TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. A I OSA - TOM. 391

ASTRONOMICA - CHEMICA - PHYSICA - MATHEMATICA

HIGHLY FUNCTIONAL BINDING SURFACE FOR MINIATURISED SOLID-PHASE IMMUNOASSAYS – A SPOT STORY –

by

Johanna Ylikotila

TURUN YLIOPISTO
Turku 2009

From the Department of Biochemistry and Food Chemistry
University of Turku
Turku, Finland

Supervised by

Professor Kim Pettersson, Ph.D.
Department of Biotechnology
University of Turku
Turku, Finland

and

Professor Timo Lövgren, Ph.D.
Department of Biotechnology
University of Turku
Turku, Finland

Reviewed by

Professor Matti Karp, Ph.D.
Department of Chemistry and Bioengineering
Tampere University of Technology
Tampere, Finland

and

Professor Hans Söderlund, Ph.D.
Department of Biotechnology
VTT, Technical Research Centre of Finland
Helsinki, Finland

Opponent

Docent Markku Viander, M.D., Ph.D.
Department of Medical Microbiology and Immunology
University of Turku
Turku, Finland

ISBN 978-951-29-3830-8 (PRINT)
ISBN 978-951-29-3831-5 (PDF)
ISSN 0082-7002
Painosalama Oy – Turku, Finland 2009

To my family

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their roman numerals (**I-IV**).

I Johanna Ylikotila, Lasse Välimaa, Markus Vehniäinen, Harri Takalo, Timo Lövgren, and Kim Pettersson (2005) A sensitive TSH assay in spot-coated microwells utilizing recombinant antibody fragments. *J Immunol Methods* **306**: 104-114.

II Johanna Ylikotila, Johanna L. Hellström, Susann Eriksson, Markus Vehniäinen, Lasse Välimaa, Harri Takalo, Anastasia Bereznikova, and Kim Pettersson (2006) Utilization of recombinant Fab fragments in a cTnI immunoassay conducted in spot wells. *Clin Biochem* **39**: 843-850.

III Johanna Ylikotila, Lasse Välimaa, Harri Takalo, and Kim Pettersson (2008) Improved surface stability and biotin binding properties of streptavidin coating on polystyrene. *Colloids Surf. B Biointerfaces*. Published online January 9, 2009. doi: 10.1016/j.colsurfb.2008.12.042.

IV Johanna Ylikotila, Lasse Välimaa, Harri Takalo, Kim Pettersson (2008) Method for preparing high capacity solid phase materials. *Patent application*

In addition, some unpublished data are included.

ABSTRACT

Several bioaffinity assays are based on the detection of an analyte which is bound on a solid substrate via biochemical interaction. These so called solid-phase assays are based on the adhesion of the primary binding partner on a solid surface, which then binds the analyte to be detected. In this thesis work a novel solid phase based assay technology, known as spot technology, was developed. The spot technology is based on combination of high-capacity solid phases, concentrated in a spot format, utilising modified streptavidin molecules and recombinant antibody fragments. The reduction of the solid phase binding surface to a size of a spot enabled denser binding of the target molecules, providing improved signal intensities and signal-to-background ratio when applied in different solid-phase immunoassays.

Streptavidin-biotin interactions are commonly utilised in numerous different bio-affinity assays and the ultimate nature of streptavidin to bind biotin is among the strongest non-covalent interaction reported between two biomolecules. In this study native core streptavidin was chemically modified to provide polymerised streptavidin molecules with altered adsorption properties. These streptavidin conjugates, when coated onto polystyrene surface, provided enhanced biotin binding capacity and surface stability when compared to a reference coating constructed with native streptavidin. Furthermore, the combination of chemically modified streptavidin, site-specifically biotinylated antibody fragments and the spot coating technology provided highly dense solid phase coating with improved binding properties.

The performance of the spot assay technology was further demonstrated in different immunoassay configurations. Human thyroid stimulating hormone (TSH) and human cardiac troponin I (cTnI) were used as model analytes to show the applicability of the highly sensitive spot-based solid-phase immunoassay for detection of very low levels of analytes. It was demonstrated that the spot technology provided an assay concept with enhanced sensitivity and short turn-around times, characteristics that are highly suitable for point-of-care applications.

ABBREVIATIONS

ALP	alkaline phosphatase
Bio-	refers to biotinylated molecule
BSA	bovine serum albumin
cTnI	cardiac troponin I
DELFIA®	dissociation-enhanced fluoro immuno-assay
DL	detection limit
DTT	dithiotreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Eu	europium
Fab / Fab'	fragment antigen binding
$F(ab')_2$	fragment of an antibody consisting two Fab fragments which are linked to each other with disulphide bridges at the hinge region
Fc	fragment crystallisable region
Fd	heavy chain portion of a Fab fragment
GA-Sav	glutaraldehyde modified streptavidin
IEF	isoelectric focusing
IgG	immunoglobulin G
IgM	immunoglobulin M
K	activity constant of a binding reaction
Mab	monoclonal antibody
PAMAM	polyamidoamine
pI	isoelectric point
PSA	prostate specific antigen
UV	ultraviolet
SAM	self-assembled monolayer
SATA	N-succinimidyl S-acetylthioacetate
Sav	streptavidin
scFv	single chain variable fragments of an antibody
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH-Sav	refers to SATA modified streptavidin or thiolated streptavidin
SPI	solid-phase immunoassay
SPR	surface plasmon resonance
TCEP	tris(2-carboxyethyl)phosphine
TSH, hTSH	(human) thyroid-stimulating hormone

1 REVIEW OF THE LITERATURE

1.1 Introduction

Immunoassay techniques utilising synthetic solid phases cover the majority of today's modern assay designs. These solid-phase immunoassays (SPIs) are based on the binding of an analyte to a solid interface, thus providing a simple method for separating the reactive and non-reactive analyte in a sample solution (Butler JE, 2000). Immunoassays on solid phases have become routine techniques in a variety of areas in the medical science, including diagnostics, immunology and microbiology. In clinical diagnostics these assays are widely applied to monitor the concentration of different biochemical markers with altered expression in various pathophysiological conditions.

Immunoassays can be divided into two main categories based on how the differentiation between the reacted and non-reacted component is accomplished. In heterogeneous immunoassays the bound analyte is separated from the unbound counterpart by an additional washing and separation step while in homogeneous assay designs the immunoreaction occurs entirely in one interface, without a separation (Davies C, 2001, Hemmilä I, 1985). Most often the separation of bound and unbound component is accomplished through an interface by immobilising either one of the immunoreactant onto a solid surface. Thus, in some extent both heterogeneous immunoassays, like ELISA in microtitration well, and homogeneous immunoassays, like those utilising nanoparticles as solid phase (Kokko L*, et al.*, 2004), can be considered as solid-phase assays. The homogeneous and heterogeneous assay types can be further characterised as competitive (reagent limited) or non-competitive (reagent excess) assays (Davies C, 2001) (Figure 1). In competitive immunoassay the unlabelled analyte is competed with its labelled counterpart for the binding to the capture surface. The non-competitive assay utilises two antibodies, one bound to the solid phase and one carrying a detectable label, both binding to the analyte of interest. In principle, non-competitive systems are capable of yielding assay sensitivities order of magnitude greater than competitive assay designs (Lövgren T*, et al.*, 1990).

The solid phase properties play a crucial role in both heterogeneous and homogeneous assay designs, conducted either in competitive or non-competitive manner. The SPIs on polystyrene have become the "assay of choice" over 30 years ago and already at that time (partially driven by straightforward adsorption of binding molecules) it was evidenced that passive adsorption of capture antibodies and other proteins on a solid surface results in molecular alteration, such as change in the three dimensional structure (Bull HB, 1956, Kochwa S*, et al.*, 1967, Oreskes I*, et al.*, 1961). In solid-phase assays, the chemical and physical properties of the solid phase itself as a binding surface highly determine the assay functionality.

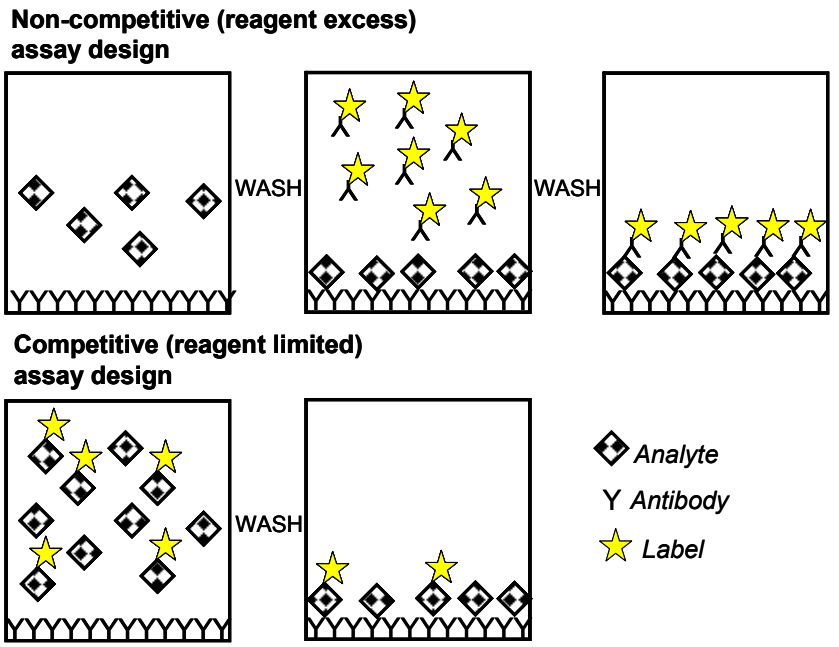


Figure 1. The basic principles of non-competitive (reagent excess) and competitive (reagent limited) heterogeneous immunoassays. In non-competitive immunoassays the fractional amount of antibodies that are occupied by the analyte is measured, thus reflecting directly the amount of analyte present in the sample. In competitive immunoassays the detectable signal is generated through those antibodies that are not occupied by the analyte, and thus the measured signals reflect indirectly the amount of analyte present in the sample.

This review of the literature will concentrate on the physical and chemical properties of solid phase materials (particularly polystyrene) and discuss how these characteristics affect on the solid-phase immunoassay performance. Furthermore, different immobilisation methods to obtain a functional and well-oriented solid phase binding surface will be reviewed.

1.2 Solid-phase immunoassay design

The properties of the binding surface lay the initial foundation in solid-phase assay designs and heavily influence the results relating to the analytical sensitivity of the particular method. Thus, an important step in the solid-phase immunoassay development is the selection of an appropriate solid phase material and the choice of the coating method that is to be applied to immobilise the capture agents to provide a fully functional capture layer. A multitude of different solid phase materials have been developed and utilised in numerous different applications. The quantitation of different biochemical markers by SPIs have been reported to be performed typically in plastic tubes, in microtitration wells, on spherical microparticles or on glass slides (Fenton JJ,

et al., 1984, Hackler L, Jr*., et al.*, 2003, Lövgren T*, et al.*, 1996, von Lode P*, et al.*, 2003a). All these different assay approaches have different geometries and thus have unique physical dimensions as well as chemical properties, which makes the solid-phase immunoassay highly adaptable technique providing variable assay characteristics (in terms of binding kinetics, turn-around-times, processability).

1.2.1 Sensitivity and detection limit

Several authorities have given their own definitions for the term "sensitivity", when applied in the analytical method's performance. For example the international union of pure and applied chemistry (IUPAC) defines the sensitivity of an analytical system as the slope of the calibration curve or, if the curve is not a straight line, as the function of analyte concentration or amount (McNaught AD*, et al.*, 1997). The international federation of clinical chemistry (IFCC) defines the assay sensitivity in terms of the detection limit (DL) relying on the general statement that the assay sensitivity is the smallest amount of the analyte that can be truly measured (Büttner J*, et al.*, 1979). In addition, Ekins and co-workers have defined the assay sensitivity as "the resolving power of zero dose" proposing that maximal sensitivity can be achieved when the imprecision of the zero dose is least (Ekins R*, et al.*, 1997). A widely accepted method to calculate the assay DL is based on the determination of the concentration reflecting the standard deviation of the zero-dose blank signal multiplied by three (ICH guidance for industry; Q2B Validation of analytical procedures: Methodology, 1996). This definition necessitates that the assay sensitivity can be improved, and thus reduced DL values can be obtained, by lowering the amount of non-specific binding. For example detergents (such as Tween 20 or Triton X-100) have been widely utilised to reduce the background signal caused by non-specific interactions between antibody and antigen adsorption in immunoassays (Engvall E*, et al.*, 1972, Esser P, 1990).

In every immunoassay, the overall performance of the method is influenced by the equilibrium constants of the binding reactions (i.e. the avidity of the antibody binding to the analyte), the signal measurement error, the accuracy of reactant addition and the extent of misclassification of the free labelled reactant as bound and vice versa (Jackson TM*, et al.*, 1986). All of these directly affect to the assay sensitivity. For heterogeneous immunoassays the assay sensitivity is largely determined by the specific activity of the label (Hemmilä I*, et al.*, 1984, Jackson TM*, et al.*, 1986). Different labelling techniques have been developed during the last few decades, radioisotopic tracers being the first label-molecules, that were adopted to immunoassays in 1960s (Yalow RS*, et al.*, 1960). Since then also more specific nonisotopic labelling techniques have been employed, among which the fluorescent labels (such as lanthanide chelates), colorimetric substrates (such as alkaline phosphatase, ALP), and chemiluminescent labels have been the most widely exploited (Diamandis EP, 1990, Hemmilä I*, et al.*, 1984, Johannsson A*, et al.*, 1986). Attachment of many signal generating groups to the labelling reagent (i.e. multilabelling) have been shown to improve the immunoassay sensitivity especially in time-resolved fluoroimmunoassays (Hemmilä I, 1985).

In addition to the above mentioned issues related to the assay components, the assay sensitivity can also be improved by careful design of the geometry of the assay reaction compartment and the selection of the adequate measurement technology applied to the particular system. For example the detection limit of a time-resolved fluorescence immunoassay have been shown to improve through immobilisation of the capture agent within the area where the analyte captured on a solid surface coincides with the excitation beam of the detection signal on a U-shaped immunoassay well surface (Christopoulos TK*, et al.*, 1990). Alternatively, microparticles in a form of magnetic beads, instead of planar surface, can be utilised as solid surfaces to enhance the assay sensitivity (Al-Abdulla IH*, et al.*, 1989, Chapman RS*, et al.*, 1982, Zhang H*, et al.*, 2006). Typically, these particles are made of polystyrene with an iron oxide core, having functional groups on the particle outer surface to facilitate the biomolecule immobilisation process. Since microparticles can be widely dispersed within the sample solution, the mass-transfer distance of the analyte and reagents to the immobilised antibodies is greatly reduced, which enables the antibody-analyte equilibrium state to be reached more rapidly than if the antibodies are immobilised on a planar surface. However, the use of microparticles requires highly integrated systems where the beads are collected with high efficiency after sequential equilibration and washing steps, and thus the usability of the particle based assays is somewhat limited.

1.2.2 Coating density

The importance of antibody orientation, both on planar surface and in terms of position of binding sites is obvious to obtain maximum assay sensitivity. Coating density, meaning how the immobilised binding molecules are distributed over the coated surface, is one of the most critical parameter when considering the solid phase properties affecting the solid-phase assay performance. Generally, the more capture molecules are adsorbed to the solid support, the more analyte molecules can be captured. The optimal adsorption density may thus correspond to the solid phase saturation. However, a very dense surface may not always be worth striven, because the optimal surface density of a solid-phase assay also largely depends on the size of the captured analyte. For example, when considering a closest-packed monolayered surface prepared from antibody Fab fragments, a surface coverage of 220-440 ng/mm^2 have been reported, depending on the antibodies surface orientation (i.e. side, edge, or end-orientation) (Buijs J*, et al.*, 1995). However, if the dimensions of an antigen bound to such closely packed Fab surface is larger than the dimensions of an individual Fab bound to the solid phase, one antigen will cover more than one capture Fab, leading to decreased antigen binding efficiency (i.e. the percent of immobilised antibodies that bind antigen) (Brogan KL*, et al.*, 2003), which may again limit the potential assay sensitivity. With very closely packed surface coverage the total surface binding capacity easily falls down due to the repulsive intermolecular interactions between the captured molecules in near vicinity (Butler JE*, et al.*, 1992, Norde W, 1986).

1.3 Solid phase materials and their chemical properties

Coating of a biologically active molecule onto a solid surface plays an important role in diagnostics methodology. The primary surface properties (i.e. the chemical and physical properties of the synthetic solid surface) are critical for the assay performance of the surface-capture assays. Protein adsorption on the primary solid phase material is influenced by numerous different factors, including properties of the solid phase, such as surface roughness, hydrophobicity, charge, chemical composition, and structure. Furthermore, properties related to the protein itself, such as size, shape and charge, also affect to the efficiency of the overall coating process (Castner D*, et al.*, 2002). The available material diversity provides possibility to apply numerous different immobilisation techniques to the solid phase coatings, and this highly determines the selection of the most applicable surface material for the particular assay. Some of the widely used solid phase materials in SPIs are thermoplastics, such as polystyrene, polypropylene, polysulfone, or polycarbonate, which can be moulded into an unlimited variety of shape (Mahajan S*, et al.*, 2007, Penney CL*, et al.*, 1989, Pesce AJ*, et al.*, 1977, Young DB*, et al.*, 1985). This formability enables the production of reaction compartments with different physical dimensions, thus providing different surface to volume ratios, which further influences the assay performance, especially the assay kinetics (i.e time in which the reaction equilibrium can be reached). This multiformity is realised when two different solid phase geometries, for example spherical microparticles and planar surface of a microtitration well and their functions in solid-phase immunoassays are compared. The reactive surface area of a microparticle is much larger than that of a microtitration well, leading to noticeable differences in the assay kinetics between these two assay types. Thus a careful consideration is to be made, not only on the immobilisation technique, but also on the primary selection of the synthetic solid phase material (and dimensions) that is to be used for adsorption and immobilisation of the capture agents, when developing new solid-phase assay systems.

