a second ligand structure (the enhancement chelate) forming a fluorescent complex. Solution measurement was performed using DELFIA Enhancement solution (Wallac Oy). This measurement method represented an integrated signal from the entire area of the captured reporter molecule and enabled the straightforward quantification of the bound molecules by measuring the appropriate lanthanide calibrators. Since the labeled reporter molecules were directly and abundantly bound to the surface, the instrumental

response had to be decreased by reducing the excitation intensity and replacing a smaller emission slot compared with regular Eu measurement.

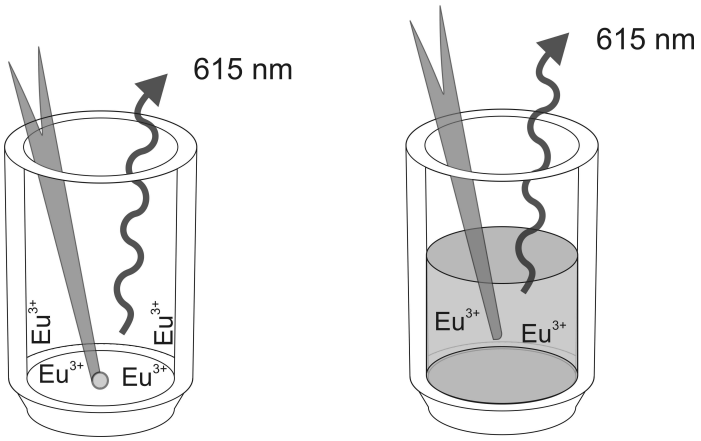

Figure 9. The principles of surface readout measurement (left) and solution measurement (right).

Development in lanthanide label technology has led to inherently fluorescent chelates (Takalo *et al*., 1994; von Lode *et al*., 2003) where the coupling and sensitizer functionalities are introduced in the same structure. These labels can be measured directly from a washed surface without an additional development step. This provided the basis for the other measurement method used in the studies, the surface readout measurement, where the excitation beam is focused on the bottom of the well and the response represents a fraction of the bound label. The signal reflects the density of the labeled molecule under the beam projection rather than the total capacity of the well. The wells were regularly dried under warm air to eliminate the excess moisture before being subjected to surface measurement. Of the chelate types used, the seven-dentate Eu-chelate (Eu ion coordinated by seven bonds in the chelate) was utilized both in the surface readout and dissociation-enhanced measurements, the nine-dentate Eu-chelate was used mainly in surface readout measurement and Tb-chelate in dissociation-enhanced measurement only.

3.4 Characterization of streptavidin-coated surfaces

3.4.1 Binding capacities (I - IV)

The biotinylated and labeled reporter molecules were prepared in the assay buffer (Innotrac buffer solution red, Innotrac Diagnostics Oy, Turku, Finland) and subsequently bound into the streptavidin-coated wells, typically by shaking for one hour at room temperature. After washing and drying, the plates were subjected to surface readout and solution measurement. The molar quantification of the immobilized reporter molecules was based on the determination of the lanthanide ion concentration in solution measurement against the respective lanthanide ion calibrators. The lanthanide concen-

tration measured was divided by the degree of labeling defined for the reporter molecule to calculate the quantity of the bound molecule.

3.4.2 Protein assay (II)

A sensitive Micro BCA protein assay was used to quantify adsorbed streptavidin. The assay is based on the reduction of the Cu^{2+} ion to Cu^{1+} ion by the protein. Consequently, the Cu^{1+} ion forms a complex with bicinchoninic acid (BCA) which exhibits strong absorbance at 562 nm (Smith *et al.*, 1985). Adsorbed streptavidin was quantified against streptavidin calibrators dried in the wells. The drying of the calibrators was intended to mimic the conditions of the adsorbed protein. The absorbance was read with the Victor device using a 545 nm filter which was closest to the specified optimal 562 nm wavelength. The sensitivity of the assay was, however, well preserved in the slightly deviating wavelength.

3.4.3 Desorption assay (III)

Desorption, or leaching, of adsorbed streptavidin from the surface was studied under regular assay conditions. Assay buffer (Innotrac Diagnostics Oy) was shaken in the streptavidin-coated wells after which an aliquot of the buffer was transferred to a biotinylated surface. The biotinylated surface consisted of a biotinylated IgM antibody coated into the 96-well plate. The plates were prepared and optimized in-house. Desorbed streptavidin, which bound to the Bio-IgM surface, was detected using Eu-biotin. The quantification was enabled by employing a series of defined streptavidin concentrations in parallel Bio-IgM-coated wells.

3.5 Immunoassays

3.5.1 Time-resolved immunofluorometric assays (I, III, IV)

Several immunoassays were used as models to study various aspects of the streptavidin-coated surfaces to immunoassay performance. The time-resolved immunofluorometric (TR-IFMA) assays consisted of separate immobilization of the biotinylated capture antibody to the streptavidin-coated surface and subsequent incubation of the antigen and the labeled tracer antibody.

The TR-IFMA of PSA (I) utilized biotinylated capture antibody H117 or alternatively a recombinant Fab fragment of it. The Fab fragment was cloned, produced and biotinylated site-specifically at the Department of Biotechnology (Eriksson *et al.*, 2000). The input quantities of the antibodies used in the assays were 400 ng/well of the intact antibody and 600 ng/well of the Fab fragment. The detection antibody was Eu-labeled 5F7 (Nurmikko *et al.*, 2000), which was used at 200 ng or 600 ng in the assay.

The hCG-assay (I) utilized a biotinylated antibody E27 as the immobilized capturing antibody (400 ng/well) and an Eu-labeled antibody 8D10 as a tracer (300 ng/well). The antibodies were from Wallac Oy.

The TSH-assays (III, IV) were carried out using various antibody combinations. One set consisted of biotinylated 5409 and Eu-labeled 5405 and the other comprised biotinylated 5404 and Eu-labeled 5409. The quantities of the capture antibodies used for immobilization varied from 100 ng to 600 ng in a regular assay and 32 - 160 ng in spot-assays. The Eu-labeled detection antibodies were used at 50 ng per reaction. All antibodies of the TSH-assays were from Medix Biochemica Oy (Kauniainen, Finland).

3.5.2 Enzyme immunoassays (III)

The enzyme immunoassay (EIA) kits were gifts from Fujirebio Diagnostics AB (formerly CanAg Diagnostics AB, Gothenburg, Sweden). The PSA assay was intended for a quantitative determination of total PSA. The assay comprised a simultaneous incubation of the biotinylated antibody, antigen and the horseradish peroxidase (HRP) conjugated tracer antibody in one mixture. The PSA-assay was a sandwich type assay in principle, but it deviated from the above-mentioned TR-IFMA assays which used separate incubation steps for the biotinylated capture antibodies.