The chemical composition of a solid surface (i.e. the surface electronic nature) as well as the net charge of the protein to be coated plays an important role in the immobilisation efficiency of the solid phase coatings and the overall adsorption process. It has been generally observed, that the adsorption of a protein to a solid surface is at maximum at the pH close to the protein isoelectric point (Höök F*, et al.*, 1998, Koutsoukos PG*, et al.*, 1982). At pH below the pI value or pH above the pI value the protein surface charge is increased, which causes repulsive electronic interactions between the protein and the charged solid surface (as well as between the neighbouring proteins) and thus decrease in the protein adsorption level is seen at these conditions (Norde W, 1986). It has been further shown that the adsorption of proteins to a hydrophobic surfaces is dominated by hydrophobic bonding, whereas on a hydrophilic surface the electrostatic interactions are the main forces for protein adsorption (Buijs J*, et al.*, 1995). Bujis and coworkers (1995) found that at hydrophobic surfaces both IgG and $F(ab')_2$ fragments adsorb spontaneously, even when the protein net charge repel with the surface net charge. However, at hydrophilic surfaces the amount of protein ad-

sorbed vanish completely under electrostatically repelling conditions, which indicate that the adsorption of proteins on a hydrophilic surface occurs only if there are enough charges on the protein surface that are attracted by charges on the solid phase.

One of the most typical solid support material used in solid-phase immunoassays is polystyrene, which is hydrophobic in nature, when untreated. There are different approaches for immobilising biomolecules onto polystyrene, among which the passive adsorption and covalent coupling (when modified polystyrene material is used) are the most widely known. Polystyrene materials in the form of microtitraton wells are usually plasma-treated or gamma-irradiated, which reveal more hydrophilic groups and free radicals on the surface, providing improved surface capacity to adsorb proteins. The passive adsorption of a protein to a polystyrene is dominated by the intermolecular attraction forces, such as van der Waals forces (Esser P, 1997). In addition, the hydrophobic interactions and formation of hydrogen bonds between the adsorbed protein and the solid surface are utilised, thus favouring the hydrophilic chemical groups such as –OH, =O, $–NH_2$, =NH, ≡N, found in the protein and surface, which are capable of participating the hydrogen bond formation.

The covalent coupling of protein to the solid support minimises the leaching of the immobilised molecules from the surface, which is a generally recognised phenomenon for passive protein adsorption (Howell EE*, et al.*, 1981). However, to achieve the covalent attachment of antibodies (or other proteins) onto a polystyrene surface, the polystyrene must first be chemically modified to provide suitable chemical groups. Primary amines are convenient groups for cross-linking of proteins, and several chemical or physical treatments to provide covalent linkage or coating of amine groups on the polystyrene surface have also been described in the literature. One method to provide amino-groups onto the polystyrene surface is by nitration with sulphuric acid and nitric acid, followed by reduction of the nitro groups (Page JD*, et al.*, 1998). Furthermore, direct graftin methods, like gamma irradiation (Varga JM*, et al.*, 1990), plasma treatment (Matson RS*, et al.*, 1994), pre-coating with polymers such as phenylalanine-lysine copolymer (Karir T*, et al.*, 2006), and electromagnetic irradiation (Elsner H*, et al.*, 1989) are also some examples of methods that can be applied to introduce reactive amine groups onto the polystyrene surface. These methods have been applied to produce functional binding solid surfaces useful for bioaffinity based solid-phase assays. For example electromagnetic irradiation is used to functionalise a Covalink™ NH surface, a commercial amine-containing polystyrene material manufactured by NUNC A/S (Roskilde, Denmark) (Zammatteo N*, et al.*, 1996). The Covalink polystyrene surface contains ten carbon length spacer arms carrying terminal amino groups which are free to react with active carboxylic or phosphate groups available in proteins or other biomolecules (Esser P, 1992a, Rasmussen S, 1990).

Amine groups can be introduced also indirectly onto the polystyrene surface containing carboxyl, carbonyl or hydroxyl functional groups by means of water soluble car-

bodiimide (Niveleau A*, et al.*, 1993). Zammatteo *et al* (1996) reported a method for grafting secondary amines on polystyrene microtitration well surface by first carboxylating the polystyrene surface with permanganate, followed by the activation of the carboxylic groups with water-soluble carbodiimide and finally coupling with secondary amine (N-methyl-1,3-propane diamide). Furthermore, the amine groups generated onto a surface through different grafting methods can be cross-linked with polyethylene glycol to introduce additional surface characteristics with respect of the biomolecule immobilisation (Sun X*, et al.*, 2008). In addition also other methods that provide functionalised polystyrene surfaces capable for covalent attachment of biomolecules, have been reported and utilised in different assay setups. Among others, polyvinylbenzyl lactonoylamide (PVLA; poly[*N*-*p*-vinylbenzyl-4-*O*-β-*D*-galactopyranosyl-*D*-gluconaminde), a polystyrene derivate with pendant lactonoylamide chains (a material mainly utilised in coatings of cell culture dishes) has been used in covalent immobilisation of different proteins onto a microtitration plate surface (Kang IK*, et al.*, 2003, Kobayashi A*, et al.*, 1992, Suzuki N*, et al.*, 1997).

1.4 Immobilisation techniques

In addition to the direct adsorption of biomolecules to a synthetic solid surface, the immobilisation of the binding reagents on a bioactive secondary layer is commonly utilised in different solid-phase assays. Proteins can be immobilised on a solid support in many different ways. However, it is crucial that upon the immobilisation proteins retain their native conformational structure and, therefore, their bioactivity. Optimisation of immobilisation strategies for biomolecules is thus the key point to address before fully functional coatings can be prepared. Direct attachment of capture agents through passive adsorption would greatly simplify the immobilisation process, but may also suffer from several problems associated with e.g. surface stability. However, these methods are especially useful in applications where fast and inexpensive manufacturing process is necessary (e.g. one-use, disposable solid phases), providing a simple and fast immobilisation and patterning strategy. A variety of immobilisation methods based on different chemical cross-linking chemistries or protein attachment via special binding proteins have been developed (Turkova J, 1999). However, some immobilisation strategies, like covalent attachment and cross-linking can suffer from problems, such as loss of bioactivity and thus result in reduced assay sensitivity in the solid-phase capture assays.

1.4.1 Oriented versus random immobilisation

Optimal immobilisation process involves attachment of capture molecules to the solid surface in a way that provides stable and oriented binding surface. When larger complex molecules (e.g. antibodies) are passively coated on a solid surface, the orientation of the molecules will be random so that all of the adsorbed proteins will not be able to capture the analyte from the sample solution. Antibodies and other biologically active binding agents are conventionally adsorbed to the solid support by passive methods,

utilising primarily non-covalent interactions, such as hydrophobic forces and hydrogen bonds. This method provides antibody attachment on the binding surface in randomly oriented manner, resulting in high possibility of one or both of the antibody binding sites positioned in such a way that the binding of the antigen is sterically hindered. Despite the simplicity and speed of the passive adsorption technique, the analytical performance in immunoassays is quite poor. The lack of control over the orientation of the adsorbed binding molecule on the solid surface may lead to loss of binding activity and thus worsen the solid-phase assay performance. Butler *et al* (1993) reported that only 5 to 10 % of the adsorbed antibodies are found to be active after passive coating.

The attachment of antibodies onto a solid support by physical adsorption can lead to partial or complete loss of the antigen binding activity as a result of several reasons: 1) antibodies are attached to the surface at a region close to the binding site and this causes a steric hindrance for the antigen binding, or 2) antibodies may subject to conformational changes and loose their activity during immobilisation or 3) multi-site attachment of the antibody to the solid support restrict the intramolecular flexibility of the antibody (van Erp R*, et al.*, 1992). The immobilised solid phase capture antibodies may also have different physico-chemical and biological properties than in solution (Butler JE*, et al.*, 1992). Furthermore, deposition of multilayers of the immobilised proteins, obtained after unspecific and random immobilisation, may increase the protein desorption from the coated surface and thus lead to unstable coating and irreproducible assay results. The loss of binding activity is not acceptable in many analytical systems, especially in those, which have limited surface area, including assays based on miniaturised systems integrated with microfluidics.

If the immobilisation is done with a specific (site-selective) orientation, the binding regions of the antibodies (or other biomolecules) can be made readily accessible to antigens by directing the antigen binding sites upwards and away from the solid support. The molecular orientation of the adsorbed protein may be controlled to some extent by the buffer composition (i.e. buffer pH and ionic strength) and by the choice of the solid support material. For example, improved binding activity of the physically adsorbed monoclonal antibodies (Mabs) can be obtained by exposing the antibodies to a low pH environment prior to adsorption (van Erp R*, et al.*, 1992). The acidic treatment of Mabs changes the structural integrity facilitating an improved orientation of the molecule, meaning that the antigen binding region of Mab is more oriented towards the solvent after acidic treatment, favouring antigen binding capacity. At higher ionic strengths the adsorption of proteins to the solid surface is usually enhanced, due to reduced lateral repulsion between the proteins (Buijs J*, et al.*, 1995).

A more precise orientation control (in addition to the buffer composition and selection of the solid support material) may be achieved by using covalent coupling through chemical linkages. The covalent immobilisation results in better assay reproducibility and stabilises the solid phase properties due to minimised desorption of the immobi-

lised proteins from the solid surface, as compared to passive coatings (Howell EE*, et al.*, 1981). The covalent attachment of protein to a solid support can be achieved using different conjugation chemistries and cross-linking reagents between the solid support and the biomolecule that is to be immobilised. For example antibodies possess several different functional groups that are readily suitable for modification or conjugation allowing site-oriented attachment of these molecules (Figure 2).

Traditional covalent immobilisation methods between a biomolecule and a solid surface utilise links to amino acid side chains of the biomolecules (O'Shannessy DJ*, et al.*, 1987). One common method for covalent antibody coupling to the solid support is the utilisation of lysine ε-amine groups or N-terminal α-amine groups. In addition, carboxylate groups may be used for covalent coupling with another molecule using C-terminal, aspartic acid, and glutamic acid residues. However, both amine and carboxyl groups are abundant in the antibodies (as well as they are in most proteins) thus frequently providing non-oriented immobilisation if coupling techniques via amine and carboxylic groups are applied (Hermanson GT, 1996a).

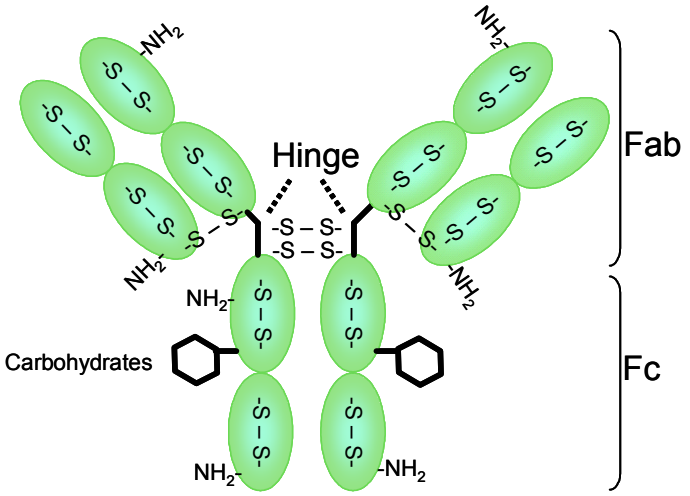


Figure 2. Schematic representation of antibody structure showing the functional groups that could be used to attach antibodies onto a surface. Primary amine groups (NH_2) are found on lysine side-chains and at the amino terminus of each polypeptide chain. Sulphydryl groups (-SH) can be generated by reducing disulphide bond in the hinge region. Carbohydrate residues containing *cis*-diols can be oxidised to create active aldehydes. Modified from Hermanson (1996a).

Conjugation reactions are more successful to preserve the binding activity if the functional groups that are utilised for conjugation are present in limiting quantities and expressed only at certain positions on the molecule surface. Examples of such func-

tional groups are the antibody hinge SH-groups, which can be produced by selective cleavage with a reductant, 2-mercaptoethylamine (DeSilva BS*, et al.*, 1995). Alternatively, smaller antigen binding fragments can be produced from antibodies through enzymatic digestion with for example pepsin or papain (generating $F(ab')_2$ fragment or Fab' fragments) (Kurkela R*, et al.*, 1988, Mariani M*, et al.*, 1991). Both of these methods provide exposed sulphydryl groups in the protein structure that can be targeted for conjugation using sulphydryl-reactive cross-linkers.

Immobilisation of antibodies via sugar residues have also shown to provide functional well-oriented binding surface. Carbohydrate residues are largely located on the C_H2 domain of the Fc portion of an IgG molecule (Hermanson GT, 1996a) and only 10% of IgG have carbohydrate groups on their Fab' fragments (Malan Borel I*, et al.*, 1990). This finding has allowed the development of site-specific immobilisation techniques, that utilise either amine or hydrazide groups and aldehyde groups produced from oxidizing oligosaccharide moieties (Batalla P*, et al.*, 2008, Gering JP*, et al.*, 2002). The only limitation of the carbohydrate mediated immobilisation techniques is that not all antibodies posses polysaccharides in their Fc-portion (Hermanson GT, 1996a, Malan Borel I*, et al.*, 1990).

A more sophisticated method to produce oriented immobilisation is the utilisation of different carrier molecules between the solid support and the primary binding molecule on solid phase coatings. Several studies have reported enhanced surface binding capacity and surface stability when the capture molecules have been immobilised to the solid substrate through a carrier molecule. The coating through a secondary molecule can serve as a spacer between the surface and the capture protein, thus providing a binding surface where the active binding agent can be located a distance away from the solid support. In this way denaturing effects evident for contacting with a synthetic support materials can be avoided. One example of such technique is the use of a more hydrophobic carrier protein (for example heat-treated BSA) to immobilise a less hydrophobic capture molecule (for example streptavidin) to a synthetic solid support (Tischer W*, et al.*, 1991). Furthermore, König *et al.* (1998) reported the utilisation of human serum albumin coated micrtotitration plates, to which recombinant antibody fragments possessing a single albumin binding domain were further immobilised in site-oriented manner and utilised in an ELISA assay.

Examples of assays that have gained improved characteristics through the oriented immobilisation techniques are represented in the Table 1.

Table 1. Examples of utilisation of oriented binding surfaces in different solid-phase based assays.

Analytical method / analyte	Surface coating		Reported advantages obtained with the oriented binding surface compared to the reference surface	References
	Oriented binding surface	Reference binding surface		
SPR / anti-hT Mab	Covalent immobilisation of Fab fragments via SH-groups, produced after enzymatic or chemical fragmentation, on a Ti/Au coated gold substrate	Random covalent immobilisation of Fab fragments via NH_2-groups (in lysine residues) on a Ti/Au coated gold substrate	> 2-fold increase of the antigen binding signals	(Bonroy K, *et al.*, 2006)
sandwich ELISA / CRP	Fab' fragments immobilised on gold-coated polystyrene magnetic particles by self assembly	Covalent attachment of Fab' fragments on commercial tosylated magnetic polystyrene particles via the tosyl reaction with amine groups of the Fab'	Improved detection limits (DL): DL = 0.14 ng/ml (oriented binding) DL = 1.9 ng/ml (reference surface)	(Zhang H, *et al.*, 2006)
sandwich ELISA / cTnI	Site-directly biotinylated $F(ab')_2$ fragment or Mab immobilised on a streptavidin-coated polystyrene microwells via the biotin-streptavidin linkage.	Randomly biotinylated Mab immobilised on a streptavidin-coated polystyrene microwells via the biotin-streptavidin linkage.	Improved detection limits (DL): DL = 0.13 ng/ml (oriented $F(ab')_2$) DL = 1.01 ng/ml (oriented Mab) DL = 2.65 ng/ml (reference surface)	(Cho IH, et al., 2007)
one-step sandwich ELISA/ D-dimer	IgG immobilised on a silica surface via Si-tagged protein A	IgG immobilised on a silica surface by passive adsorption	4- to 5-fold increase in the antigen binding activity	(Ikeda T, *et al.*, 2008)
Protein microarray/ IL-8, IL-2	PLL-PEG-biotin-Sav surface immobilised with site-oriented biotinylated Fab-fragments or with site-oriented biotinylated Mab.	PLL-PEG-biotin-Sav surface immobilised with randomly biotinylated Mab or Fab-fragments.	Higher binding capacity and thus improved signal intensities.	(Peluso P, *et al.*, 2003)
SPR / PSA	anti-PSA Mab immobilised through a cysteine-fused protein G on a gold surface by site-directed covalent attachment	anti-PSA Mab immobilised through a Tag-free protein G on a gold surface by passive adsorption	> 10-fold improvement in the amount of the bound analyte	(Lee JM, *et al.*, 2007)

Abbreviations CRP, C-reactive protein; hT, holo-transferrin; IL-2, interleukin-2; IL-8, interleukin-8; PSA, prostate specific antigen

1.4.2 Utilisation of streptavidin-biotin interaction

Perhaps the most commonly utilised biochemical interaction for protein immobilisation is the interaction between streptavidin and biotin (Diamandis EP*, et al.*, 1991, Schetters H, 1999). Streptavidin is a tetrameric protein which can bind simultaneously up to four biotins. Interaction between streptavidin and biotin is among the strongest biochemical interaction known today featuring an affinity constant (K_a) of 2.5 x 10^{13} M^{-1} (Green NM, 1990). This ultimate binding characteristic have provided the possibilities to apply streptavidin-biotin interactions in solid phase coatings, enabling oriented and functional binding surfaces for solid-phase immunoassay applications.

The immobilisation technique through the streptavidin- (or avidin-) biotin interaction is one of the most commonly utilised methods to obtain a solid phase binding surface where the capture agents are immobilised in an oriented way. The process in which one or more biotins are covalently coupled to a secondary molecule is called biotinylation. This coupling reaction results in biotin-containing molecules, which can be specifically coupled to streptavidin coated surfaces. Because of the small size of biotin (244 Da) it can be conjugated to many different biomolecules, such as proteins, carbohydrates, lipid molecules and nucleic acids, without significantly altering the biological activity of the target molecule (Della-Penna D*, et al.*, 1986).