The EIA-assay of the cancer associated antigen 125 (CA125) started with a simultaneous incubation of the biotinylated capture antibody (Ov197) and the antigen. The detection antibody (Ov185) was added after washing. The detection technologies of both EIA-assays were based on the HRP-conjugated tracer antibodies and the photometric measurement of the color formed upon the substrate conversion at 620 nm.

4 SUMMARY OF THE RESULTS

Detailed descriptions of the results are reported in the original publications. Here is a summary of the results accompanied with some previously unpublished data.

4.1 Characterization of streptavidin used (unpublished)

One batch of streptavidin from the supplier was subjected to N-terminal sequencing. The sequencing showed a main N-terminal sequence of Ala-Glu-Ala-Gly-Ile-Thr-Gly, which confirmed the preparation as core streptavidin. In the SDS-PAGE analysis the protein appeared as a pure single band at approximately 14 kD when temperatures of +80 - 100 °C were used for the preparation of the samples, which further verified the presence of core streptavidin.

4.2 Modeling and quantification of the adsorption (II)

4.2.1 Theoretical modeling of the adsorbed layer

The three-dimensional structure and the dimensions of tetrameric streptavidin were disclosed in diffraction studies in the late 1980s (Hendrickson *et al*., 1989; Weber *et al*., 1989). The dimensions of a single molecule are approximately 54 × 58 × 48 Å (5.4 × 5.8 × 4.8 nm). These values were employed to calculate the theoretical maximum monolayer coverage of adsorbed streptavidin considering two quadratic patterns (end-on and side-on orientations) and a globular adsorption pattern (Fig. 10).

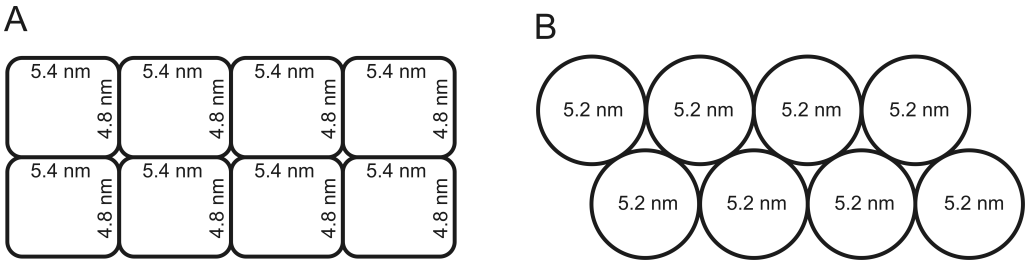

Figure 10. Two possible models for the organization of the streptavidin monolayers. A) A quadratic pattern with an end-on orientation yields a coverage of 6.41 pmol/cm^2 (380 ng). B) A globular pattern with an estimated molecular diameter of 5.2 nm yields a maximum monolayer coverage of 7.09 pmol/cm^2 (430 ng). The patterns were used for the modeling of the surfaces in original publication II.

Since the actual adsorption pattern is unknown, an average of the models conferring to the highest (globular) and the lowest (side-on with 5.4 × 5.8 nm side to the surface) adsorption capacities was employed. The calculated value, 6.20 pmol/cm^2 (370 ng) was subsequently used as an estimate of the maximal monolayer adsorption capacity for streptavidin. Consequently, the maximum capacity of a microtiter well coated with a typical 200 µl volume utilizing a binding area of 1.54 cm^2 equals to 9.55 pmol (570 ng).

4.2.2 Quantification of adsorbed streptavidin

Adsorbed protein was quantified using two independent methods; either by Tb-labeled streptavidin or by measuring adsorbed streptavidin using a sensitive protein assay. The Micro BCA assay is one of the most sensitive colorimetric protein assays providing sufficient sensitivity for measuring adsorbed streptavidin directly from the well. The results of the fluorescent assay and the protein assay were consistent with respect to maximum adsorption, the 350 ng/well with the Tb-labeled streptavidin and the 380 ng/well with the protein assay (Fig. 11 A). The quantities represented 61 - 67 % of the maximum monolayer coverage. The monitoring of adsorbed streptavidin using Eu-biotin (Fig. 11 B) complemented the direct measurement of adsorbed protein and gave estimates of the average biotin-binding sites available per adsorbed streptavidin molecule. Table 1 summarizes the calculated monolayer capacities and experimentally observed results.

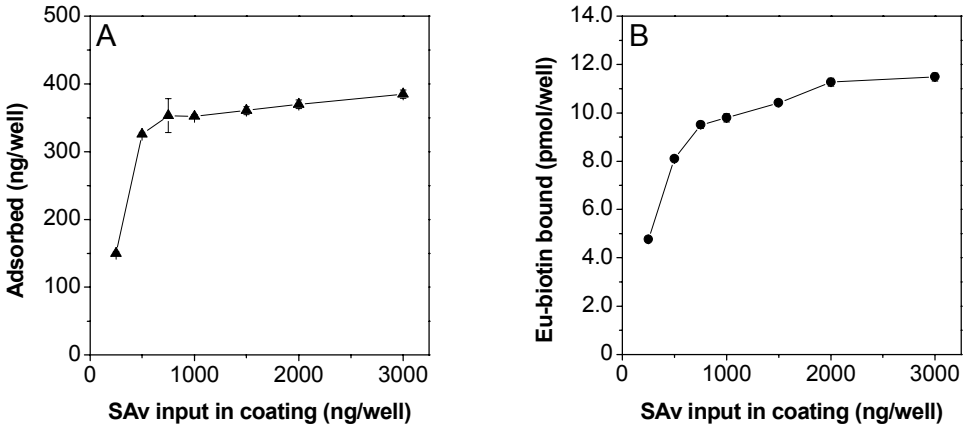

Figure 11. A) The quantification of streptavidin adsorbed in the microtiter well by means of a sensitive protein assay (Micro BCA). B) Monitoring of adsorbed streptavidin through the immobilization of Eu-biotin to the coated wells. The graphs are modified from original publication II.

Table 1. The theoretical monolayer adsorption capacities of streptavidin and the actual adsorbed quantities measured from the microtiter wells. Based on the data from original publication II.

	Per cm^2		In the well [a]	
	pmol	ng	pmol	ng
Maximum monolayer	6.2	370	9.6	570
Measured	4.1	250	6.3	380

[a] Well of C-geometry with a coated area of 1.54 cm^2

The adsorbed quantities of streptavidin observed in the microtiter wells using labeled streptavidin and the protein assay for the monitoring were consistent with the previous results of maximal quantities (250 ng/cm^2, 49 - 63 % coverage) observed using scanning probe microscopy and quartz crystal microbalance (Ihalainen and Peltonen, 2004).