The basic design of a biotinylation reagent (Figure 3) includes the bicyclic biotin ring at one end of the structure and a reactive functional group at the other end that can be used to attach the biotin to a secondary molecule (Hermanson GT, 1996c). There is a great variety of different biotinylation reagents available which can be used for modification of proteins and other biomolecules. In some applications, biotinylation reagents with extended spacer arms could potentially provide improved assay sensitivity. For example the binding site for biotin on avidin is located approximately 9 Å beneath the surface of the protein (Green NM*, et al.*, 1971).

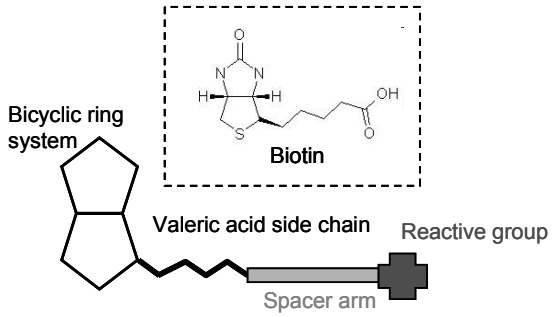
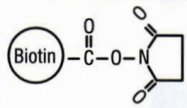
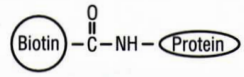
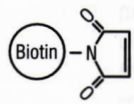
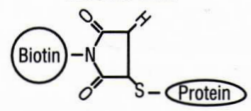
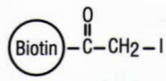
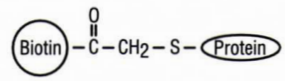
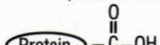
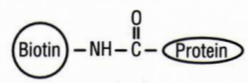
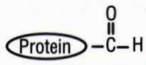
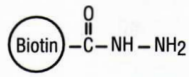
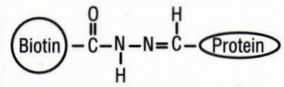
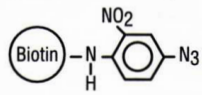
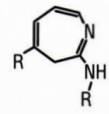


Figure 3. The molecular structure of biotin and the basic design of a biotinylation reagent. The biotinylation reagent includes the bicyclic ring structure and the valeric acid side chain of biotin in one end and a reactive group able to couple the target group at the other end.

Biotin can be attached to antibodies through several different chemical groups (Table 2). The most frequently used biotinylation reagents react with the primary amines, such as α-amino groups of the N-terminal amino acids and aliphatic ε-amines of the amino acid

lysine. Examples of such reagents are biotins containing NHS-esters able to react with the primary amino groups in proteins. Although the number and type of the functional groups available for conjugation can hardly be known, with most proteins it is, however, safe to assume that these primary amines are available and accessible for conjugation reactions. Lysines are usually present to some extent in proteins and are often quite abundant. For example tetrameric streptavidin contains up to 16 lysines (Gitlin G, *et al.*, 1988) whereas insulin contains only a single lysine (Sanger F, 1959). There is usually at least one α-amino acid in a protein, which is able to react with the NHS-ester of the biotinylation reagent. Although the N-terminal amines or lysines are almost always present in a protein, and these groups are easily reacted, modification of proteins or other biomolecules with the amine-reactive biotin derivates is not always eligible, especially if site-specific biotinylation of the biomolecule is desired.

Table 2. Examples of reaction chemistries for protein biotinylation. (Table is adapted from Avidin-biotin technology brochure, Pierce biotechnology Inc., 2003, USA).

Functional Group	Reactive Group	Linkage Formed
Primary Amine (lysine residue) 	NHS-Ester/Sulfo-NHS Ester 	Amide Bond 
Sulfhydryl (cysteine residue – not disulfide bonded) 	Maleimide 	Thioether Bond 
	Iodoacetyl 	Thioether Bond 
Carboxyl (glutamate or aspartate residues)  (Reaction requires EDC cross-linker)	Amine 	Amide Bond 
Oxidized Carbohydrate 	Hydrazide 	Hydrazone Bond 
DNA/RNA, Protein, Carbohydrates	Azido (Photoactivatable) 	 Ring expansion followed by coupling with primary amine or insertion into double bonds

Antibodies that possess reactive amine groups near to the antigen binding region, and thus are susceptible to loss of antigen binding activity when reacted with amine-reactive biotin derivates, are preferably biotinylated with other biotin derivatives, such as those reacting with sulphydryl groups. In proteins, the amino acid cysteine contains free thiol group, which is generally the most reactive functional group in proteins (Brinkley M, 1992). In addition to the sulphydryl groups present in the amino acid cysteine residues, free sulphydryl groups can also be generated by reduction of disulphide linkages present in some biomolecules, using mild reducing conditions (Karyakin AA*, et al.*, 2000). Monoclonal antibodies can also be cleaved to smaller antibody fragments possessing free sulphydryl goups via enzymatic digestions to form $(Fab')_2$ fragments, which can then be further reduced to provide Fab' fragments containing free sulphydryl group at the cleaved hinge region. Furthermore, aldehyde reactive biotinylation reagent can be utilised for antibody biotinylation if the carbohydrates of the antibodies are first oxidised with periodates. Because carbohydrates are selectively found in the Fc fragment of antibodies, the biotinylation is restricted from occurring near to the antigen binding region, thus providing possibility for site-oriented attachment of these antibodies to a solid support through streptavidin-biotin linkage. (Hermanson GT, 1996b).

1.4.3 Utilisation of antibody fragments

Recombinant antibody technology has provided the possibility to synthesise antibody fragments with desired functionalities. The recombinant antibody production provides numerous advantages over the hybridoma methods, including increased diversity of specificities that are obtainable and greater speed of production without need to immunise animals. Over the last decades the N-nitrilotriacetic acid (NTA)/ His_6-tag chelator system has become a powerful tool for the one step isolation and purification of gene products (Crowe J*, et al.*, 1994, Hochuli E*, et al.*, 1987). Furthermore the histidine tag in a protein sequence can be utilised to provide site-specific attachment of other chemical groups which can be further applied for site-directed immobilisation of the protein to a solid support either directly or indirectly (Walter JG*, et al.*, 2008).

Many advantages are involved in the use of antibody fragments as solid phase capture agents, compared to intact antibodies. These advantages include diminished non-specific binding and apparent low reactivity against heterophilic antibodies, as well as the ability to be immobilised in a more defined orientation (Bjerner J*, et al.*, 2002, Warren DJ*, et al.*, 2005, Väisänen V*, et al.*, 2006). Assay systems that require high fractional activity and surface functional density have been shown to gain improved characteristics when utilising smaller antigen binding fragments, instead of full-size antibody, for capturing the analyte of interest. Peluso and coworkers (2003) have performed an extensive study to investigate the influence of surface orientation and binding density of the capture layer to the assay characteristics in a microarray system. They found that oriented immobilisation of Fab fragments enabled increased antigen

capturing, compared to an oriented Mab surface, providing three fold improvement in the detected signal intensities in a fluorescence based immuno-sandwich assay.

1.4.4 Utilisation of antibody binding proteins

One method to produce a properly oriented antibody immobilisation on a solid surface is the utilisation of selective binding proteins, e.g. protein A or protein G, which bind to the heavy chain constant Fc region of antibodies. Protein A is a 42 kDa protein found in the cell wall of *Staphylococcus aureus*. It contains five highly homologous Fc-binding regions having high affinity ($K_d \approx 10^{-8}$ M) for the Fc-regions of immunoglobulins from various species (Aybay C, 2003, Goding JW, 1978). The principal molecule that can be bound is IgG, although the classical Fc-binding capability of the protein A is restricted to certain IgG subclasses only (subclasses 1, 2 and 4) (Björck L*, et al.*, 1984). In addition to the Fc-selectivity, protein A is also able to bind the Fab-fragment of human IgM (Aybay C, 2003). Protein G, found in the surface of streptococcal cell, contains three highly similar amino acid regions that are capable of binding the Fc-region as well as the Fab portions of IgG molecules from various species (Nomellini JF*, et al.*, 2007). Antibodies from different species (including human, mouse, rat, bovine, sheep, goat and horse) and subclasses that do not well bind to protein A generally bind to protein G.

Several studies have reported successful antibody immobilisation by using protein A or protein G layers on a solid surface. For example Lee *et al.* (2007) reported a well oriented protein G film on a gold surface utilising genetically engineered protein G, that contained cysteine residues able to react with the gold surface. When the surface functionality of the cysteine-tagged protein G was compared with a tag-free protein G, it was revealed that approximately 4-fold more human IgG was able to bind to the cysteine-tagged protein G layer compared to the reference tag-free protein G layer. The improved antibody binding capacity of the cysteine-tagged protein G layer was resulted from the formation of a well-oriented binding surface on the gold surface. Utilisation of protein A as capture linkage in solid-phase assays has been reported for example by Marquette *et al*. (2008) and Renberg *et al.* (2005).

1.4.5 Self-assembly and self-assembled monolayers

One embodiment of the self-assembly process is the formation of so called self-assembled monolayers (SAMs), which are “thin ordered assemblies that are formed spontaneously by the adsorption of a surfactant with a specific activity of its head group to the substrate” (Schreiber F, 2000). Self-assembly, in general, is a spontaneous and reversible organisation of molecular units into ordered structure by non-covalent interactions. The primary driving force for the self-assembly process is physisorption, i.e. the adsorption energy generated from weak hydrogen bonds and van der Waals interactions (Churbanova IY*, et al.*, 2006, Khatri OP*, et al.*, 2008). Self-assembly is a common phenomenon in nature and it is exhibited in the formation of

lipid membranes, in DNA double helixes and in the proteins quaternary structures, thus being crucial to the cell function. Another common example of self-assembly process is the formation of micelles by surfactant molecules, a phenomenon closely related to the cleaning action of soap. Self-assembly has been used in numerous different industrial and technical applications, such as in the production of dendrimeric particles generated by self-assembly of smaller subunits to larger structures. Dendrimers are spheroid or globular nanostructures that are precisely engineered to carry molecules encapsulated in their interior void spaces or attached to the surface (Matthews OA*, et al.*, 1998). Size, shape, and reactivity of the dendrimeric structures are determined by generation (shells) and chemical composition of the core, interior branching, and surface functionalities. In addition to the control over size, shape and surface functionality, their physical and chemical properties, such as high solubility, high surface density and high affinity, makes the dendrimeric molecules highly applicable to a variety of analytical systems, including immunoassays (Klajnert B*, et al.*, 2001). Highly effective immunoassays, based on antibodies covalently attached to fifth generation PAMAM dendrimers have been reported by Singh and co-workers (Singh P*, et al.*, 1996, Singh P*, et al.*, 1994).

SAMs were first discovered by Bigelow *et al.* (1948) who noticed that eicosyl alcohol was able to adsorb from a solution to the interior walls of an Erlenmeyer flask. In that time they reported first time the formation of monomolecular coatings by self-assembly of surfactants onto platinum support. Because of the ease of preparation, good orientation and high stability of the SAMs, they have been utilised in various different technical applications. One classical examples of SAMs is the assembly of thiols on gold surface (Karyakin AA*, et al.*, 2000). The utilisation of SH-group containing antibody fragments on a gold surface to provide a functional solid phase has been reported by several groups. Karyakin *et al.* (2000) used native immunoglobulin thiol groups, liberated through splitting the intact antibody into two half-IgG fragments, for simple adsorption onto a gold support to generate a functional solid phase for bioaffinity assay. This approach can be utilised generally with all immunoglobulins. Zhang *et al.* (2006) used gold-coated magnetic particles coated with thiol-containing antibody Fab fragments. The attachment of the thiol containing Fab fragments by self-assembly onto a gold surface provided enhanced binding efficiency and analytical performance of a solid-phase immunoassay: a 2–fold improvement in the dose-response slope, enabling up to 13-fold improvement in the detection limit of the particular assay setup was reported. In addition, an engineered cysteine containing B-domain of Staphylococcal protein A has been utilised to provide an oriented immobilisation by the self-assembly process on a gold surface (Kanno S*, et al.*, 2000). It has been also shown that thiolated protein G, when immobilised to a gold electrode, provides faster antibody immobilisation and improved orientation for the analyte binding, compared to a conventional covalently bound nonthiolated protein G surface on an alkanethiol coated gold electrode (Fowler JM*, et al.*, 2007).

The most widely utilised biotechnical applications of SAMs are the usage of such coatings in surface plasmon resonance (SPR) and in lab-on-chip devices, where they have gained numerous advantages for the assay performance. Gobi *et al.* (2007) reported enhanced sensitivity and surface stability on SAM-based SPR immunosensor for the detection of a small molecular weight compound, benzaldehyde. Also other groups have reported improved surface functionality when SAM based SPR surfaces have been compared with surfaces prepared by covalent coupling methods (Sigal GB*, et al.*, 1996). In addition to the immunoassay applications, SAMs on SPR have been widely used to study the adsorption properties of biomolecules to organic surfaces (Prime K*, et al.*, 1991).

Self-assembly peptides are one example of self-assembling biomolecular materials that can be utilised in the modification of surface properties. Ionic-complementary peptides have a unique molecular structure of alternating positive and negative charge distributions, thus leading to ionic-complementarity. The amphiphilic nature of the ionic-complementary peptides allows them to interact with both hydrophobic and hydrophilic surfaces, which is advantageous for different surface modification applications. In addition to the hydrogen bonding and hydrophobicity, these peptides contribute to the peptide self-assembly (Yang H*, et al.*, 2007). The self-assembly process is highly dependent on the pH of the solution, and thus by simple adjustment of the solution pH the charge state of the peptides can be altered leading to altered interaction with the surface and peptide, providing excellent chemical stability. Self-assembling peptides have been found to be very stable over a wide range of pHs, at high temperature and in the presence of denaturing agents and proteases (Zhang S*, et al.*, 1993, Zhang S*, et al.*, 1994). Furthermore, the peptide sequence can be designed and synthesised to contain desirable functions, such as residues containing COOH or NH_2 groups for protein binding, which provides further advantages for their usage in the surface modification purposes (Shervedani RK*, et al.*, 2006, Zhang S*, et al.*, 2005).

Another example of surface active self-assembly proteins applicable for solid-phase assays, is fungal hydrophobins. The high surface activity and the characteristic property of hydrophobins to form an amphipathic membrane upon contact with a hydrophilic-hydrophobic interface allow them to change the nature of a surface. These properties make hydrophobins potential tools in many biotechnological applications. For instance, hydrophobins have been utilised as priming layers to attach proteins to hydrophobic surfaces, such as silanized glass or Teflon (Linder M*, et al.*, 2002). In addition, variety of proteins, such as antibodies or enzymes, can be immobilised to a solid supports using hydrophobin-protein fusions, allowing high concentration and ordered packing of the protein of interest (Linder MB*, et al.*, 2005).

1.5 Miniaturised assay systems

The development of non-radioactive, highly specific labels in the 20th century enabled the immunoassays to become more sensitive and turned the interest of immunoassay

designs towards miniaturised, multi-analyte assay concepts. The miniaturisation of the assay designs offers many practical possibilities to develop functional point-of-care devices especially for assays where the measurement of at least two analytes is clinically desirable (for example in thyroid disease), offering reduced sample volumes and running costs. One example of miniaturised assay technology is microarrays, which have been utilised in numerous different formats ranging from arrays of a few elements (Mendoza LG*, et al.*, 1999) to high density arrays (MacBeath G*, et al.*, 2000). Despite the advantages related to the reaction volume, and possibilities for automation and parallelisation, the miniaturised assay system has also its limitations contributing mostly to the surface functionality.

1.5.1 Ambient analyte theory

The first truly highly sensitive miniaturised assay concept was represented by Ekins and coworkers (Ekins R*, et al.*, 1992, Ekins R*, et al.*, 1990, Ekins RP, 1989). This so called ambient analyte immunoassay is an assay system that measures the analyte concentration in the sample solution in ambient analyte condition, i.e. in conditions where the amount of the analyte is not a limiting factor. When an antibody is introduced to a sample medium containing the analyte, the binding sites of the antibody are occupied by the analyte to a fractional extent that reflects both the binding reaction equilibrium and the final analyte concentration that is unbound in the sample mixture. Generally the final concentration of the free analyte is dependent both on the concentration of the capture antibody and the concentration of total analyte present in the sample. However, when the capture antibody concentration in the assay system is less than 0.01/K (where K is the activity constant of the binding reaction) the resulting "fractional" occupancy of the antibody binding sites reflects only the ambient analyte concentrations in the medium and is thus independent of the total antibody present. Thus the ambient analyte assay theory demonstrates that miniaturised ligand-binding assays are able to achieve a superior sensitivity.

1.5.2 Microarray technology

Microarray systems consist of a large number of regularly arranged discrete spots (< 250 μm in size) of capture molecules, which are transferred onto a solid substrate using spotting robots (Glökler J*, et al.*, 2003). Depending on the assay application, different capture molecules, such as antibodies (Haab BB*, et al.*, 2001), DNA (Wacker R*, et al.*, 2004), peptides (Min DH*, et al.*, 2004) or aptamers (Lee M*, et al.*, 2000) can be used. One crucial factor in the generation of microarrays for different purposes is the choice of the surface coating used for immobilisation of the capture agents (Kersten B*, et al.*, 2005). The extent and kind of interaction evolved between surface and the capture agent differs widely between molecules and therefore, achieving a low degree of unspecific binding is extremely complicated in microarrays, where usually a complex sample containing thousands of molecules is to be analysed.

The solid support material used in the generation of microarrays must provide optimal binding conditions and an environment that promotes functionality of the immobilised substances. In addition to the thermoplastic materials (like polystyrene), used in the conventional solid phase assay coatings, several other materials are more favoured in the microarray production. Silica is the most often used material in microarrays, since it has great chemical resistance against solvents, it is mechanically stable and it has low intrinsic fluorescence properties (Benters R*, et al.*, 2001). Additionally, the surface silanol groups of silica are sufficiently reactive to allow covalent modifications (Afanassiev V*, et al.*, 2000, Beier M*, et al.*, 1999). However, a general drawback on the silica supports is frequently poor surface coverage resulting limited immobilisation capacity and relative low assay sensitivity (Afanassiev V*, et al.*, 2000). The different support medias that have been utilised in the production of microarray immunoassays have been recently reviewed by Kusnezow and Hoheisel (Kusnezow W*, et al.*, 2003).

2 AIMS OF THE STUDY

The main objective of this thesis work was to establish a new assay technology for solid-phase immunoassays and by this technology to improve the characteristics of solid-phase immunoassays utilising novel binding surfaces.

More specifically the aims were:

I. To establish and evaluate a new spot assay technology and its potential, using mini wells with different spot dimensions and solid phase coating chemistries based on streptavidin-mediated immobilisation.

II. To further evaluate the spot assay technology and its applicability to a novel immunoassay utilising site-oriented attachment of the capture agents to provide highly dense binding surface for analyte capturing. Furthermore the importance of the utilisation of several capture antibodies was investigated.

III. To disclose the properties of a chemically polymerised streptavidin and demonstrate its ability to improve surface stability and biotin binding capacity compared to a native streptavidin surface when coated on a solid support.

IV. To investigate potentials of another patterning approach for spot-shaped binding surface production, utilising contact printing technique in combination with chemically polymerised streptavidin and optimised coating solution composition.

3 SUMMARY OF MATERIALS AND METHODS

A detailed description of the materials and methods are presented in the original publications **I-IV**. A brief summary and some additional information is presented in the following chapters.

3.1 Preparation and characterisation of chemically modified streptavidin

Native streptavidin (Sav) and chemically modified streptavidin were used in solid phase coatings. Amine-reactive glutaraldehyde (I) and N-succinimidyl S-acetylthioacetate (III, IV) were used to cross-link streptavidin molecules to form larger protein conjugates. The characterisation of the modified streptavidin was performed using size-exclusion chromatography, SDS-PAGE and isoelectric focusing.

3.1.1 Cross-linking chemistries (I, III, IV)

The bis-aldehyde homobifunctional cross-linking compound glutaraldehyde (from J.T Baker, Deventer, The Netherlands) was used to conjugate the individual streptavidin units into larger protein polymers. The conjugation of streptavidin molecules was performed according to Välimaa *et al.* (2003) and it briefly contained the following steps: prior to conjugation lyophilised streptavidin was dissolved in pure water. A reaction mixture containing 2 mg/ml Sav, 10 mM NaH_2PO_4/ Na_2HPO_4 (pH 7.0), 150 mM NaCl and 1.0 % glutaraldehyde was prepared followed by incubation at + 4 ^oC for 2 hours. The excess of glutaraldehyde was removed by eluting the reaction mixture in TSA buffer (50 mM Tris-HCl pH 7.75, 150 mM NaCl, 0.05 % NaN_3,) through NAP-5 and NAP-10 desalting columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The molecular concentration of the purified glutaraldehyde cross-linked streptavidin (GA-Sav) was determined by measuring absorbance at 280 nm or alternatively with a dye-binding assay (Micro BCA assay from Pierce Biotechnology).

To introduce sulphydryl residues (thiolation) to Sav molecules, streptavidin was treated with amine reactive N-succinimidyl S-acetylthioacetate (SATA, from Pierce, USA) as described in III. Briefly, streptavidin was first dissolved in pure water and diluted further in phosphate buffer (pH 7.5) to obtain a final streptavidin concentration of 37-148 µM (1-4 mg/ml) in the solution. Various molar excesses (5-, 20-, 40-, 60- and 80-fold) of SATA over Sav were used to optimise the cross-linking reaction of the native streptavidin to form larger protein conjugates. After the reaction at room temperature the protected sulphydryl groups that were bound to streptavidin were de-protected with hydroxylamine. The final reaction product containing the cross-linked streptavidin (referred as SH-Sav or thiolated streptavidin) was purified through desalting column and eluted in borate buffer (pH 8.3) containing EDTA. The incorporated thiol groups were monitored with Ellman's reaction (Ellman GL, 1959, Riener CK*, et al.*, 2002) and the protein concentration of the purified SH-Sav was determined through measuring absorbance at 280 nm. More specific characterisation of the SH-Sav was done using chromatographic and

electrophoresis techniques. The chemical characterisation of GA-Sav has been previously reported elsewhere (Välimaa L*, et al.*, 2003).

3.1.2 Characterisations of modified streptavidin (III)

Size-exclusion chromatography

To determine the polymerisation levels of the thiolated cross-linked streptavidin (SH-Sav) and to monitor the chemical stability of the conjugates, size-exclusion chromatography on Superose 200 HR 10/30 column using Äkta explorer system (both from GE Healthcare Bio-Sciences AB) was performed. Basic buffers, such as Tris-HCl or borate buffers with pH 8.4-8.8, were used for the isocratic elution of the streptavidin from the size-exclusion column. The size-exclusion chromatography analyses were monitored by measuring absorbance at 280 nm.

SDS-PAGE and IEF

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in reducing and non-reducing conditions was run to determine the molecular weight of the polymerised SH-Sav molecules. SH-Sav and the reference native streptavidin (~1 mg/ml) was diluted in buffers containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 12.5 % SDS, 0.05 % bromofenol blue and, when reducing conditions were used, additional 25 % β-mercaptoethanol. The SDS-PAGE analyses were run using PhastGel 4-15 and PhastGel 8-25 gradient gels (GE Healthcare Bio-Sciences AB) in PhastSystem™ electrophoresis device (GE Healthcare Bio-Sciences AB). The proteins were visualised in the gel using the silver staining program for SDS-PAGE analysis.

The pI values of SH-Sav were determined for SH-Sav molecules treated with different molar excesses (5-, 20-, 40 and 80-fold) of SATA. To determine the pI values of the SH-Sav by isoelectric focusing (IEF), reference native streptavidin and SH-Sav molecules were first diluted in the sample buffer in the concentration of 1 – 3 mg/ml and analysed on PhastGel IEF 3-9 gradient gel using PhastSystem™ electrophoresis apparatus. The proteins were visualized in the gels using the IEF silver staining program.

3.2 Design and manufacturing of the spot-shaped solid phases

During this thesis study more detailed optimisation of the composition of the coating solution for spot coatings was performed to provide a functional and stable streptavidin coating with improved biotin binding properties. The conventional microtitration wells coated with streptavidin or with chemically modified streptavidins (GA-Sav or SH-Sav) were used as reference surfaces throughout the studies.

Modified immunoassay wells (spot wells), containing a small circular indentation ("mini well") of either 2.5 mm, 3.4 mm or 4.5 mm in diameter and approximately

0.2 mm in depth, moulded in a bottom of a conventional single microtitration well, were served as solid supports for optimising the coating chemistries and the spot assays (I, II). The purpose of the indentation was to give physical boundaries for liquid droplet used for coatings to produce a spot-shape binding surface for analyte capture in solid-phase immunoassays (Figure 4). The wells were manufactured in collaboration with NUNC A/S (Roskilde, Denmark) and were made from low-background fluorescent polystyrene, irradiated to obtain the maximal protein adsorption capability.

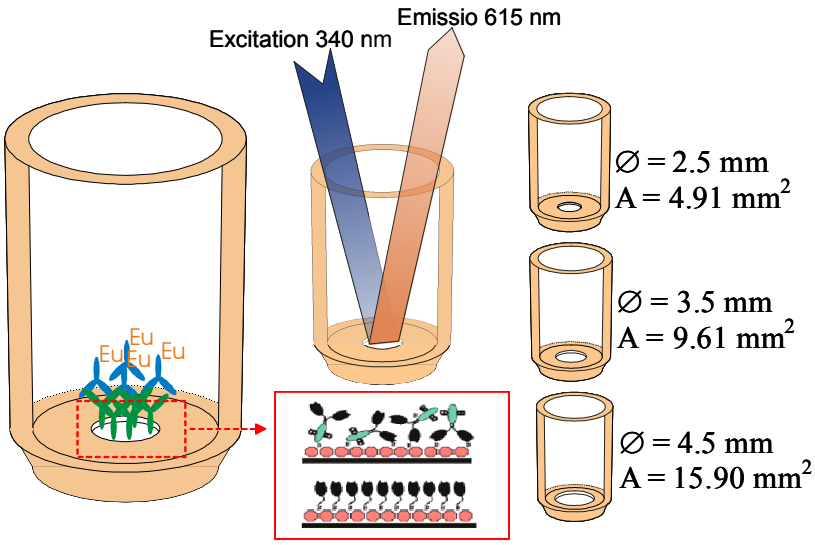


Figure 4. Spot well designs. Spot wells containing moulded spot areas with different sizes. In each well the hollow of the spot is 0.2 mm in depth. The diameters of the moulded spots are 2.5 mm, 3.5 mm or 4.5 mm, corresponding to total binding areas of 4.91 mm^2, 9.62 mm^2 and 15.90 mm^2, respectively. Respective surface areas including the bottom and the walls of the mini wells are 6.5 mm^2, 12 mm^2 and 19 mm^2.

In addition to the moulded spot wells (mini wells) described above, special spot wells possessing the spot-shaped binding area in the bottom of conventional microtitration wells (Maxisorp microtitration wells in single well format, from NUNC A/S) were produced with contact printing technique (Flexys device from Genomic Solutions™, USA) utilising solid pins (IV). This technique enabled the production of spot-shaped binding surfaces without physical indentations and provided faster coating processes originated from reduced droplet volumes.

3.2.1 Coating of conventional microtitration wells (I-IV)

Conventional polystyrene microtitration wells in single-well format or in C12 plate-format were coated with native streptavidin or with cross-linked streptavidins (III, IV). In addition to the wells coated in-house, commercial streptavidin coated plates (manu-

factured by Innotrac Diagnostics Oy, Finland) were used (I, II). In general, the coating procedure included the following steps: first streptavidin was diluted in selected coating buffer to given protein concentration. Buffered solutions with pH 5 – 8 were used. A volume of 200 µl of the coating solution was applied to each of the wells and the coating was performed over night incubation at + 37 °C. In some cases the coating solution was allowed to dry during the coating process (IV). After over night incubation the wells were washed with the washing solution containing additional 0.05 g/l Tween 20. The uncoated regions were blocked with BSA during over night saturation at room temperature. Finally the dried wells were used directly or packed in closed chambers with desiccant and stored at 4 °C.

3.2.2 Coating of the spot wells (I, II, IV, unpublished)

Spot wells (mini wells) with circular indentations of 2.5 mm, 3.5 mm or 4.5 mm in diameter, were coated with Sav (I, II), GA-Sav (I), or SH-Sav (unpublished) using coating buffer (pH 5.0) containing 20 µg/ml of the protein. The coating volumes applied to the spot wells were 4 µl, 10 µl and 15 µl for 2.5 mm, 3.5 mm and 4.5 mm spots, respectively. Coatings were performed as over night incubation at 35 °C in closed humid chambers to prevent evaporation of the small droplets. After overnight incubation the spot wells were washed and incubated with 100 µl of saturation solution containing BSA. After saturation the wells were allowed to dry before use. Wells that were not used immediately were packed in closed containers with desiccant and storage at 4 °C.

Spot-shaped binding surfaces into the regular microtitration wells (IV) were manufactured using Flexys device (Genomic Solutions, USA) utilising contact printing technique. Coating buffer containing 1.5 mg/ml of SH-Sav or 10 mg/ml of Sav diluted in 100 mM Na_2HPO_4, 50 mM citric acid and 0.5 M betaine monohydrate was used. The printing was performed with solid pins (manufactured in collaboration with Innotrac Diagnostics Oy, Turku, Finland) having a diameter of 2.0 mm, which provided a patterned circular spot-shaped binding area of approximately 4.2 mm^2 (and 2.3 mm in diameter) in the bottom of a polystyrene microtitration well (Maxisorp). Before and after each printing action, the solid pins were washed with water by dipping the pins in ultrasonic bath for 10 seconds and rinsed with 20 % ethanol and finally dried using the specific washing and drying unit incorporated in the Flexys device. After printing the surfaces, the created spots were allowed to dry 5 to 24 hours at 35 °C, followed by a saturation step and final washing with the wash solution and drying. The contact printing process described above was also utilised to coat the spot wells with antibodies or antibody fragments (IV).

3.3 Biotinylation and labelling with fluorescent labels

3.3.1 Biotinylation of monoclonal antibodies (I-IV)

Biotinylation of monoclonal antibodies anti-hTSH Mab 5404 (Medix Biochemica, Finland) (I), anti-cTnI Mab 7F4 (Hytest Ltd., Finland) (II), anti cTnI Mab 4C2 (Hytest

Ltd.) (II) and anti-cTnI Mab 19C7 (Hytest Ltd.) (II), were performed using isothiocyanate modified biotin (Mukkala V-M*, et al.*, 1993). Anti-hTSH Mab was labelled with 40-fold molar excess (147 µM) of biotin over the antibody in a sodium carbonate buffer (pH 9.8). To ensure sufficient remaining affinity of the cTnI specific antibodies, the biotinylation of these was performed with lower proportional molar excess of biotin over the antibodies. A molar excess of 10-fold (75 – 145 µM) of biotin over the cTnI specific antibodies were used for biotinylation in a sodium carbonate buffer (pH 9.8). After four hours of incubation each of the biotinylation reactions were reduced in volume by ultrafiltration through a 50 kDa cut-off membrane and purified through two consecutive desalting columns.

The portion of biotinylated antibodies was determined using an in-house test performed as follows: first biotinylated antibody was diluted in assay buffer (Assay solution, red, Innotrac Diagnostics) to the concentration of 2 ng/µl and applied to the streptavidin coated plates as a volume of 200 µl/well. After one hour incubation with shaking a portion of this solution was transferred to anti-mouse-IgG coated wells, together with additional bio-Mab dilutions (0.04 – 0.4 ng/µl), and incubated two hours with shaking. After the incubation the wells were washed with wash solution and the bound antibodies were detected using europium labelled anti-mouse-IgG. The portion of biotinylated antibodies were determined against a standard dilution prepared from the same biotinylated antibody. Concentrations of the antibody populations were determined using the Coomassie blue dye-binding assay (Bradford MM, 1976).

3.3.2 Labelling with lanthanide chelates (I-IV)

Intrinsically fluorescent 7-dentate (Takalo H*, et al.*, 1994) or 9-dentate (von Lode P*, et al.*, 2003b) europium (Eu) chelates containing amine reactive isothiocyanate moieties were used to label the monoclonal antibodies anti-hTSH Mab 5409 (Medix Biochemica) (I) and cTnI specific Mab 8I7 (Spectral Diagnostics Inc., Canada) (II, IV) and Mab 7B9 (Hytest Ltd.) (II) and cTnI antigen (native human cardiac troponin I-T-C complex, from HyTest Ltd., Turku, Finland) (II). Europium chelate of 2,4-(isothiocyanatobenzyl)di-ethylenetriaminetetra acetic acid (Mukkala VM*, et al.*, 1989) was used to label the human TSH antigen (Scripps Laboratories, San Diego, CA) as described in I. For the labelling of the antibodies or antigens a suitable molar excess (10 – 35-fold) of the selected fluorescent label was dissolved in carbonate buffer (pH 9.8) and reacted with the desired protein during an over night incubation at room temperature or at 4 ^{o}C. The labelled proteins were purified using size-exclusion chromatography. The labelling degree (i.e. Eu/protein ratio) was determined against a europium calibrator in DELFIA® enhancement solution (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland). The labelling degrees for monoclonal antibodies were found to be 6 – 10 Eu / Mab. For labelled antigens a labelling degree of 1 – 2 was determined.

3.3.3 Production and biotinylation of antibody fragments (I, II)

TSH-specific monoclonal antibody cell line 5404 was cloned as a Fab fragment (M_w 49 300 Da) and as an scFv fragment (M_w 28 200 Da) using standard procedure (Brockmann EC*, et al.*, 2005). cTnI specific hybridoma cell lines 7F4 and 4C2 (Hytest Ltd.) were cloned as Fab fragments (Fab 7F4, M_w = 49.02 kDa, Fab 4C2, M_w = 47.8 kDa) by reverse transcription PCR. The production of cTnI specific Fab fragments were performed as follows: Fab fragments were separately cloned into a pAK400 expression vectors (Krebber A*, et al.*, 1997) containing a Cys-Pro-His_6 coding sequence at the carboxyterminal end of the Fd chain providing a site for affinity purification and site-specific attachment of EZ-link maleimide PEO_2-biotin (Pierce, Rockford, USA). The cells were grown in SB-medium containing 25 mg/l of antibiotic substance (chloramfenicol). When the cells reached a suitable optical density, the production of the Fab fragments were induced by adding 0.4 M isopropyl-β-D-thiogalactopyranoside. After 4-8 hours from the induction, the cells were harvested by centrifugation and the Fab fragments were extracted form the periplasmic space of the *Escherichia coli* cells by Tris-Cl extraction based on osmotic shock. The purification of Fab fragments from the cell lysate was performed using cation exchange chromatography (Streamline SP 25, GE Healthcare Bio-Sciences) followed by a more specific purification with a Ni-NTA superflow column (Qiagen, Venlo, The Netherlands). Finally the produced and purified Fab fragments were dialysed to PBS-buffer (10 mM $NaHPO_4$ / NaH_2PO_4, 154 mM NaCl) and stored at 4 °C. The characterisation of the Fab fragments was performed with SDS-PAGE analysis run in PhastSystem™ electrophoresis apparatus (GE Healthcare Bio-Sciences). The biotinylated Fab fragments were further characterised for portion of biotinylation using an in-house test described above (in the chapter 3.4.1) and for their antigen binding properties using Biacore X instrument (Biacore, Uppsala, Sweden).

3.4 Characterisation of the solid phase properties

3.4.1 Reporter molecules (III, IV)

Biotin or biotinylated macromolecules (myoglobin or full-size antibody) containing Eu-chelate as detectable fluorescent label were used to monitor the surface binding capacity and surface binding density of the streptavidin coated wells (conventional microtitration wells and spot wells). A monoclonal antibody (αPSA H117) (Piironen T*, et al.*, 1998) was used for determination of the surface binding capacities for a large molecule ($M_w \approx$ 160 000 Da), whereas myoglobin (horse skeletal muscle myoglobin from Sigma-Aldrich) represented a smaller macromolecule ($M_w \approx$ 117 000 Da). These reporter molecules were biotinylated with 80-fold molar excess of isothiocyanate modified biotin and labelled with 7-dentate fluorescent lanthanide chelate. The labelling degrees of these reporter molecules were adjusted to be approximately 1 – 2. The synthesis of the smallest reporter molecule (Eu-biotin), a modified biotin product of 4-[2-(D-Biotinamido)-1-ethyl]phenylamine containing 7-dentate Eu chelate (M_w 1036

Da), has been previously described elsewhere (Välimaa L*, et al.*, 2004), and it was used to monitor the surface binding densities and biotin binding capacities.