4.3 Binding capacities (I - IV)

4.3.1 Steric effects

Size-dependent steric hindrance to the binding of the reporter molecules was clearly recognized when comparing the binding of the three reporter molecules representing different molecular sizes. For example, a regular streptavidin surface bound approximately 8 - 11 pmol of Eu-biotin, 2.8 pmol of biotinylated myoglobin and 2.2 pmol of biotinylated antibody. In addition, the masking effect was observed when the reporter molecules were immobilized to the streptavidin-coated surfaces of different binding capacities. For example, the ratios of the bound quantities between the GA-SAv-surfaces and the regular streptavidin surfaces were 1.6 with antibody, 1.8 with myoglobin and 2.4 with Eu-biotin (Fig. 12). Furthermore, the comparison of the streptavidin-coated plates from different manufacturers (III) showed the binding-capacity ratios of more than 30-fold with Eu-biotin but only 5.3-fold with the antibody between the plates of the highest and the lowest binding capacities. The results indicate that a larger proportion of the biotin-binding sites of the streptavidin-coated surface are utilized when immobilizing small molecules and the increased binding capacities and densities provided by the streptavidin layer are not completely benefited when immobilizing large molecules.

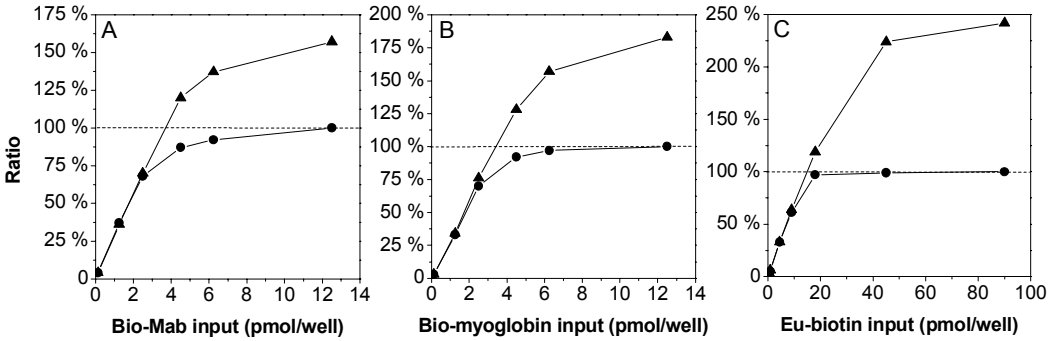

Figure 12. The relative binding capacities of a regular streptavidin-coated surface (●) in comparison with the high-capacity surface of polymerized streptavidin (GA-SAv) (▲) with respect to the size of the reporter molecule. A) Biotinylated antibody (MW = 160 000). B) Biotinylated myoglobin (MW = 17 000). C) Eu-biotin (MW = 1 036). Graphs A and B are modified from original publication I, graph C contains unpublished data.

4.3.2 Total capacities and binding site densities

The total binding capacities of the streptavidin-coated microtiter wells (the 96-well format) defined in the studies of this thesis ranged from 4.4 to more than 150 pmol/well for Eu-biotin and from 1.2 to 6.4 pmol/well for the biotinylated antibody. The total binding capacity of the well is dependent on the coated surface area, and it is not actually a good indicator of the performance of the coating chemistries used. Therefore, it was necessary to employ another parameter, the area-corrected specific capacity, or the binding density, to assess a universal value for convenient comparison of various coating chemistries and conditions (III, IV). The binding site density was calculated by dividing the defined total capacity (pmol) by the coated surface area (mm^2). The 96-well plates were coated using volumes of either 100 µl, 200 µl or 300 µl per well and the respective surface areas covered in the wells were approximately 90 mm^2, 150 mm^2 and 210 mm^2. The binding areas were based on the specifications by Esser (1997a) and experimental measurements of the filled wells. The calculated binding densities varied from 0.040 to more than 1.00 pmol/mm^2 for Eu-biotin and 0.011 to 0.061 pmol/mm^2 for the antibody.

The plates representing the highest binding capacities and densities were found among the commercial plates when comparing the features of the commercial and in-house 96-well plates (III). The coating chemistries used for the plates were determined as extensively as could be found in the scientific literature, patent files and manufacturer's technical notes. Some of these data are reviewed in section 1.4 of this thesis, though without indications to any particular plate.

4.3.3 Spot surfaces (IV)

Regular streptavidin (referred as SAv in the study, IV) and polymerized streptavidin (GA-SAv) were successfully coated in the droplet format yielding functional and evenly distributed spot surfaces. The binding capacities of the SAv-coated and GA-SAv-coated spots ranged from 0.5 to 5.5 pmol for Eu-biotin depending on the spot size and the coating type. The respective binding site densities of the spots were 0.076 - 0.47 pmol/mm^2 which were at least equal to those obtained with the ordinary method using larger volumes. The GA-SAv-surface further provided a close to 6-fold increase in binding site densities compared with the surface prepared from unmodified streptavidin (SAv).

4.4 Desorption (III, and unpublished)

The desorption (or leaching) of streptavidin from the streptavidin-coated wells was quantified by subjecting the surface to a mobile buffer solution followed by the transfer of an aliquot of the solution to a biotinylated surface (Bio-IgM) to serve the binding of desorbed streptavidin and detection using Eu-biotin. The dose-response curve of the streptavidin calibrators on the Bio-IgM surface appeared slightly sigmoidal in shape and the assay allowed the quantification of 0.1 - 80 ng streptavidin when bound to the Bio-IgM surface (Fig. 13). The concave shape of the curve in the low region evidently

originates from the proportionally higher occupation of streptavidin's four biotin-binding sites when using small streptavidin quantities. The biotin-binding sites are readily occupied by the biotin residues from the Bio-IgM surface while a smaller proportion remains detectable by Eu-biotin compared with the more crowded situation of the higher streptavidin quantities. The occupation of the biotin-binding sites was experimentally confirmed since the low-end lag region was significantly extended after increased amounts of the coated Bio-IgM. Therefore, the construction of the assay required careful optimization to compromise between the sensitivity and adequate dynamic range of the assay.

Desorbed streptavidin was calculated using an appropriate fitting of the sigmoidal curve or alternatively by dividing the graph into smaller fragments and applying quadratic fitting to the sections of the curve. The desorbed quantities from various streptavidin-coated microtiter wells (commercial and in-house plates) ranged from 0.5 to 76 ng/well. These represented approximately 0.003 - 3.4 % of the total biotin-binding sites of the wells. The washing of the streptavidin-coated wells before the desorption assay typically resulted in decreased leaching. Some surfaces exhibited rather low desorption even from unwashed wells proving their inherently stable characteristics.