3.4.2 Binding capacity of the streptavidin-coated surfaces for biotinylated reporter molecules (I-IV)

For the characterisation of the binding efficiency of conventional immunoassay wells 0.5-18 pmol of Eu-biotin or 0.5-90 pmol of biotinylated macromolecules were used, while 0.01-15 pmol of Eu-biotin or 0.1-60 pmol of biotinylated macromolecules were used for spot wells. The biotinylated and labelled molecules were diluted in the assay buffer (Assay solution, red) to the desired concentrations and immobilised on solid surface through streptavidin-biotin linkage. The immobilisation was performed at room temperature under shaking, followed by washing and drying steps. The fluorescence of the bound biotinylated molecule was first measured directly from the dried surface and after that upon dissociation of the fluorescent label to a homogeneous solution through DELFIA® enhancement. The surface readout measurement reflected the local surface binding density of the particular binding areas, whereas the DELFIA® enhancement represented an integrated signal from entire area and reflected the total binding capacity of the particular solid phase.

3.4.3 Antigen binding features of immobilised antibodies (I, II, IV)

Biotinylated anti-hTSH Mab clone 5404 (I), biotinylated recombinant Fab and scFv fragments specific against hTSH (I), anti-cTnI Mab clones 7F4 and 4C2 (II) and biotinylated recombinant anti-cTnI Fab fragments (II) were used to determine the antigen binding capacities of the streptavidin coated spot surfaces. In brief, the binding assays were performed as follows: biotinylated antibodies or antibody fragments were diluted in the assay buffer (Assay solution, red) prior to dosing into the streptavidin coated spot wells. Input amounts of 0.05–1.0 pmol per spot well for a Mab and 0.125–2.5 pmol per spot well for antibody fragments were used. The immobilisation reactions of the biotinylated antibodies or antibody fragments onto the streptavidin coated surfaces were performed at room temperature (anti-hTSH antibodies) or at 35 ^{o}C (cTnI specific antibodies). After one hour incubation the wells were washed and 4-6 pmol of Eu-hTSH or Eu-cTnI was added into the wells and incubated for additional 30-60 minutes. The fluorescence signal of the bound Eu-hTSH antigen was measured using Victor multilable counter (PerkinElmer Life and Anlytical Sciences – Wallac Oy) after DELFIA® enhancement. The 9-dentate Eu chelate of the labelled cTnI antigen enabled the fluorescent measurement to be performed directly from the dried well surface and thus additional enhancement step was not required.

3.5 Solid-phase Immunoassays

Several heterogeneous solid-phase immunoassays were performed to study the immunoassay performance in streptavidin coated surfaces. Briefly, the assays were conducted by first immobilising the biotinylated capture antibodies or antibody Fab or scFv fragments onto a streptavidin coated polystyrene microtitration wells (conventional wells or spot wells), followed by an incubation step with an analyte and a tracer antibody, which was labelled with intrinsically fluorescent europium chelate. Time-resolved fluorescence measurement was performed either directly from the dried surface or through DELFIA® enhancement.

TSH immunoassays (I)

TSH immunoassays on spot wells (and on conventional microtitration wells as reference assay) were performed either in a format of normal solution based assay design or utilising all-in-one dry chemistry technology (Lövgren T*, et al.*, 1996). Capture antibody amounts of 0.2 – 1.0 pmol (for bio-Mab 5404), 0.5 – 1.25 pmol (for bio-Fab 5404) and 0.8 – 2.0 pmol (for bio-scFv 5404) were used to achieve the immobilisation of the capture agents on spot surfaces through Sav-biotin linkage. On conventional microtitration wells coated with native streptavidin 2.8 pmol of Mab was immobilised. Eu-labelled anti-hTSH antibody 5409, recognising different epitope of hTSH than the capture antibodies (bio-Mab 5404, Bio-rFab 5404, Bio-rScFv 5404), was used as the tracer molecule for quantitative measurement of the bound analyte.

cTnI immunoassays (II, IV)

For cTnI immunoassays three different capture antibodies (clones 7F4, 4C2 and 19C7), specific against different epitopes of cTnI (N-terminal, C-terminal or mid-fragment epitopes) were immobilised on streptavidin coated spot surface to provide a binding surface for cTnI capturing. Eu-containing labelled antibody, which was specific either to cTnI (clone 8I7) or to cTnC (clone 7B9) was used as tracer molecule in different assay configurations. The fluorescent signal of the bound analyte-tracer complex was measured either directly from the dried binding surface and/or after DELFIA® enhancement. The cTnI immunoassays were conducted on spot wells either in mini well format or wells produced with contact printing technique, providing a spot-shaped binding are on a regular microtitration well surface.

4 SUMMARY OF THE RESULTS AND DISCUSSION

4.1 Characterisation of thiolated streaptvidin

SATA is a versatile reagent which reacts with available amine groups in proteins via the NHS-ester end to form protected sulphydryl derivates. Amines that are located on the surface of the protein and are thus most accessible for conjugation are suggestively modified first while those that are in more buried positions are poorly available for conjugation. The primary targets for modification in streptavidin are the N-terminal α-amines in the polypeptide chains and the NH_2-groups (ε-amines) in the lysine side chains. The tetrameric streptavidin is composed of four identical monomeric sub-units, which each contains identical polypeptide chains with up to four lysine-residues providing the reactive ε-amines for conjugation. Thus, up to 16 ε-amines in the lysine side-chains plus four N-terminal α-amines of the polypeptide chains (i.e. totally 20 amines), can be found in one tetrameric streptavidin, which are possible targets for conjugation reaction with SATA (Gitlin G*, et al.*, 1988).

Protein Data Bank files for streptavidin structures (entry 1SWQ, Figure 5) were reviewed to disclose the positions of lysines. Three of the four lysines (Lys121, Lys132 and Lys134) found in each of the four sub-units of streptavidin are located on the surface readily available for conjugation. The fourth lysine (Lys80) found in each of the protein sub-units are located in more buried positions. It was revealed that at least 70 % of the possible target groups were activated in the conjugation reaction with excess of SATA during SH-Sav production, which is inline with the above mentioned observation about the amount of possible target groups.

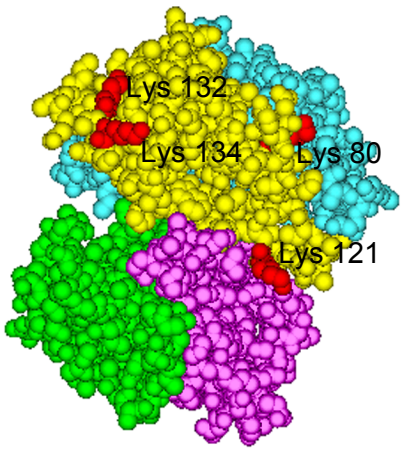


Figure 5. The three dimensional structure of streptavidin. The four identical sub-units are shown in different colours (purple, green, yellow and blue). Lysines (Lys80, Lys121, Lys132 and Lys134) found in the surface of one sub-unit are shown in red. The image was generated from the RCSB Protein Data Bank entry 1 SWQ using Discovery Studio Visualizer (Accelrys, Inc) program.

An average of 2 SH/Sav was found when 5-fold molar excess of SATA was used in the activation reaction and up to 12 SH/Sav was repeatedly measured when the activation reaction was conducted with 40 to 80 –fold molar excesses of SATA (Figure 6) (III). Thus the polymerisation level was correlated well with the activation degree (i.e. with the amount of thiols per Sav), which was measured directly after the SATA activation, using Ellman's reaction.

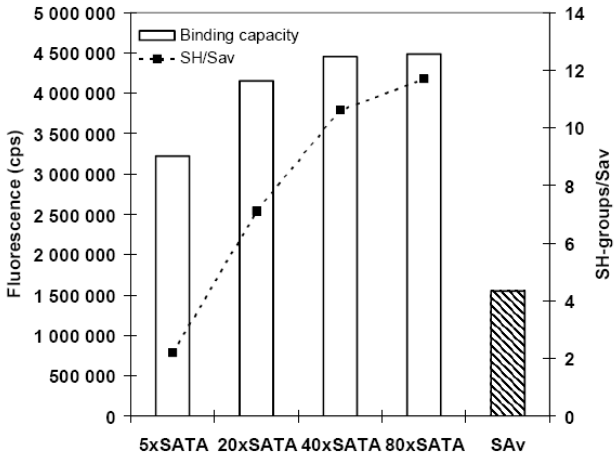


Figure 6. The binding densities of surface coated with the thiolated streptavidin (white columns) and level of sulphydryl incorporation (dotted line) of SH-Sav prepared with increasing concentrations of SATA reagent. SH-Sav was prepared using four different molar excess (5-, 20-, 40 and 80-fold) of SATA. For reference, the binding capacity of native streptavidin (Sav) coating is shown (filled column). Saturated amount of Bio-Eu (90 pmol/well) has been used. Fluorescence signals have been measured from dried surface, reflecting the local binding densities of the coated surfaces. (III)

Gel filtration chromatograms of SH-Sav produced with different molar excesses of SATA revealed that when more thiol groups (measured with Ellman's reaction) were incorporated to the streptavidin structure through the SATA activation, the polymerisation level was increased providing larger protein complexes (Figure 7) (III). Corresponding results were also obtained with SDS-PAGE analysis. Furthermore, it was shown that the formation of larger polymeric units was enhanced with increasing excess of SATA used for conjugation, while the proportion of the smaller monomeric units decreased. This reflects enhanced polymerisation through disulphide linkage formation achieved with increased excess of SATA.

The chemical properties of SH-Sav were found to be different from that of the native SAv molecule. The positive charge of the amino-groups was neutralised by introducing more thiol residues to the Sav structure with higher excess of SATA, resulting in lower apparent pI-value (pI 3-5) of the protein, compared to native streptavidin (pI ~7). Thus the decrease in the pI-value of Sav after SATA treatment most likely derives from the formation of covalent amide bonds and thus leading to neutralization of the positively charged amines and the reduction in the protein net charge. The increased concentrations of negative thiol residues achieved by the deacetylation reaction also increases the negative charge of the SH-Sav, which causes further decrease in the pI-value compared to the native protein.

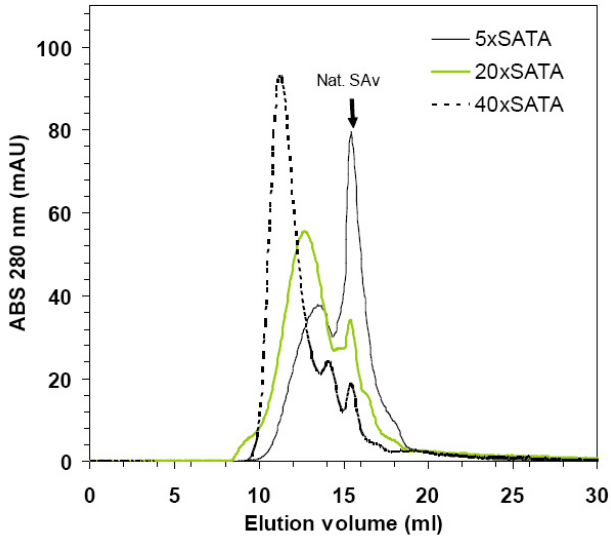


Figure 7. Gel filtration chromatograms of polymerised streptavidin molecules, produced by activating the native streptavidin with different molar excesses (5-, 20- or 40- fold excess) of SATA reagent (III).

4.1.1 Adsorption properties of SH-Sav (III, unpublished)

The change in pI had an influence on the adsorption behaviour of SH-Sav and thus it mainly contributed to the suitable coating buffer composition to obtain the best possible protein adsorption onto polystyrene. The most efficient adsorption of the thiolated streptavidin was observed at acidic pH region, where the net charge of the polymer was neutral. For comparison, the optimal coating condition for native streptavidin (pI 7) is at pH 7 (unpublished). Adsorption of proteins to a solid surface is typically at maximum in the isoelectric region of the protein (Koutsoukos PG*, et al.*, 1982). This is likely due to several reasons: the stability of a globular protein structure decreases with increasing net charge on the molecule, and hence structural changes are more likely to occur when proteins are adsorbed at a pH away from their isoelectric point. In contrast, at the isoelectric region the positive and negative charges of the protein are more or less evenly distributed over the molecule, resulting in intramolecular electrostatic attractions, which favours a compact structure, thus stabilising the molecule and leading to improved functionality when adsorbed. Furthermore, at the isoelectric point, where the protein net charge equals to zero, the proteins exhibit least lateral repulsion between the neighbouring proteins, thus facilitating the adsorption. (Norde W, 1986).

The electrochemical interactions emerging between the solid surface and the protein to be adsorbed are one of the key driving forces behind the adsorption process. Thus one could expect that the protein adsorption on a solid surface is higher when the protein and surface are oppositely charged and that lower adsorption will be achieved with

equally charged patterns. This has been shown to be true in several studies (Ladam G*, et al.*, 2002, Lubarsky GV*, et al.*, 2005, Soltys-Robitaille CE*, et al.*, 2001). However, some of the studies suggest that the surface charge does not influence the protein adsorption efficiency at all (Blanco EM*, et al.*, 2008, Ladam G*, et al.*, 2002). Upon adsorption process conformational changes of the protein may occur, which may lead to hydrophobic or hydrophilic forces becoming dominant interaction in the adsorption process independent of the surface charge. At pH 5.0 SH-Sav molecules carry a neutral net charge, and thus it may be speculated that the adsorption of the polymerised SH-Sav molecules onto the amphiphilic polystyrene (Maxisorp) surface is mainly controlled by the intermolecular attraction forces (that is van der Waals interactions), rather than electrostatic interactions (that is covalent bonding or ionic bonds). Furthermore, if the formation of the adsorbed layer of SH-Sav was mainly driven by electrostatic interactions, it should be influenced by the ionic strength of the coating solution in contact with the solid phase. However, increase in the ionic strength of the SH-Sav coating solution (achieved with sodium chloride addition) had only a minor effect on the adsorption efficiency of the SH-Sav coatings, supporting the statement that the main driving force in the SH-Sav adsorption is mainly intermolecular attraction forces, caused by the combined action of hydrogen bonding and hydrophobic interactions rather than the electrochemical interactions generated between the solid phase and the protein.

It is widely known that larger proteins are more readily adsorbed compared to the smaller ones. The polymerisation of streptavidin molecules into larger units through SATA activation was, however, helpful to certain extent only. This was realised as the the size of the protein (SH-Sav) and thus the polymerisation level were found to increase during the storage (Figure 8), but the binding capacity of the surface prepared from more extensively polymerised thiolated streptavidin was not further increased compared to the freshly prepared thiolated streptavidin when coated on polystyrene (unpublished). As the conjugation proceeded during the storage, the free thiol level (when measured with Ellman's reaction) was decreased as they were consumed in the disulphide bridges (Figure 8, inserted graph). This indicates that the free thiol level did not have immediate effect on the adsorption of advanced polymers. However, to control the effect of the polymerisation, SH-Sav was kept and coated also under reducing conditions (DTT) where disulphide formation was inhibited. This resulted in slightly (but clearly) higher adsorption compared to Sav, indicating that the reactive thiols are somewhat helpful, for contacts between the protein and surface, either directly (contacts between the thiols and surface) or indirectly (through polymerisation).

The improved adsorption properties of SH-Sav were found to be related to the improved adsorption kinetics of the protein onto the solid surface. Already after 15 min coating time with SH-Sav on polystyrene, equal binding capacity was obtained compared to a reference SAv surface, which was prepared with over night incubation. Furthermore, the increase in the protein concentration in the coating solution was

found to contribute to faster coating process: increase of the SH-Sav amount from 0.5 to 3 µg/well using 15 min coating time resulted up to 3.3-fold increase in the immobilisation efficiency of the coated surfaces, although the same increase in the protein concentration with 20 hour coating time did not show any remarkable differences in the surface binding capacities. The gained improvement in the coating efficiency obviously enables the production of streptavidin coated plates in shorter times, thus potentially lowering the costs of the manufacturing process.

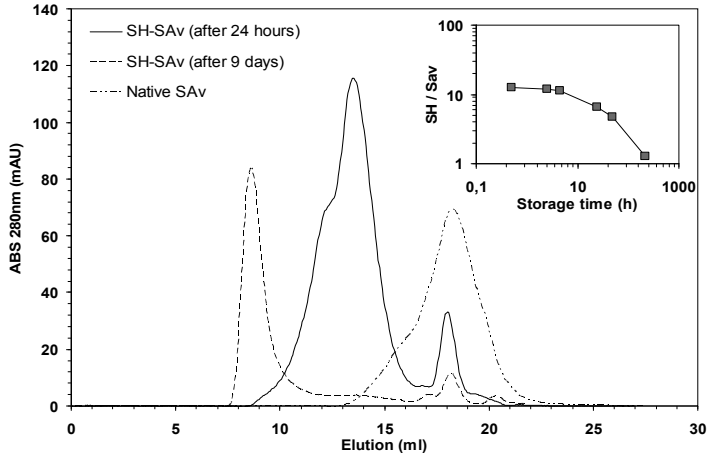


Figure 8. Gel filtration chromatograms of SH-Sav obtained after 24 hours and 9 days (216 h) storage at 4 °C. As reference an elution profile of native streptavidin is also shown. The inserted graph shows the change in the thiolation degrees (SH/Sav) during storage time, measured with the Ellman's reaction. (modified from original publication III)

4.1.2 Binding properties of the SH-Sav coatings (III, unpublished)

The biotin binding capacities of the surfaces coated with thiolated streptavidin increased after the excess of SATA used in modification (Figure 6). Based on this finding it can be concluded that the formation of large polymeric units through the S-S-linkage are mainly responsible for the improved binding properties achieved. In addition to the ability to bind small biotin, the surface ability to bind biotinylated macromolecules was studied. These results are illustrated in the Figure 9. The Eu-derived fluorescence signals reached the saturation level after addition of 5 pmol of the biotinylated macromolecule (myoglobin or monoclonal antibody) while addition of up to 20 pmol of Eu-labelled small biotin was needed to reach the saturation level. The differences in the saturation levels between small biotin and the biotinylated macromolecules on corresponding surfaces are derived from the steric hindrance evident for the macromolecules. Furthermore, the difference in the binding capacities between the native Sav coating and the SH-Sav coating derives from the improved surface binding density of the SH-Sav surface, as compared to the Sav surface. This was demonstrated by the surface readout measurement, which gave improved detectable signals on the SH-Sav surface, compared to the native Sav coatings (unpublished).