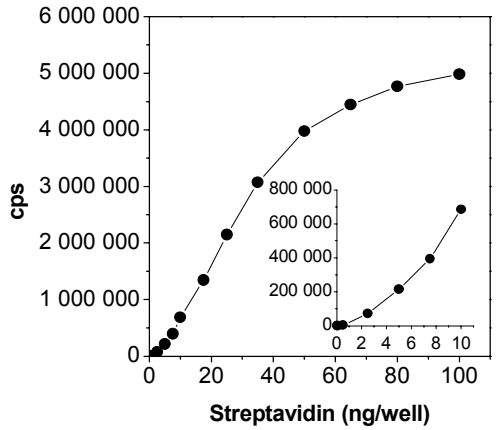

Figure 13. The calibration curve of streptavidin on the Bio-IgM surface, the assay that was used for the quantification of streptavidin desorbed from the streptavidin-coated wells. The inset graph shows the dose-response relation with low streptavidin quantities (0.5 - 10 ng). Unpublished figure, drawn according to data from original publication III.

A prerequisite of the desorption test used is that the dissociating units consist of at least subunit dimers of streptavidin. The test is not responsive for single subunits (unless the subunits are refolded in the solution). One of the two dimeric interfaces of streptavidin is weaker than the other, at least without bound biotin (see sections 1.2.1 and 1.2.3). In addition, the superficial protein layers are typically dissolved more readily than those which are in direct contact with the solid substrate (section 1.3.3). Therefore, it may indeed be true that the dissociating units are the subunit dimers. The data could not, however, distinguish between the dimeric and intact tetrameric structures.

4.5 Modifications (I, III, IV, and unpublished)

4.5.1 Polymerized streptavidin (I, and unpublished data)

Streptavidin was successfully polymerized (GA-SAv) using chemical treatment as indicated by size-exclusion chromatography analysis (Fig. 14). The polymerization degree could be adjusted depending on the conditions used. For example, the increased protein concentration resulted both in larger sizes of the polymer and a higher proportion of the polymerized protein. The largest conjugates represented molecular weights above 200 kD. Before the analysis and the fractionation of streptavidin and GA-SAv by size-exclusion chromatography, efforts were made to find appropriate conditions for the elution. The regular starting conditions frequently used for proteins, such as a buffer of pH 7.0 - 7.5 containing 150 mM NaCl, were found totally inappropriate for streptavidin. The elution profile appeared as a flat and broad peak and several column volumes of the solution were required to run the protein completely out from the column. The non-specific adhesion of the protein to the matrix was found to originate from hydrophobic forces rather than ionic interactions, since the removal of NaCl from the elution buffer and the raising the pH to 8.4 - 8.8 resulted in a more efficient resolution.

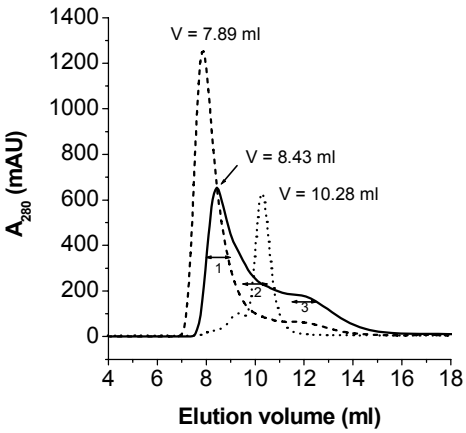

Figure 14. The elution profiles of polymerized streptavidin (GA-SAv) and BSA on a Superose 12 size-exclusion column. GA-SAv was produced using protein concentrations of 2 mg/ml (36 µM, solid line) and 4 mg/ml (72 µM, dashed line) in the polymerization reaction. BSA was run as a molecular weight control (dotted line). The three fractions used for the coating from the 2 mg/ml reaction are shown with bars. The figure is modified from original publication I.

The highest binding capacities of the coated surfaces were obtained when a non-fractionated mixture containing varying molecular sizes was used for the coating. Neither larger polymers nor any fraction alone from the separation exceeded the capacity of the surface made from the mixture when monitored using the reporter antibody and myoglobin.

In the time when the work with the polymerized streptavidin was made (I) the Eu-biotin reporter molecule was not available and the surfaces were monitored using protein reporters (antibody and myoglobin) which were conflicted by steric restrictions. Subsequent characterization of the GA-SAv-surface using Eu-biotin revealed that a higher input quantity of polymerized streptavidin was required in the coating in order to reach maximum binding capacity. The surfaces coated with polymerized strepta-

vidin showed close to a 10-fold increase in the signal of the surface readout measurement (Fig. 15 A) compared with the regular surface when assayed using Eu-biotin. The increase in the total binding capacity was close to 5-fold as determined by the solution measurement (Fig. 15 B). The total binding capacity of the GA-SAv-coated well for Eu-biotin yielded 40 pmol/well.

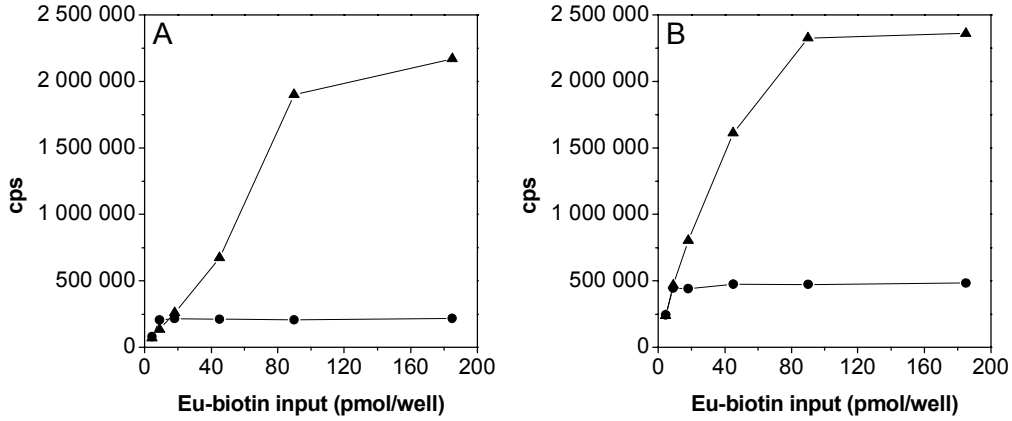

Figure 15. The binding capacity comparison of the streptavidin-coated surfaces produced from polymerized streptavidin (GA-SAv) (▲) and unmodified core streptavidin (●). Eu-biotin was used as the reporter molecule. A) Surface readout measurement. B) Solution measurement. Unpublished results.

A special feature observed with the surface readout and the solution measurements using each reporter molecule was the larger signal ratio of the surface readout measurement between the surfaces made from polymerized and regular streptavidin. This implied the higher density of binding sites on the bottom surface which is beneficial for applications using surface measurement. Another specific feature was the slight non-linearity in the binding isotherms of the high capacity surfaces (both in the regular wells and the spots) with surface measurement. Solution measurement, which integrated the signal from the whole area, showed equal binding and linearity within the sub-saturated region. The apparent deviation in binding behavior originated essentially from the uneven distribution of the bound reporter molecules outside the measurement beam in surface measurement. The liquid motion induced by shaking resulted in more efficient binding of the biotinylated reporter molecules outside the illuminated central region. This feature was clearly pronounced below the saturating reporter molecule quantities.