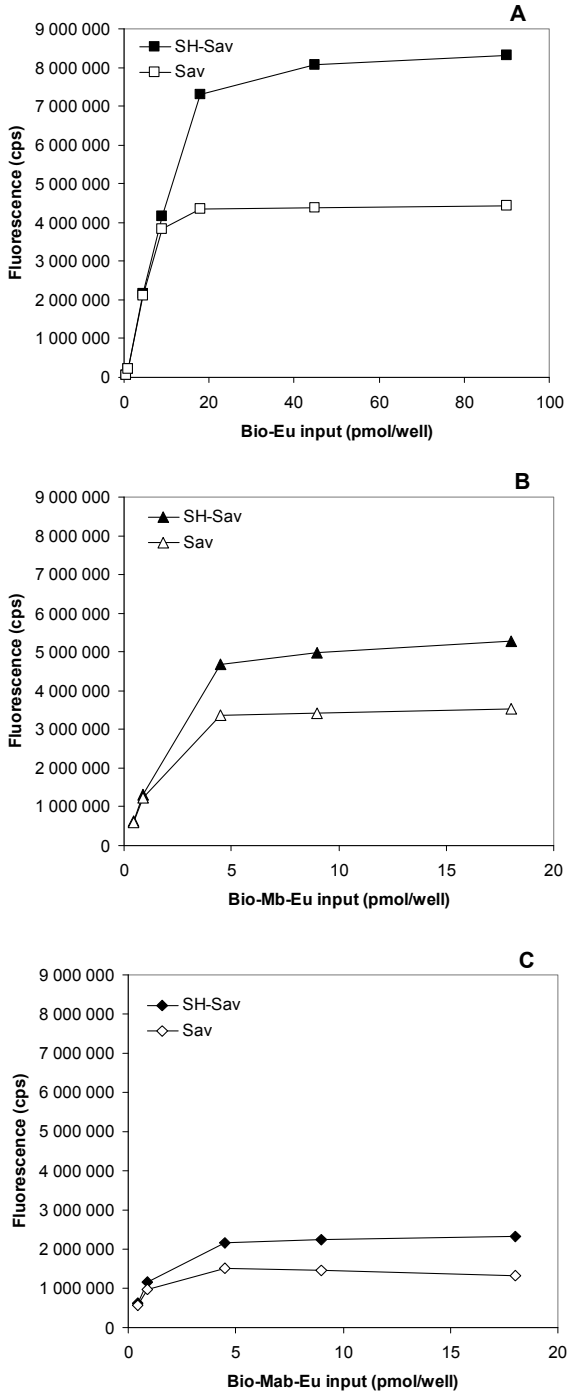


Figure 9. The binding efficiencies of the Sav and SH-Sav coated surfaces. Fluorescence signals of europium labelled biotin (A) or biotinylated macromolecules (myoglobin; Mb (B) and monoclonal antibody; Mab (C)) bound on SAv and SH-Sav coated surfaces are measured with Victor multilabel counter after DELFIA® enhancement. (Unpublished)

4.1.3 Stability of the SH-Sav coatings (III)

In addition to the improved biotin-binding properties of the SH-Sav surface, the surface stability against desorption in solid-phase bioaffinity assay conditions was enhanced, compared to the reference Sav surface. The improved surface stability of the SH-Sav coating is most probably due to the covalent attachment of the Sav molecules into larger conjugates followed by the reduction in the amount of more readily dissociating monomeric units. Adsorption of protein occurs in the first place through the sites present directly in the plastic surface. For uniformly assembled streptavidin monolayer the theoretical maximum adsorption capacity is 570 ng/well, but only 60 % of this has been experimentally reached with native streptavidin due to random assembly (Välimaa L*, et al.*, 2004). Typically, an excess of protein is added (like 1 µg / well / 200 µl) and the generation of weakly bound secondary layers is evident. In the case of SH-Sav coatings the secondary layers are held by covalent bonds which contributes to the lower amount of proteins released during incubation and to the maintenance of the high binding properties of the surface. Furthermore, the surface coated with SH-Sav was able to tolerate harsh conditions, such as addition of denaturing (such as guanidine hydrochloride, urea and SDS) or reducing agents (such as TCEP or DTT). This property is highly valuable especially in applications utilising buffers or samples containing surfactants or chaotropic agents.

4.2 Performance of the spot coatings

The high surface density gained with the modified streptavidin (SH-Sav and GA-Sav) coatings is a valuable characteristic especially in applications where the integrated signal of the bound tracer molecule is measured directly from a dry surface to assay very small and unsaturated amount of analytes. When the whole binding area of a capture solid phase is coinciding with the excitation beam of a measuring instrument, the change in detectable signals derived from the analyte binding can be more precisely observed providing improved detection limits for the particular assay. This has been previously shown to be true in the measurement of α-fetoprotein (Christopoulos TK*, et al.*, 1990) and is probably most clearly exploited within the ambient analyte immunoassay concept represented by Ekins and co-workers in the early 1990s (Ekins RP*, et al.*, 1991). Although the basis of the spot assay technology developed during this thesis work is different from that of the Ekins's microspot theory (the spot assay being a normal reagent excess, "mass sensing" assay), the dependence of the assay performance on the reduced size of the coated area and on the capture surface density and binding activity of the immobilised antibodies to obtain improved detection limit is well demonstrated.

4.2.1 Biotin binding capacity of spot surfaces in mini well format (I, II)

Binding of biotinylated reporter molecules on the spot coated mini wells (used in the studies described in the original publications I and II) showed linear increase in binding followed by a rapid leave near to the saturation-level. Typical binding isotherms of

the spot wells are shown in the Figure 10. The fluorescence signal, when measured through the surface readout directly from the dried spot surface, showed lower detectable signals within subsaturating quantities of small Eu-biotin on the high capacity streptavidin surfaces (GA-Sav or SH-Sav), compared to surface made from unmodified streptavidin, although the difference between the coating types was clearly evident in the saturation region. This is derived from characteristics of the surface readout measuring technology. The imaging of the spot surfaces has previously disclosed the uneven distribution of the bound Eu-labelled biotin onto the spot coatings, due to the liquid streams and high local velocities induced by the shaking motion during the binding steps (Välimaa L*, et al.*, 2008). This was the main reason to gain lower detectable signal levels at the subsaturating quantities. It was further speculated that some lateral diffusion and clustering of the surface bound molecules may be relevant causes as well (Välimaa L*, et al.*, 2008).

The total biotin binding capacity was measured after dissociating the Eu ion of the fluorescent chelate into homogeneous solution through DELFIA® enhancement. The total binding capacity of the spot wells were increased with the increasing spot size (Figure 11, inserted graph). This is most likely derived from the increased capture area gained with the increasing spot size. Furthermore, the lower signal levels obtained at subsaturated levels in the surface readout measurement was not detected in the measurement based on the dissociation enhancement, and rather identical signal levels were obtained with the Sav and high capacity surfaces already at the lower Eu-biotin input levels (unpublished). This further supports the argument stated by Välimaa *et al.* (2008) that the low signal detected at subsaturated levels are derived form the uneven distribution of the labelled analyte due to shaking mode, rather than irregular coating pattern of the high capacity streptavidin layer obtained inside the mini wells.

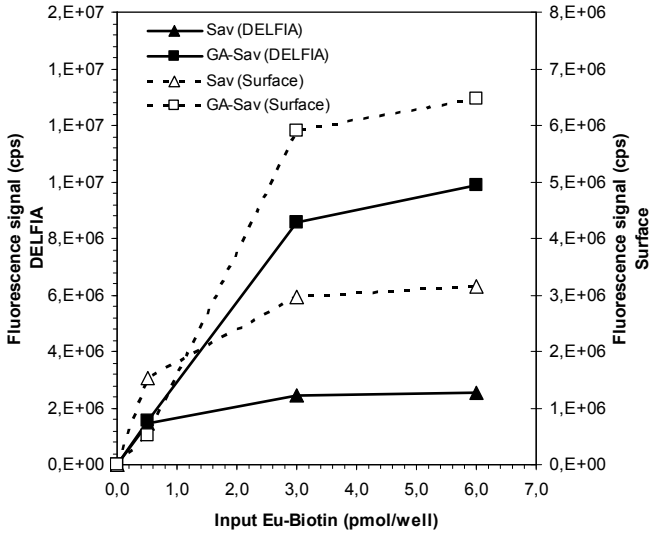


Figure 10. Binding isotherms of the Eu-biotin binding to the 2.5 mm spots coated with Sav or GA-Sav. Surface readout measurement (dotted lines) and DELFIA® measurement (solid lines) are presented.

4.2.2 Antigen binding capacity of spot surfaces in mini well format (I, II)

The intensity of Eu-derived fluorescence signals detected from the Eu-labelled hTSH antigen bound to streptavidin coated spot surfaces immobilised with biotinylated antibody, were increased when modified streptavidins and/or smaller site-directed biotinylated antibody fragments were used for coatings (Figure 11).

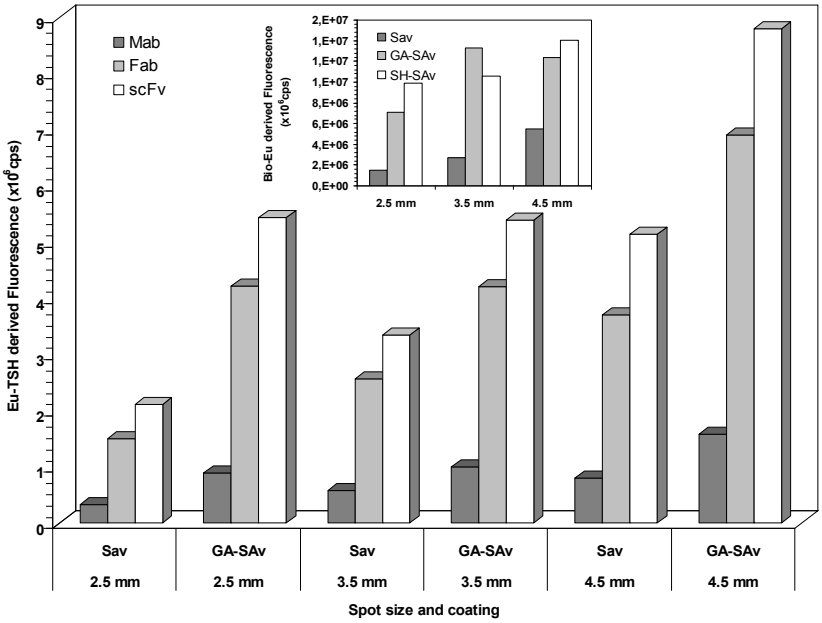


Figure 11. Maximal antigen (hTSH) binding capacities of streptavidin (Sav) and high-capacity streptavidin (GA-Sav) coated spot-surfaces in mini-well format, immobilised with different biotinylated capture antibodies (Mab, Fab and scFv). The inserted graph shows the maximal biotin binding capacities of the native Sav, SH-Sav or GA-Sav coated spot surfaces, measured after DELFIA® enhancement. (I with unpublished data)

When chemically modified Sav (generated either by glutaraldehyde linkage or by SATA activation) was adsorbed onto the spot-shaped solid phase, the antigen binding capacity was improved up to 6-fold, compared to the native Sav coatings, depending on the spot size and the capture antibody format used. The utilisation of small antibody fragments on streptavidin coatings contributed more clearly to the improved antigen binding efficiency, compared to the utilisation of modified streptavidin coatings. The gained improvement in the binding capacity of the surfaces utilising biotinylated Fab or scFv fragments, instead of Mab, on Sav surface was up to 6.8–fold, while only 2 – 3-fold further improvement was gained with the GA-Sav surface immobilised with corresponding biotinylated capture agents.

The increases in the antigen binding capacities along with the different coatings on spot surfaces are presented in the Table 3. The increase in the amount of bound antigen along with the increasing spot size more likely originates from the larger binding surface, thus providing enhanced binding capacity. However, the increase in the binding capacity obtained on the modified streptavidin surfaces was not directly correlated with the coated surface area (and the spot size) and was slightly emphasised on the smaller (2.5 mm) spot surfaces, as compared to the larger spots (3.5 mm and 4.5 mm). One reason for the non-linear correlation of the antigen binding capacities between different spot surfaces could be the differences in the surface-to-volume ratios of different spot sizes. For 2.5 mm spot coatings (coating volume 4 µl, total surface area of 6.5 mm^2) the surface-to-volume ratio is 15.8 cm^{-1}, while for the larger spots the corresponding surface-to-volume ratio is ~12 cm^{-1} (surface-to-volume ratios calculated for the area covering the bottom and the walls of the mini well indentation), providing shorter diffusion distance for the protein adsorption from the liquid onto the solid phase in the 2.5 mm spots, compared to the larger spots. On the Sav coatings the improvement in the binding capacities correlated well with the increasing surface areas. It is known that the adsorption behaviour of GA-Sav is different from the native Sav in coatings perfomed on regular microtitration wells (coating volume 200 µl, surface area 154 mm^2) and more favourable adsorption in the bottom of the wells have been noticed for GA-Sav (Välimaa L*, et al.*, 2003). This is likely true also in the case of SH-Sav molecules. The different coating practice of the spot production, compared to the conventional coating of regular microtitration wells, could add further discrepancy to the Sav and GA-Sav and/or SH-Sav adsorption behaviours.

Table 3. Average improvement in antigen binding capacities obtained with spot-shaped binding surfaces utilising different streptavidin coatings and biotinylated capture molecules (full size antibody or antibody fragments). The average values presented (as *X*-fold compared to a reference surface) are calculated for the mini wells having diameter of 2.5 mm, 3.5 mm and 4.5 mm.

Reference surface		Surface with advanced properties		Improvement in antigen binding capacity (*X*-fold)
streptavidin coating	biotinylated capture molecule	streptavidin coating	biotinylated capture molecule	
Sav	Mab	GA-SAv	Mab	1.8 – 2.7 -fold
Sav	Fab	GA-SAv	Fab	1.7 – 2.9 -fold
Sav	scFv	GA-SAv	scFv	1.7 – 2.7 -fold
Sav	Mab	Sav	Fab	4.5 – 4.8 -fold
Sav	Mab	Sav	scFv	6.0 – 6.8 -fold
GA-Sav	Mab	GA-SAv	Fab	4.4 – 4.9 -fold
GA-Sav	Mab	GA-SAv	scFv	4.7 – 6.5 -fold

Site-oriented attachment of the capture agents in solid-phase immunoassays has provided improvements in the overall assay performance (see Table 1 in the literature review section for examples). In this theses work site-specific biotinylation of recombinant antibody fragment was utilised to provide well oriented binding surface for

solid-phase spot assays. The functionality of the site-oriented attachments of the biotinylated Fab and scFv were compared to the randomly biotinylated Mabs, to reveal the advantages involved in the site-oriented binding (I, II). The improvement in the antigen binding efficiency of the streptavidin surfaces immobilised with biotinylated Fab or scFv fragments (shown in the Figure 11) are obviously derived from the dense functional binding surface formed through the site-oriented attachment of these fragments to the streptavidin coatings. This was most clearly demonstrated with the binding of fluorescently labeled cTnI antigen on the streptavidin/capture antibody coatings. The inherently fluorecent 9-dentate Eu-chelated used for the cTnI antigen labeling enabled the fluorescence signal to be measured directly form the dried spot surface, thus reflecting the local antigen binding density, rather than the total antigen binding capacity of the measured surfaces.

The antigen binding density of the immobilised Bio-Fab surface was up to 4.3 times higher than the corresponding values of the immobilised randomly biotinylated Mab surface (Figure 12). This indicates that the site-specifically biotinylated Fab fragments are more oriented upon the binding to the streptavidin coated polystyrene due to biotin-Sav linkage. In addition to the direct orientation of the Fab-immobilised surface, the smaller size of the Fab fragments compared to the full-size Mab also facilitate the surface coverage providing increased binding densities.

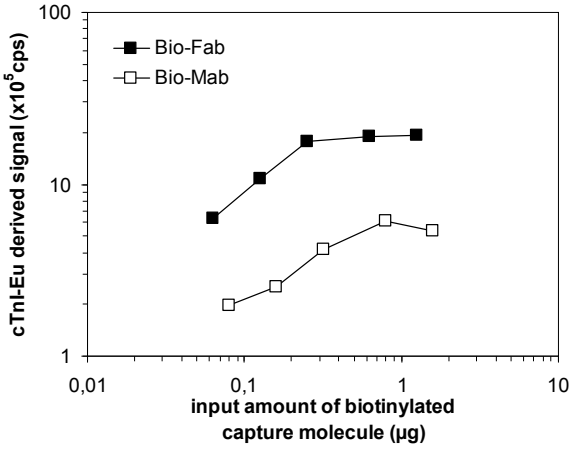


Figure 12. Antigen binding activities of site-specifically biotinylated Fab fragment and randomly biotinylated Mab as a function of surface coverage. Immobilisation of the biotinylated capture agents (bio-Mab, bio-Fab) have been achieved through a steptavidin coated polystyrene spot surface (mini well ∅ 3.5 mm). (Modified from the original publication II).

The amount of antigen binding was significantly greater for the site-directed biotinylated Fab on streptavidin surfaces at every antibody fragment surface coverage studied. However, it is important to note that while the antigen binding increased linearly up to a certain point (0.25 µg Fab input), further increase in the immobilisation amounts did not lead to significant improvement in the amount of antigen bound by the corresponding surface, indicating that the relative antigen binding efficiency of Sav-Bio-Fab surface decreases with surface coverage. This is most likely due to steric crowding at high surface loadings. The utilisation of SH-Sav or GA-Sav further im-

proved the functional binding density (and thus the total binding capacity) of these surfaces, probably since more biotin-binding sites are available due to three dimensional branched structure of the polymerised Savs formed through chemical linkage.