4.5.2 Thiolated streptavidin (III)

An alternative means to modify streptavidin for improved adsorption capability and better surface stability was tentatively introduced in one of the studies (III). Reactive thiols were introduced to the protein through amines. This contributed to a partial po-

lymerization of streptavidin and tight binding to the surface. An average of 10 - 12 thiols per streptavidin were repeatedly coupled based on the quantification by Ellman's reaction. The surfaces coated with this thiolated streptavidin yielded 2 - 3-fold higher binding capacities for Eu-biotin compared with the surfaces made from unmodified streptavidin. The leaching of adsorbed streptavidin from this surface was substantially reduced in comparison with several other high-capacity surfaces and the regular streptavidin surface. The preparation and optimization of this modified type will be discussed more specifically elsewhere (Ylikotila *et al*., manuscript in preparation).

4.6 Immunoassays (I, III, IV)

4.6.1 Linearity and the dose-response relationship

The high capturing capacity of the surface obviously increased the high-end dynamic range of the TR-IFMA assays and avoided or postponed the high-dose hook effect towards the higher concentrations. This was most clearly observed when using a combination of the high-capacity streptavidin surface and the site-specifically biotinylated Fab fragments (I). In the low-end region of the dose-response curves the higher total binding capacity did not guarantee higher signal levels.

4.6.2 Characteristics of various assay types

Though the TR-IFMA- and the EIA-assays were in principle similar sandwich-type assays they differed in the reagent incubation sequences. The TR-IFMA-assays employed a separate immobilization of the biotinylated capture antibody prior to the actual assay. The differences between the plates possessing various capacity and desorption features were actually quite small in the linear region of the assay (Fig. 16 A). The IFMA-assay, with separate immobilization of the capture antibody, represented a relatively reliable reaction setup in terms of the equilibrium reactions taking place in the well. Furthermore, the preceding immobilization and washing steps evidently eliminated some unwanted surface characteristics related to leaching and variation.

The EIA-assays (III) using a one-step incubation of the capture antibody and the antigen (CA125-assay) or a one-step incubation of all binding partners (PSA-assay) represented more demanding systems with more complicated equilibrium reactions. More substantial differences were observed between the streptavidin-coated surfaces in the linear assay range when using the EIA-assays (Fig. 16 B). This is most probably explained by the different reaction schemes compared with the IFMA-assays and the absence of the prior immobilization and washing steps which would have relieved some of the surface defects. The features of the surfaces observed in the EIA-assays could be to some extent, but not definitely, related to the leaching characteristics of the plates.

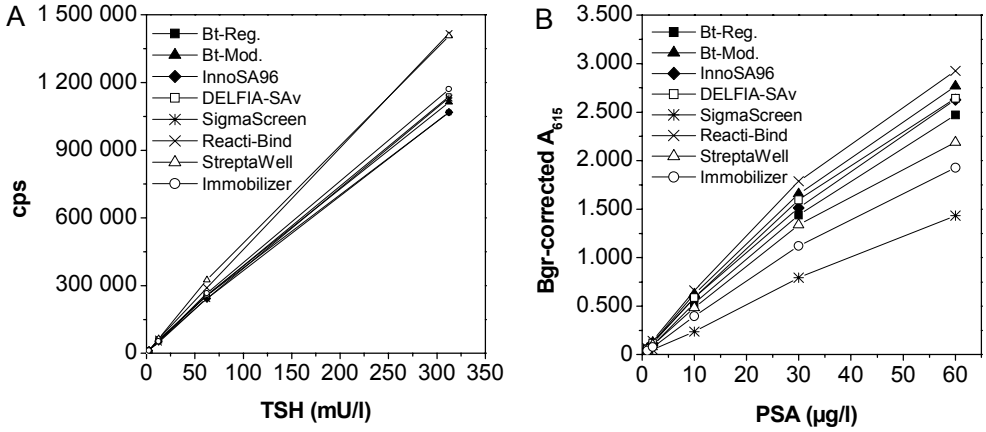

Figure 16. The features of various streptavidin-coated surfaces in the immunoassays. A) An IFMA-type assay of TSH utilizes a separate immobilization of the biotinylated capture antibody prior to the actual assay. B) In the EIA-assay of PSA all reagents are simultaneously mixed in the well. More substantial differences are recognized between the surfaces in the EIA-assay. Modified from original publication III.

4.6.3 Non-specific binding and the sensitivity

The background responses (absorbance or fluorescence) measured from the control reactions without an analyte consist of both the material background and the non-specific binding of the labeled reagents. Various inorganic materials exhibit different absorption and autofluorescence features. This is evident even within one material type, for example, polystyrene may have variable fluorescence properties depending on the source and treatment. Therefore, a plain comparison of the total background signals from different surfaces is only partially indicative of the degree of non-specific binding to the surface. Preferably, a number of unused wells should be measured for reference in order to distinguish the background signal of the material from the background caused by the non-specific binding. However, it is the total background response that is routinely employed to determine the specific net responses from the calibrators and the samples and, therefore, the distinction between non-specific binding and material background is typically omitted. This was also practiced in this work (I, III, IV).

Various streptavidin-coated surfaces used in this work showed different background responses. The surfaces with the higher binding capacities tended to exhibit higher background levels, but the differences were no more than approximately 2-fold at the highest in comparison with the regular streptavidin surfaces. The analytical sensitivities of the assays (the term sensitivity generally referring to the detection limits in this work), when defined by the method based on the standard deviation of the replicate background readings, are especially vulnerable to variation in background responses. The analytical detection limits of the assays were affected by the streptavidin-coated

surfaces; the effect was mainly related to the magnitude and variation of the background readings.

4.6.4 Kinetics (I, IV)

The principal definer of the kinetics of an assay is the ratio of the binding surface area to the liquid volume of the reaction (Esser, 1992). The regular microtiter well presented relatively beneficial surface-to-volume ratios so that the higher binding capacity provided by the streptavidin surface did not remarkably improve the assay kinetics, particularly when using intact antibodies for the capture of the antigen. The spot surfaces introduced new challenges, with highly reduced surface-to-volume ratios. This was also well recognized in assay kinetics. The decreased surface area was compensated for by increasing the binding capacity of the streptavidin-coated surface and consequently increasing the immobilization capacity of the biotinylated capturing antibody. The benefit remained marginal when using intact antibodies. A more pronounced effect has been observed by using combinations of high capacity streptavidin surfaces with site-specifically biotinylated fragments of antibodies which evidently exhibit the correct orientation on the surface (Ylikotila *et al.*, 2005).