4.2.3 Characteristics of the spot surfaces created on a planar surface (IV)

The formulation of the spot-shaped binding surface on the bottom of a microtitration well through contact printing technique represented other approach for creating condense binding surfaces compared to the spot wells manufactured in a mini well format. One of the advantages of the contact printing technique was associated to the manufacture point of view. The preparation of the spots in mini wells required a careful handling of the wells to avoid the breakage of the fragile liquid droplet placed in the moulded areas of the spot wells during processing and this was emphasised for larger spot sizes (especially for 4.5 mm). In addition, the uneven distribution of the reporter molecules in a bioaffinity assay, which was evident for the mini well approach, was avoided when spots were patterned on a planar surface instead of coating inside the mini wells. Although the patterned spot areas, produced through the contact printing, was not defined with any physical boundaries (like in the mini wells), the very small coating volume (still enabling sufficient binding characteristics) facilitated the handling of the spot wells during processing. The surface binding area of 4.15 mm^2 achieved through the contact printing provided binding characteristics comparable to the conventional microtitration wells (154 mm^2).

The surface binding density of the bound Eu-biotin (when measured using surface readout) was found to be remarkably enhanced on the SH-Sav spots produced with additional betaine when compared to a reference streptavidin surface in a regular microtitration well (Figure 13). For some reason the spot coatings utilising native streptavidin molecules and betaine in combination to the contact printing technique did not produce improved solid phase binding properties compared to the other coatings prepared with native streptavidin. The improved binding densities of the SH-Sav/betaine surface enabled almost equal total binding capacities (when measured after DELFIA® enhancement) of the 4.15 mm^2 spots compared to the 154 mm^2 reference surface.

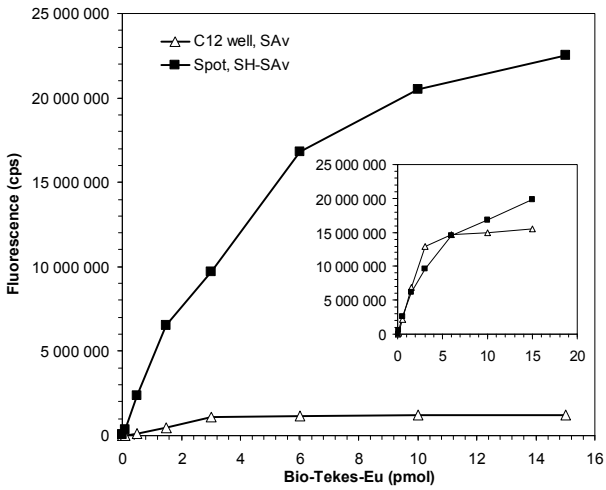


Figure 13. Surface binding capacities and surface densities of a reference Sav coated regular microtitration wells and contact printed spot wells coated with SH-Sav. The main picture shows the signals of surface readout measurement, reflecting the surface binding density. The inserted graph shows the fluorescence signals derived form the bound Eu-biotin after DELFA® enhancement, thus representing integrated signal from the whole binding surface. (unpublished)

Betaines are amphoteric surfactants (zwitterionic), which at pH above 3 are present in their internally compensated betaine-like form and at pH below 1 are completely protonated showing properties of a cationic surfactant (Holmberg K, *et al.*, 2003). Betaines have been utilised in many different industrial applications as well as in biotechnology. Betaine has been for example found to improve the amplification reactions in polymerase chain reactions (Miller GA, 1998). In this thesis study a betaine-like compound of 2-trimethylammoniumacetate (also known as trimethylglycine or TMG) was applied in coatings at pH 5.0, where the zwitterionic form is expected to be predominant. The molecular structure of trimethylglycine is presented in the Figure 14.

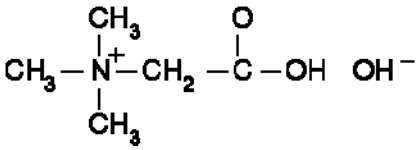


Figure 14. Molecular structure of betaine monohydrate (2-trimethylammoniumacetate).

SH-Sav spots were created using additional 1.5 M betaine in the coating solution. Based on the data by Diehl *et al*. (2001) this concentration has been found to be most suitable for the production of DNA microarrays on poly-*L*-lysine slides. They reported enhanced spot homogeneity and signal intensities at subsaturating analyte concentrations with the addition of 1.5 M betaine, but no improvement in the total binding capacity was reported. The actual mechanisms related to the enhanced solid phase binding properties of the SH-Sav/betaine spots compared to a reference streptavidin surface produced with the conventional methods remain somewhat unclear, although some speculative proposition can be made: one mechanism for the increased binding

efficiency is most likely the physical characteristics of betaine to reduce the evaporation rate. At high concentrations betaine is known to increase viscosity and reduce evaporation, without affecting the surface tension (Diehl F*, et al.*, 2001). During the course of evaporation betaine concentrates into the droplet until reaching a limiting value of 8 M, at which point the evaporation ceases (Diehl F*, et al.*, 2001). By this way, the streptavidin molecules concentrate in the solution during spot formation and the immobilisation may thus become enhanced, providing improved biotin binding properties of the spot coatings.

4.3 Immunoassays using spot surfaces

To study the applicability of the spot-shaped solid surfaces for novel solid-phase immunoassays, two different biomarkers, thyroid stimulating hormone (TSH, thyrotropin) and cardiac troponin I (cTnI), were applied as model analytes in spot assays. In the diagnosis of thyroid diseases TSH plays an important role being the primary biomarker of choice for the screening purposes when differentiation between the hypothyroidism and hyperthyroidism is aimed (Dussault JH*, et al.*, 1976, Kaplan MM, 1999). The screening of neonatal hypothyroidism has in recent years been focused in the measurement of TSH (LePage J*, et al.*, 2004, Spencer CA*, et al.*, 1993) and therefore more sensitive assays for TSH analysis are needed, that provides quantitative measurements of TSH concentrations at very low levels (≤ 0.01 mU/l).

4.3.1 TSH immunoassays on mini wells (I)

The TSH assays conducted in the spot wells, described in the original publication I, reached assay detection limits of 0.002 – 0.006 mU/l, providing 4th generation assay sensitivity (Spencer CA*, et al.*, 1993). Examples of dose-response curves of TSH immunoassays conducted on different solid phase binding surfaces utilised are presented in the Figure 15.

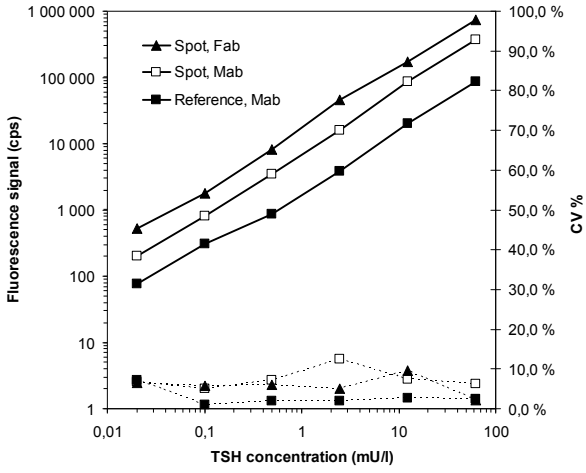
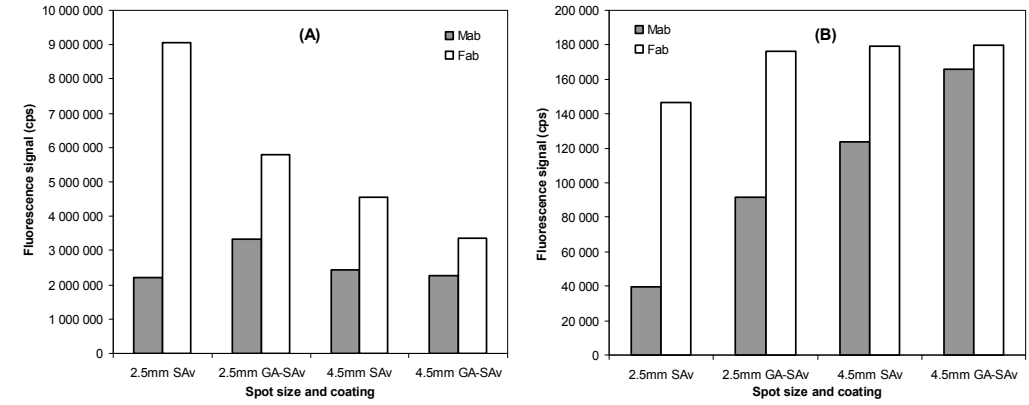


Figure 15. Dose-response curves of the TSH immunoassay performed in a reference assay and in two different spot assays. The reference assay is conducted on conventional microtitration wells, coated with native streptavidin and further immoblised with biotinylated Mab. The spot assays are conducted in streptavidin coated mini wells (∅ 2.5 mm) utilising either biotinylated full size antibody (Mab) or biotinylated Fab-fragment for solid phase capturing. (Unpublished figure).

The most sensitive THS immunoassay was obtained with the smallest spot area (mini well, diameter 2.5 mm), which was coated with native streptavidin and immobilised with the site-specifically biotinylated Fab fragments. The slopes of the dose-response curves for the spot-based assays, compared to a reference assay performed in conventional microtitration wells, was increased providing up to 8-fold improvement in the detectable signal levels (Figure 15). Surprisingly, no further improvements in the assay sensitivity was gained with the high capacity streptavidin coatings (GA-Sav or SH-Sav) and, contrast to the expectations, lower signal intensities were obtained with these coatings, compared to the coatings produced with the native streptavidin. This is inconsistent with the data showing improved biotin binding capacity and antigen binding efficiency in high capacity coatings performed in larger spots (I).

The reason for lower signal levels (when measured at surface readout) in larger high-capacity spots, compared to smaller ones, in the TSH immunoassay measurements is largely originated from uneven distribution of the bound analyte on the capture surface and for this reason the measuring technology applied (surface readout) is not capable of reading the overall signal intensities derived from the total binding area. The total antigen binding capacity of these surfaces in a TSH assay was revealed after dissociating the bound fluorescent label of the capture antibody from the chelating structure to a homogeneous solution through the DELFIA® enhancement (Figure 16, unpublished).

It was shown that increased signal intensities in DELFIA® enhancement were obtained with increasing surface capacity, which was contradictory to the results obtained with the surface readout measurements, but related to the actual total binding capacity of these surfaces. The mechanism behind this phenomenon has been previously discussed in more detail by Välimaa *et al.* (2008). They showed that the insufficient allocation of the measuring beam within the surface readout to cover the surface binding area more than ~5 mm^2, contributed to the signal drop in surface readout with the high capacity binding surfaces of larger spots. The preferred binding of the captured compounds to the edge of the spot area (due to incubation geometries and fluid dynamics) further facilitates the uneven distribution of the labelled molecules within the spot-shaped binding areas.



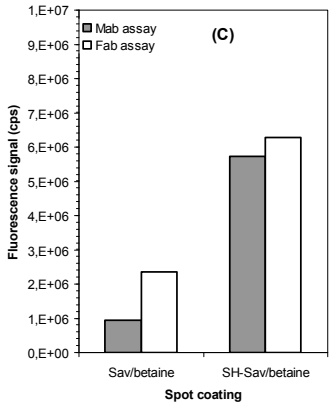


Figure 16. The signal drop seen through the surface measurement (A) on larger spot areas (4.5 mm compared to 2.5mm) and on high capacity surfaces (GA-Sav compared to Sav) in TSH immunoassays is compensated by DELFIA® measurement (B). TSH assays were conducted on 2.5 mm or 4.5 mm spot wells in mini well format, coated with either Sav or GA-Sav and immobilised with biotinylted Mab or biotinylated Fab. In cTnI immunoassay (C) the signal levels were detected directly from the dried spot surfaces. cTnI assays were conducted in spots produced with contact printing technique, thus providing a smooth bindinig surface without indentations. These spots were coated with either Sav/betaine or SH-SAv/betaine solution and immobilised with either biotinylated capture antibodies (Bio-Mab 19C7 and Bio-Mab 7F4; Mab assay) or with biotinylated Fab fragment (Bio-Fab 7F4; Fab assay). Note that the label molecules used in the TSH assay and in the cTnI assay are different and thus the signal levels represented in the figures (A) / (B) and (C) are not directly comparable. (Unpublished).

4.3.2 cTnI immunoassays on spot surfaces (II, IV, unpublished)

Low detection limits are prerequisite also for the measurement of cardiac troponins (including cTnI), which are released into blood circulation after necrosis of heart muscle cells and are thus considered as highly specific biochemical marker of myocardial infarction (Katrukha AG*, et al.*, 1997, Wu AH*, et al.*, 1998). During this thesis work, different capture antibodies, individually or as in combination (each having specificity against different epitopes of cTnI antigen) were studied in the cTnI immunoassays, conducted in streptavidin coated 3.5 mm spot-wells, to obtain the best possible cTnI assay performance (II). The solid phase capture antibodies were used either in a form of randomly biotinylated Mab or site-oriented biotinylated Fab-fragment. It was shown that the utilisation of several capture antibodies, instead of a single capture antibody, provided improved signal intensities and enhanced assay sensitivity (Table 4). Furthermore, the utilisation of the site-oriented attachment of the capture agents onto the solid surface provided further improvement in the surface binding capacity and density, reaching an assay detection limit of 0.002 µg/l (Table 4). The difference

in the binding affinities between the biotinylated Mab and Fab (revealed through Biacore analysis) most likely contributed to the obtained improvement in the overall performance of the cTnI assay when the site-specifically biotinylated Fab-fragments, instead of Mab, were used for capturing the analyte. The advantage of the smaller Fab fragments, over the full-size Mab, in solid phase coatings was not only limited to the obtained improvement in the detectable signal intensities providing assay sensitivity of 0.002 µg/l, but also contributed to reduction of non-specific interference with the sample matrix (II). This feature is highly important in immunoassays utilising solid phase antibodies susceptible to negative or positive interference caused by the sample matrix (e.g. complement binding or interference caused by autoantibodies). The observed serum related interference with one of the capture Mab was only detectable when this particular Mab was used alone for capturing and the interference was not detected when combined with other antibodies or used as Fab fragment on the capture surface (II). Thus, the utilisation of more than one capture antibody, specific to different epitopes of the captured analyte can be highly recommended if the analyte is easily exposed to proteolytic cleavage, being heterogeneous in structure, like that of cardiac troponins.

Table 4. Detection limits of cTnI immunoassay performed in 3.5 mm spot coated microwells or in regular C-format microtitration wells. The assay numbering (#) corresponds to the numbering used in the original publication II. The represented detection limits are obtained from the experiments discussed in the original publication II and from additional unpublished data.

				Detection limit* (µg/l)	
Assay #	Capture antibody	Tracer antibody	Streptavidin coating	in spot wells	in C12-wells
1	Mab 7F4[a]	8I7-Eu [1]	native Sav	0.016	-
2	Mab 4C2[b]	8I7-Eu [1]	native Sav	0.21	-
3	Mab 7F4[a], Mab 4C2[b]	8I7-Eu [1]	native Sav	0.003	0.011
3	Mab 7F4[a], Mab 4C2[b]	8I7-Eu [1]	SH-SAv	0.005	-
4	Mab 7F4[a], Mab 4C2[b], Mab 19C7[c]	7B9-Eu [2]	native Sav	0.004	-
5	Fab 7F4[a]	8I7-Eu [1]	native Sav	0.011	-
6	Fab 4C2[b]	8I7-Eu [1]	native Sav	0.061	-
7	Fab 7F4[a], Fab 4C2[b]	8I7-Eu [1]	native Sav	0.002	0.007
7	Fab 7F4[a], Fab 4C2[b]	8I7-Eu [1]	SH-SAv	0.008	-
8	Fab 7F4[a], Fab 4C2[b], Mab 19C7[c]	7B9-Eu [2]	native Sav	0.006	-

[1] *cTnI specific monoclonal antibody*
[2] *cTnC specific monoclonal antibody*
[a] *specificity against C-terminal epitopes of cTnI (a.a.r. 190-196)*
[b] *specificity against N-terminal epitopes of cTnI (a.a.r. 23-29)*
[c] *specificity against mid-fragent epitopes (a.a.r. 41-49)*
** The detection limits correspond to the cTnI concentration calculated from 3-times of the standard deviation of the zero dose*

In addition to the mini wells, spot surfaces prepared through contact printing were applied for cTnI immunoassays. This alternative approach for spot well manufacturing provided advanced properties for measuring technology point of view: contrary to the immunoassays conducted on mini wells, the signal levels measured with the surface readout measurement in cTnI immunoassays performed on spot surfaces produced with contact printing technique (thus providing a smooth binding surface without indentation), showed signal levels consistent with the surface antigen binding capacity

(Figure 16, unpublished), and thus in these assays the inadequacy of the measuring technique to cover the whole binding area was eluded. The importance of adapting a proper measuring technology in particular assay design to obtain a substantial advantage of the solid phase properties to the assay sensitivity was thus demonstrated.

The cTnI immunoassay on contact printed spots utilising SH-SAv/betaine coating provided approximately 4-fold improvement in the specific signal levels compared to a reference assay performed on conventional microtitration wells with Sav coating (Figure 17). Furthermore a detection limit of 0.005 µg/l was obtained, providing a 2-fold improvement in the assay sensitivity compared to a reference assay (DL = 0.01 µg/l). Although the specific signal level were additionally 2-3–fold higher when biotinylated Fab fragments (instead of Mab) were used on contact printed spots (utilising SH-SAv/betiane surface), no further improvement in the assay sensitivity was obtained due to equal increase in the background signal levels.