4.7 Spot-assay (IV)

The assays based on the surface readout measurement of time-resolved fluorescence from a washed surface are dependent on the density of the label on a limited surface area defined by the excitation beam. The principle of the spot-assay was to condense the binding of the labeled antibodies into a dense layer under the measuring beam (Fig. 17). The regular binding surfaces manufactured using large volumes of the coating solution have been non-optimal in terms of signal collection due to the spread of labeled detection antibodies outside the measuring beam. In the TR-IFMA-assay of TSH, condensed binding provided a 5 - 6-fold increase in the signal-to-background ratios and an equivalent improvement in the detection limits of the assay (Fig. 18). The positive gain in the signal-to-background ratio was essentially due to the increased response from the actual reactions containing the analyte while the background signals beneficially remained equal to the background levels of the reference assays employing conventionally coated surfaces.

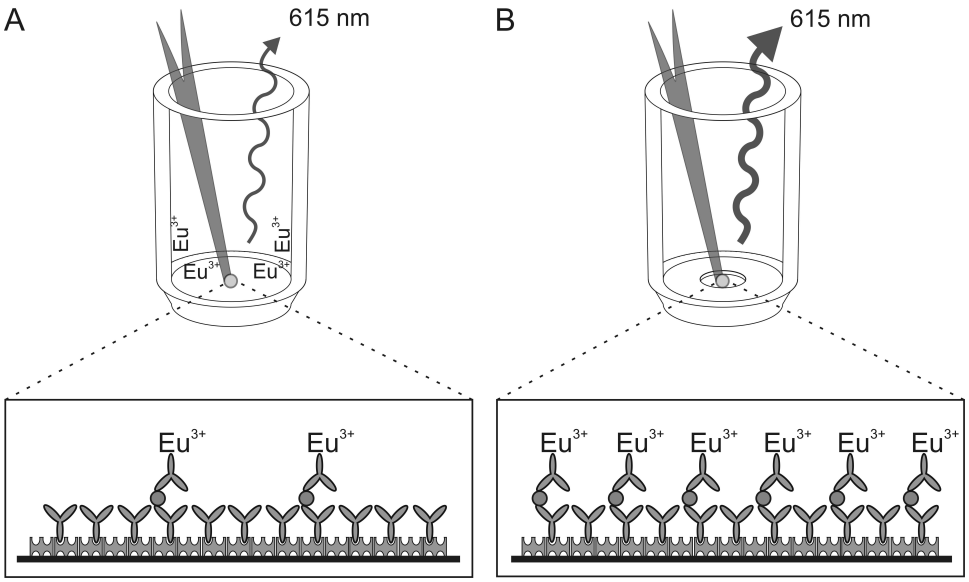

Figure 17. A) In the regular assay the binding surface area is large and, consequently, a significant proportion of the labeled antibody is bound outside the measured area. B) In the spot-assay the binding area is confined to a small spot and the labeled antibodies are captured more densely under the excitation beam. Modified from original publication IV.

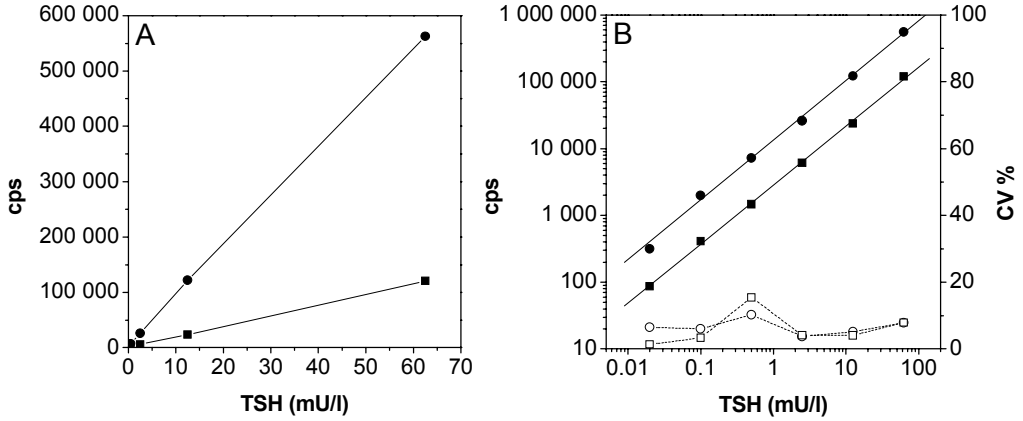

Figure 18. The dose-response curves of the TSH-assay using the reference assay employing a large binding area (■) and the spot-assay utilizing condensed binding (●). Axes in the linear (A) and logarithmic (B) scales. The variation (CV %) of the replicate wells in the reference assay (□) and the spot-assay (○) is shown in graph B. The results are from original publication IV.

5 DISCUSSION

There is ongoing active research and development accompanied by extensive literature in the field of the protein coatings. However, most research has reported the properties and performance of surfaces narrowly. Various surface coating methods have been used and reported in a number of studies but when the binding surface itself is not an issue, further evaluation and discussion on the essential features of the surface are omitted. Thereby, it is problematic, or almost impossible, to extract information about the suitability of a given method to the specified needs of other applications. The literature review section of this thesis presented the use of streptavidin as a binding surface in solid-phase assays as well as introduced various advanced coating chemistries reported in the literature. The experimental part examined the coating and modifications of streptavidin and studied the effects of the specific properties of streptavidin surfaces to the performance of solid-phase immunoassays.

The modeling of the surface and the quantification of adsorbed streptavidin were carried out to obtain an estimate of the surface coverage that can be reached by passive adsorption of unmodified streptavidin. One theoretical surface modeling approach, the random sequential adsorption (RSA) (Feder, 1980; Brosilow *et al.*, 1991; Evans, 1993; Loscar *et al.*, 2003) defines the jamming limits for the maximum adsorption of molecules into a monolayer. When the densest, well-ordered packing of a particular shape into a monolayer is represented as 100 %, the jamming limits defined by the RSA-model, 54.7 % for spheres and 56.2 % for squares, represent the limits beyond which adsorption into a single layer is no more possible in a random process. Streptavidin from one supplier yielded a 61 - 67 % coverage (II) which was fairly consistent with the RSA model. Streptavidin from the other supplier showed 93 % coverage which implies that the protein is adsorbed in multiple layers or it is organized in an ordered manner. The jamming limit for streptavidin, based on this 54.7 - 56.2 % occupancy and the defined maximum monolayer density of 6.2 pmol/cm^2, should be equal to approximately 3.5 pmol/cm^2 ($\approx$ 200 ng), which corresponds to about 5.4 pmol (320 ng) in a microtiter well with a coated area of 1.54 cm^2. Consequently, assuming that two of the four binding sites of adsorbed streptavidin are available for the binding of biotin, the maximum biotin-binding capacity of the well attainable by plain adsorption should be equal to approximately 11 pmol. Interestingly, the maximum capacity for the binding of Eu-biotin was assayed approximately 10 - 11 pmol/well with the surface prepared by adsorption of unmodified core streptavidin (II).

The biotinylated, Eu-labeled reporter molecules provided a direct means to monitor adsorption, determine binding capacities and even to study the localization of the molecules by imaging (IV). The determination of the total binding capacity was based on the quantification of the bound Eu concentration in the saturated region of the binding isotherms against the Eu-calibrators and by proportioning it to the defined labeling degree of the reporter molecule. Due to the high-affinity interaction (the biotinylated molecule binding to streptavidin, where practically 100 % is bound in the linear re-

gion), a graphical determination from the turning points of the binding isotherms would also have been feasible. This method should be, however, considered with caution especially among low affinity interactions, since the turning point of the binding isotherm indicates input concentration where the maximum binding is reached rather than the actual bound quantity. It is also vulnerable to fluctuations in input quantities of the reporter molecule. The Eu concentration based method is dependent on the correct determination of the reporter molecule's degree of labeling. This can be facilitated by a fixed chemical structure as employed in Eu-biotin which provided only one site for the coupling of the chelate. When the degree of labeling is accurately defined, this is, however, a reliable and reproducible method of capacity determination.

The polymerization of streptavidin resulted in an up to 5-fold increase in biotin-binding capacity compared with surfaces of unmodified streptavidin when integrated from the entire coated area. The local binding site densities at the bottom surface of the wells were further increased as shown by the surface readout measurements. The fractionation and the gel filtration analysis of the polymerized streptavidin (GA-SAv) revealed a considerable degree of polymerization which could be adjusted by changing the reaction conditions. Further polymerization beyond a certain level did not present increased capacities when coated on the surface, but rather a mixture of monomers and polymers of various sizes proved most efficient. Alternative thiol-based modification also resulted in increased binding capacities, but above all, the stability of the surface against leaching was improved. The studies with modified streptavidin preparations showed that relatively straightforward, cost-effective methods provide substantial improvement in the binding capacities and surface densities which can be applied first before proceeding to more complicated techniques.

The higher immobilization and the capturing capacities were helpful in avoiding or postponing the high-dose hook effect of the immunoassays. This was clearly observed when using a combination of the high-capacity streptavidin surface with the Fab fragments for the capture of the antigen. The model assays employed in the tests showed the linearity advantage mainly beyond regions of clinical relevance, but nevertheless, they showed the importance of the capturing capacity to guarantee the linearity of the assay, implying favorable effects on assays that are indeed susceptible to the high-dose hook effect. The binding capacity was not similarly relevant to assay sensitivity (detection limits) and low-end performance. In a reagent-excess assay, only a small fraction of the binding sites are occupied by the antigen in the dilute region and the streptavidin surface of a moderate binding capacity binds a sufficient amount of antibodies to serve a satisfactory capture of the antigen, at least in the 96-well plate format. In the low end and the linear regions of the assays other determinants such as leaching, variation, surface homogeneity and non-specific binding were pronounced. The initially defined properties of the surfaces such as desorption and the homogeneity of the binding capacities were not, by definition, predictive of the surface's performance in the immunoassays. Some relation could be observed between the desorption degree and the responses from the EIA-type assays which utilized simultaneous binding of the bioti-

nylated capture antibody and the antigen. Variation of the replicate wells tended to increase in the plates exhibiting high desorption, most likely due to carryover by the washing device, and generally lower responses were measured because of the considerable leaching of the captured immunocomplexes.

Desorption of the adsorbed protein and its correlation with immunoassay performance has occasionally been an issue of speculation. Adsorption of the proteins, the technique employed for the preparation of the most surfaces, is generally considered an irreversible process, though some replacement has been regularly observed especially in the presence of replacing proteins (see section 1.3.3.1). The immunoassay buffer utilized in the desorption studies contained proteins, but at the same time it contained detergents which efficiently prevent adsorption and, therefore, the replacement was not obvious. The desorption observed can be considered a new process, independent of the preceding adsorption, where the rinsing and shearing forces introduced by the mobile liquid played a major role. The assays using a preceding immobilization and washing procedure did not seem particularly vulnerable to desorption. Weakly bound streptavidin was dissociated from the surface during the immobilization step and the remaining capture layer showed stable features. The assays that avoided the preceding immobilization step evidently suffered more from the instable surfaces, as shown by the EIA-type assays. An interesting observation made during this research (but which was not comprehensively studied) was the increased desorption of adsorbed streptavidin during storage. Freshly prepared surfaces clearly exhibited lower quantities of desorbed protein than those stored for months or years at +4 °C. This obstructed their use as reference wells in desorption studies. Increased desorption is likely indicative of the rearrangement and lateral diffusion processes taking place among adsorbed proteins, as reviewed for example by Dent and Aslam (1998).

The recombinant Fab fragments of the antibodies presented superior antigen-binding performance in comparison with intact, whole-size antibodies. The higher capturing capacity of the Fab surfaces is brought about by two reasons; first the smaller size of the fragments and thereby the higher density of the antigen binding sites on the surface and second, evidently correct orientation of the fragments due to site-specific biotinylation. The specific share of one or the other to binding performance was not further studied. It seems that the combinations of tailored antibody fragments with highly dense streptavidin surfaces are proving a promising binding surface option in the micro- and nano-scale assay concepts where rapid results from small sample quantities are expected.

The immunoassays based on the surface readout measurement of intrinsically fluorescent lanthanide labels from a washed surface have suffered from the distribution of the immunocomplexes and the labeled detection antibodies outside the measuring beam. The purpose of the spot approach was to improve the sensitivity of these assays through condensed collection of the labeled antibodies to the surface and consequent increase in the signal-to-background ratios. Unmodified native streptavidin (SAv) and

polymerized high-capacity streptavidin (GA-SAv) were successfully coated to the special spotted microtiter wells to serve the binding of the capture antibodies. The significantly higher biotin-binding capacities and the binding site densities obtained with the GA-SAv-coated spots were not completely beneficial in the model immunoassay of TSH. This may be partially due to the uneven distribution recognized in the binding which was more pronounced on the GA-SA-surface. Though incomplete in the sense of the measuring parameters, the increased concentration and density of the labeled antibodies under the measuring beam resulted in a significant increase in the signal-to-background ratios and improvement in the analytical detection limits. Interestingly, the central property of the microspot-based ambient analyte assay (Ekins *et al.*, 1989; Ekins and Chu, 1991; Ekins, 1998), that is to say the increased label density in the measured area conferring to a more sensitive assay, was also recognized in the spot-assay of this work.

The research methods used in the studies of this thesis provided information on surface binding capacities, binding site densities, surface stability and the effects of the binding surface on the solid-phase immunoassay. They provided little information on the other relevant surface features such as topography, layer thickness, orientation and homogeneity (except the imaging, IV) and thereby these determinants remained mostly speculative. This raises interest to proceed to the next level, to relate the contribution of the specific surface properties defined by various surface science studies to the features of solid-phase assays. This approach would, for example, disclose the actual organization of variously coated streptavidin surfaces as well as the orientation and the densities of the capture antibodies and the Fab fragments on the surface. Several potential surface research methods and technologies exist which would provide further insights into the capturing surfaces. Scanning probe microscopy (SPM) is a common designation of surface imaging techniques that are based on the scanning of the specimen by a probe. Among these, atomic force microscopy (AFM) and scanning tunneling microscopy (STM) have been used for studying biomolecular layers (Leggett *et al.*, 1996). In AFM, the probe is a sharp tip which moves over the surface and sensitively responds to the forces encountered at the interface, thus creating a three-dimensional image of the surface topography even in a sub-nanometer resolution (Davies *et al*., 1994b; Moll *et al*., 2002; Browne *et al.*, 2004; Kim J. *et al.*, 2004). In addition, AFM has been used to quantify the interactive forces between molecules on the interface, for example, between streptavidin and biotin using a biotin-coated probe (Allen *et al*., 1996; Wong *et al*., 1999). Scanning tunneling microscopy (STM) is based on the current of electrodes (tunneling) through a small gap between an atomically sharp conducting probe and a conducting surface. While based on the electronic current, the support must be conducting or the surface must be overlaid with a metal layer. STM has been used to visualize adsorbed streptavidin (Cooper *et al.*, 1994) and antigen-antibody interactions on the surface (Davies *et al*., 1994a; 1994b). Scanning electron microscopy (SEM) has also been used for studying the protein-coated solid phases (Butler *et al*., 1992; Assis, 2003).

Among the optical measuring methods, ellipsometry is an efficient method to assay layer thicknesses ranging from less than a nanometer to several micrometers. When polarized light is directed to, and reflected from a sample surface, its polarization state changes as a function of the surface thickness and the refractive index. As a quite well-established and versatile method, ellipsometry and variations of it are widely used for studying biomolecular surfaces (Malmsten, 1995; Spaeth *et al.*, 1997; Elwing, 1998; Tengvall *et al.*, 1998; Bae *et al*., 2005). Another well established optical technique, surface plasmon resonance (SPR) has definitely gained popularity in studying protein layers and biomolecular interactions (Hodneland *et al.*, 2002; Cui *et al.*, 2003; Homola, 2003; Snopok and Kostyukevich, 2006). Of the reflection based methods, neutron reflection has recently been shown to be a feasible technique for studying protein surfaces (Su *et al.*, 1998; Xu *et al*., 2006; Lu *et al.*, 2007; Xu *et al*., 2007). Spectroscopic methods such as X-ray photoelectron spectroscopy (XPS) (Ruiz-Taylor *et al*., 2001a; Pradier *et al*., 2002b; Browne *et al*., 2004) and infrared reflection-absorption spectroscopy (IRRAS) (Mendelsohn and Flach, 2002; Pradier *et al*., 2002a; Liu and Amiridis, 2004; 2005) provide insights essentially into the chemical constitution of adsorbed layers. Quartz crystal microbalance (QCM) is a technique based on a piezoelectric quartz crystal the oscillation frequency of which changes upon a mass deposited on the crystal. The change in frequency can be quantitatively related to the applied mass and thus QCM serves as a sensitive method for quantifying adsorbed material (Rickert *et al.*, 1997; Linder *et al*., 2002; Kim J. *et al*., 2004; Zhang *et al*., 2005; Shu *et al.*, 2007).

Most of the surface research techniques referred to require specific solid substrates or sample cells, such as conductive or reflective beds, and thus are not readily applicable to all desired settings, such as to polystyrene microtiter wells or array slides. They can serve, above all, as tools for studying the organization of surfaces and molecular interactions in environments which mimic the actual molecular level situation, as shown, for example, by using a polystyrene-coated SPR-chip for the study of adsorption and binding of streptavidin and antibodies (Davies *et al*., 1994b). Direct measurement from the microtiter wells is not, however, completely out of the question. Scanning electron microscopy images have been acquired from intact microtiter wells (Butler *et al*., 1992) and other, perhaps the most closely related setup, utilized coated wells from which the walls were excised and the bottom discs were subjected to AFM and STM analyses (Davies *et al*., 1994a; 1994b; Allen *et al*., 1996).

6 CONCLUSIONS

The immobilization of proteins using streptavidin-biotin technology constitutes an advantage compared with direct adsorption in terms of preserving molecular activity and natural conformation. The binding surface is a significant determinant of solid-phase assays and therefore the specific features of streptavidin-coated surfaces relevant to solid-phase immunoassays were subjected to a comprehensive evaluation in this thesis. The work was accompanied by the establishment of a number of surface characterization tools and methods. The binding site densities of the streptavidin-coated microtiter wells were improved by close to 10-fold and the leaching of the adsorbed protein was decreased using chemically modified streptavidin. Furthermore, the sensitivity and the detection limits of the immunoassays based on the surface readout measurement of time-resolved fluorescence were improved through the condensed binding of the labeled antibodies enabled by the spotted streptavidin surfaces. The properties of the streptavidin surfaces significant to immunoassay performance were found to be the surface stability, the degree of non-specific binding and homogeneity. The increased binding capacity enabled wider dynamic ranges of the assays, while it was less relevant to assay sensitivity and low-end performance at least in the regular microtiter well format. The driving forces of several surface studies are, however, the high binding capacity and coating densities which are admittedly relevant issues in certain technologies.

Some central characteristics of the future assay concepts are high sensitivity and multiplex assays as well as rapidity combined with simplicity to serve the instant point-of-care assays (some of the trends recently reviewed by Wu, 2006). Nanotechnologies such as nanoparticles and array-based approaches have an important role when working on these objectives. In addition, the development in label technologies has opened up new opportunities for separation-free homogeneous assays (Kokko *et al.*, 2004; Hemmilä and Laitala, 2005; Kuningas *et al.*, 2005), especially in high-throughput screening where assay simplicity is valued, and gradually in clinical diagnostics as the sensitivities of homogeneous assays reach those of heterogeneous assays. The interactions between proteins and solid phases will still prevail, even in several homogeneous assays, where the biomolecular interactions after all take place at a solid-liquid interface such as on the nanoparticle surface. With respect to the array-type assays using spotted surfaces, Ekins and others have concluded that microarray assays should obey the rules of the ambient analyte assay (Ekins, 1999; Saviranta *et al.*, 2004), an assay format that demands highly dense and homogeneous surfaces. Given these facts, there is no doubt that the features of the binding surface will have a dominant and even increasing influence on assay performance in the new forthcoming assay concepts.

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