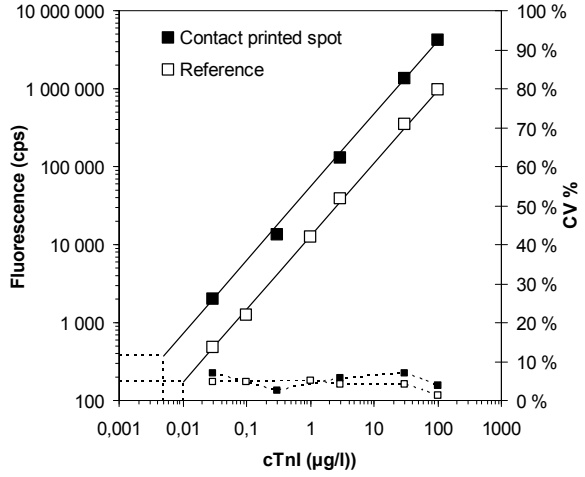


Figure 17. dose-response curves of cTnI immunoassays performed on conventional microtitration wells (*squares*) or on contact printed spots (*filled squares*). Mab 7F4 and Mab 4C2 (reference) or Mab 7F4 and Mab 19C7 (contact printed spots) were used as capture antibodies on Sav surface (reference) or on SH-SAv surface (spot). Eu-Mab 8I7 was used as tracer antibody. Fluorescence signal was measured from dried surfaces. The analytical detection limits were: 0.01 µg/l (for reference) and 0.005 µg/ml (for contact printed spots). (IV, Unpublished figure)

4.3.3 Immunoassay kinetics (I, II)

In solid-phase based assays the adsorption kinetics of the analyte binding to the solid capture surface is largely determined by the analyte concentration (mass transport and surface-to-volume ratio) and by the functional properties of the immobilised capture layer (Esser P, 1992b), and is further emphasised in miniaturised solid phases (Kusnezow W*, et al.*, 2006). In the spot assays, the surface binding density and the dimensions of the binding area correlated well with the obtained signal levels compared to the equilibrium signal at a given time-points, revealing enhanced kinetic behaviour with larger high-capacity spot coatings (I, II). By increasing the surface density of the primary streptavidin coating and/or by increasing the immobilisation density of the

capture antibody layer in a spot surface, a 2–fold improvement in the assay kinetics (in terms of the ratio of the saturated signal level and the signal level reached at different time points) was obtained, compared to a regular Sav-Mab coating performed in an identical spot geometry (Figure 18). The use of a larger spot-shaped binding areas enabled further improvement in the assay kinetic due to the increased overall surface capacity. Although the kinetic of the spot assays was obviously slower compared to the kinetic behaviour observed in a conventional assay format (i.e. assays conducted in a regular C12-geometry microtitration well), improved signal intensities were already obtained at short incubation times (5 to 15 min). This enables potentially more sensitive assay performance within short assay turnaround times (I, II).

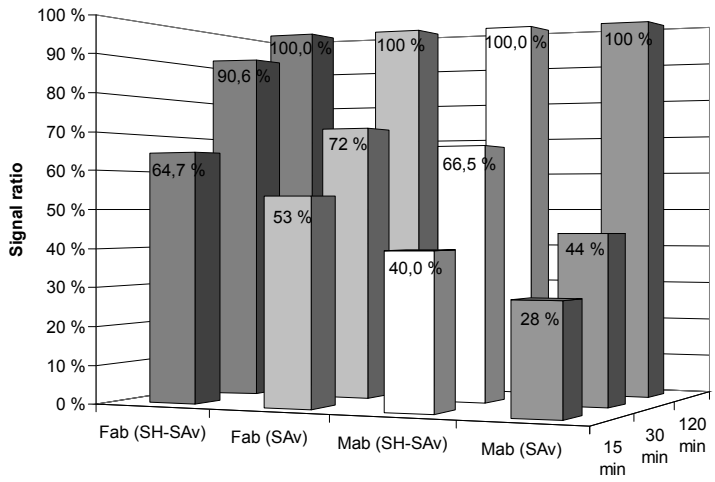


Figure 18. TSH immunoassay kinetics performed in 2.5 mm spot surface, coated with native streptavidin (Sav) or with modified streptavidin (SH-Sav) and immobilised with biotinylated monoclonal antibodies (Mab) or antibody Fab fragments. The fluorescence signal of the bound analyte-tracer complex was monitored at incubation times of 15, 30 and 120 min. (I, unpublished figure).

5 CONCLUSIONS

The increasing knowledge of disease pathophysiology and clinical diagnostics has positioned new demands for the analytical techniques utilised for the detection of novel biomarkers. This has initiated the development of new tools in the technical field of bioaffinity assays and over the last decade the research in the diagnostic field has paid extensive attention on the miniaturisation of the bio-analytical systems (Angenendt P, 2005). Miniaturisation of ligand-binding assays not only reduces costs by decreasing reagent consumption, but can simultaneously exceed the sensitivity of macroscopic techniques (Borrebaeck CA, 2000). Although the spot assay concept developed during this thesis work is not a truly miniaturised assay system in its actual meaning (the term miniaturisation refers mainly to systems encompassing devices and reaction vessels in a size of a micro- or nanoscale, for example microarrays or lab-on-chip devices), the gained improvement in the assay sensitivity compared to a conventional immunoassays performed in regular microtitration wells was clearly demonstrated. The improvement in the detection limits obtained through the spot coatings are largely originated from the dense binding surface achieved through the modified streptavidin coatings and site-oriented attachment of the antibody fragments to capture the analyte in a defined area coinciding with the excitation beam of the measuring instrument. The obtained improvements in the solid phase characteristics of the developed spot surfaces would certainly provide enhanced assay properties also for the assay concept which are based on completely miniaturised systems.

During this thesis study chemically modified streptavidin was used as the primary coatings on polystyrene surface to provide high-capacity functional binding surface for biotinylated capture molecules. The sandwich formation of the heterogeneous immunoassay, utilising site-specifically biotinylated antibody fragments on streptavidin coatings, was reduced to a size of a spot more closely coinciding with the excitation beam providing further improvement in the assay sensitivity.

The main conclusions based on the original publications are:

I The production of immunoassay wells by coating the streptavidin in a liquid droplet to provide a well-defined spot-shaped binding surface is a highly applicable approach to improve the immunoassay performance in terms of assay sensitivity. On the miniaturised binding surface the role of surface density becomes most critical for obtaining desirable assay functionality. It was shown that the increase in the binding surface density in a solid-phase immunoassay applied for quantitative measurements of biomolecules can be obtained by utilising highly dense binding area coinciding with the excitation beam of the measuring instrument in the surface readout.

II Utilisation of recombinant site-specifically oriented antibody Fab fragments as a solid phase binding agents in a cTnI immunoassay provides numerous advantages over the full-size antibody. These include improved assay sensitivity and avoidance of sample matrix derived unwanted interference. Furhermore the importance of applying several different capture antibodies immobilised onto a solid surface for capturing a highly heterogeneous analytes was highlighted.

III The chemical modification of a native biomolecule is a highly applicable method to provide altered properties for immobilisation. The immobilisation efficiency of streptavidin can be improved by cross-linking the individual streptavidin molecules into larger protein conjugates. By immobilising these polymerised streptavidin molecules onto a polystyrene surface, enhanced binding capacity (up to 2–fold) for biotinylated molecules can be obtained. Furthermore, formation of highly bridged surface structures achieved by the cross-linked streptavidin coatings clearly improves the surface stability in terms of protein desorption as well as the surface tolerability against harsh chemical conditions, such as denaturing conditions. The degree of cross-linking (i.e. the size of the formed streptavidin polymers) highly determines the immobilisation efficiency and surface stability.

IV Spot-shaped binding surface produced on the bottom of a conventional microtitration well through contact printing is a useful approach for spot-well production. The planar surface created through the contact printing technique enables spot-shaped binding surfaces, which are highly applicable for surface readout measurements. With the optimised coating buffer composition remarkable improvement (18-fold) in the biotin binding density was obtained with the contact printed spots, compared to a reference surface prepared with native streptavidin on conventional microtitration wells. This enabled almost equal total biotin binding capacities when the 4.2 mm^2 spot surface was compared with the surface area of 154 mm^2 in conventional microtitration wells.

In short conclusion, the dense and oriented binding surface, with the combination of spot assay design and surface measurement approach is a well suitable technology to be applied in solid-phase immunoassays to gain improved solid phase performance in terms of assay sensitivity and kinetics, characteristics that are very useful to improve the immunoassay performance of further point-of care assays. The utilisation of cross-linked streptavidin molecules adsorbed onto a polystyrene surface, in combination with the site-specifically biotinylated antibodies or antibody fragments and spot coating technology enables the production of highly functional solid phases capable for high capacity capturing of different analytes.

6 ACKNOWLEDGEMENTS

This doctoral thesis study was carried out during the years 2003-2007 at the Department of Biotechnology, at the University of Turku. The study was financially supported by Innotrac Diagnostics Oy and the Finnish Funding Agency for Technology and Innovation (TEKES).

First of all, Professor Timo Lövgren is greatly acknowledged for giving me the opportunity to work at the Department of Biotechnology. It was a privilege to work under his guidance, in such an inspiring and pleasant atmosphere. I also highly appreciate his confidence in me regarding to other projects outside this thesis project.

In addition, I want to express my sincere gratitude to my main supervisor, Professor Kim Pettersson, whose knowledge in the clinical diagnostics and immunoassay world and his sense of humour has made this research project to become a surprising and interesting journey. I owe my deepest thanks to him for the valuable time he spent in the numerous discussions and for providing so many new and innovative ideas.

I want to thank all my co-authors, especially Dr. Lasse Välimaa for being involved in the spot project already from the very beginning; Johanna Hellström for her excellent work with the troponin assays; Dr. Susann Eriksson for teaching me so many things about the troponins, and Markus Vehniäinen for his guidance through the recombinant antibody production. I am also grateful to our industrial collaborator, Dr. Harri Takalo, the director of the research and development at Innotrac Diagnostics Oy, for his straightforward co-operation and for providing all the different spotting devices. I thank Anastasia Bereznikova for her friendly participation.

I am very grateful to Professor Matti Karp and Professor Hans Söderlund for reviewing this thesis and for providing constructive and invaluable comments.

I wish to thank all the students; Johanna Hellström, Heidi Virtanen, Emilia Engström, Katja Laurikainen, Hannu Leino, Niina Salminen, Marja-Leena Järvenpää and Heidi Hyytiä, who have taken part in the spot project during these years. Without your effort in the laboratory this thesis would not had been accomplished. Especially Heidi Virtanen and Emilia Engström are acknowledged for their tenacious work within the printing of spots.

Furthermore, I wish to thank the entire personnel at the department of Biotechnology, the former and present staff, who have created a pleasant and enjoyable working atmosphere. Because of you I enjoyed each day at work. I wish to thank Marja-Liisa Knuuti for excellent assistance in the office matters and Mirja Jaala, Marja Maula, Veikko Wahlroos, Martti Sointusalo and Pirjo Pietilä for enabling the laboratory work running smoothly. I also appreciate Pirjo Laaksonen for providing so many practical

tips and for being the "mummu" in our AIO-lab. I am deeply indebted to Saara Wittfooth, Virve Hagren, Dr. Piia von Lode and Dr. Susann Eriksson, who have shared so many intensive moments with me in the laboratory as well as outside the work. Special thank goes to Saara for her cheerful company during the numerous congress trips and parties we have taken part in together. In addition, I am thankful to Katri Kuningas, Tiina Wikman, Hanna Polari and Noora Ristiniemi for their friendship. Finally, Lasse Välimaa and Saara Wittfooth, thank you for reading a draft of this thesis and for providing valuable feedback.

I owe my deepest thanks to all my other friends, whose companionship has given me so many happy moments and unforgettable memories. I know that these experiences will only continue to grow in the future.

My most sincere and warmest thanks belongs to my family; my parents Riitta and Kari, and my twin sister Katja, whose support and belief in me have been essential in completing this doctoral thesis. My warmest thanks belongs to my grandparents; Outi, Maire and Toivo. Thank you for being there for me. I am also very grateful to Pauli's family for their support and kindness.

Finally, this thesis would not had been accomplished without the love and understanding of my husband, Pauli, who has shared the ups and downs with me during the last years of work. Your never-ending love has guided me through the sticky moments and revealed the real meaning of life. Emilia, our dearest daughter, thank you for showing me the end-all priorities in the world.

Raisio, February 2009

Johanna Ylikotila

7 REFERENCES

Afanassiev, V, Hanemann, V and Wolfl, S (2000). Preparation of DNA and protein micro arrays on glass slides coated with an agarose film. *Nucleic Acids Res* **28**:E66

Al-Abdulla, IH, Mellor, GW, Childerstone, MS, Sidki, AM and Smith, DS (1989). Comparison of three different activation methods for coupling antibodies to magnetisable cellulose particles. *J Immunol Methods* **122**:253-8

Angenendt, P (2005). Progress in protein and antibody microarray technology. *Drug Discov Today* **10**:503-11

Aybay, C (2003). Differential binding characteristics of protein G and protein A for Fc fragments of papain-digested mouse IgG. *Immunol Lett* **85**:231-5

Batalla, P, Fuentes, M, Grazu, V, Mateo, C, Fernandez-Lafuente, R and Guisan, JM (2008). Oriented covalent immobilization of antibodies on physically inert and hydrophilic support surfaces through their glycosidic chains. *Biomacromolecules* **9**:719-23

Beier, M and Hoheisel, JD (1999). Versatile derivatisation of solid support media for covalent bonding on DNA-microchips. *Nucleic Acids Res* **27**:1970-7

Benters, R, Niemeyer, CM and Wohrle, D (2001). Dendrimer-activated solid supports for nucleic acid and protein microarrays. *Chembiochem* **2**:686-94

Bigelow, WC, Pickett, DL and Zisman, WA (1948). Oleophilic monolayers. I. Films adsorbed from solution in non-polar liquids. *J colloid Sci* **1**:513-538

Bjerner, J, Nustad, K, Norum, LF, Olsen, KH and Bormer, OP (2002). Immunometric assay interference: incidence and prevention. *Clin Chem* **48**:613-21

Björck, L and Kronvall, G (1984). Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. *J Immunol* **133**:969-74

Blanco, EM, Horton, MA and Mesquida, P (2008). Simultaneous investigation of the influence of topography and charge on protein adsorption using artificial nanopatterns. *Langmuir* **24**:2284-7

Bonroy, K, Frederix, F, Reekmans, G, Dewolf, E, De Palma, R, Borghs, G, Declerck, P and Goddeeris, B (2006). Comparison of random and oriented immobilisation of antibody fragments on mixed self-assembled monolayers. *J Immunol Methods* **312**:167-81

Borrebaeck, CA (2000). Antibodies in diagnostics - from immunoassays to protein chips. *Immunol Today* **21**:379-82

Bradford, MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248-54

Brinkley, M (1992). A brief survey of methods for preparing protein conjugates with dyes, haptens, and cross-linking reagents. *Bioconjug Chem* **3**:2-13

Brockmann, EC, Cooper, M, Stromsten, N, Vehniainen, M and Saviranta, P (2005). Selecting for antibody scFv fragments with improved stability using phage display with denaturation under reducing conditions. *J Immunol Methods* **296**:159-70

Brogan, KL, Wolfe, KN, Jones, PA and Schoenfisch, MH (2003). Direct oriented immobilization of F(ab') antibody fragments on gold. *Anal Chim Acta* **496**:73-80

Buijs, J, Lichtenbelt, JWT, Norde, W and Lyklema, J (1995). Adsorption of monoclonal IgGs and their F(ab')2 fragments onto polymeric surfaces. *Colloids Surf B: Biointerfaces* **5**:11-23

Bull, HB (1956). Adsorption of bovine serum albumin on glass. *Biochim Biophys Acta* **19**:464-71

Butler, JE (2000). Solid supports in enzyme-linked immunosorbent assay and other solid- phase immunoassays. *Methods* **22**:4-23.

Butler, JE, Ni, L, Brown, WR, Joshi, KS, Chang, J, Rosenberg, B and Voss, EW, Jr. (1993). The immunochemistry of sandwich ELISAs--VI. Greater than 90% of monoclonal and 75% of polyclonal anti-fluorescyl capture antibodies (CAbs) are denatured by passive adsorption. *Mol Immunol* **30**:1165-75

Butler, JE, Ni, L, Nessler, R, Joshi, KS, Suter, M, Rosenberg, B, Chang, J, Brown, WR and Cantarero, LA (1992). The physical and functional behavior of capture antibodies adsorbed on polystyrene. *J Immunol Methods* **150**:77-90

Büttner, J, Borth, R, Boutwell, JH, Broughton, PM and Bowyer, RC (1979). International Federation of Clinical Chemistry. Committee on Standards. Expert Panel on Nomenclature and Principles of Quality Control in Clinical Chemistry. Approved recommendation (1978) on quality control in clinical chemistry. Part 1. General principles and terminology. *Clin Chim Acta* **98**:129F-143F

Castner, D and Ratner, B (2002). Biomedical surface science: Foundations to frontiers. *Surf Sci* **500**:28-60

Chapman, RS and Ratcliffe, JG (1982). Covalent linkage of antisera to microparticulate cellulose using 1,1'-carbonyldiimidazole: a rapid, practical method with potential use in solid-phase immunoassay. *Clin Chim Acta* **118**:129-34

Cho, IH, Paek, EH, Lee, H, Kang, JY, Kim, TS and Paek, SH (2007). Site-directed biotinylation of antibodies for controlled immobilization on solid surfaces. *Anal Biochem* **365**:14-23

Christopoulos, TK, Lianidou, ES and Diamandis, EP (1990). Ultrasensitive time-resolved fluorescence method for alpha-fetoprotein. *Clin Chem* **36**:1497-502

Churbanova, IY, Tronin, A, Strzalka, J, Gog, T, Kuzmenko, I, Johansson, JS and Blasie, JK (2006). Monolayers of a model anesthetic-binding membrane protein: formation, characterization, and halothane-binding affinity. *Biophys J* **90**:3255-66

Crowe, J, Dobeli, H, Gentz, R, Hochuli, E, Stuber, D and Henco, K (1994). 6xHis-Ni-NTA chromatography as a superior technique in recombinant protein expression/purification. *Methods Mol Biol* **31**:371-87

Davies, C (2001). Introduction to immunoassay principles. *The immunoassay hanbook*. Nature publishing group, New york.

Della-Penna, D, Christoffersen, RE and Bennett, AB (1986). Biotinylated proteins as molecular weight standards on Western blots. *Anal Biochem* **152**:329-32

DeSilva, BS and Wilson, GS (1995). Solid phase synthesis of bifunctional antibodies. *J Immunol Methods* **188**:9-19

Diamandis, EP (1990). Detection techniques for immunoassay and DNA probing applications. *Clin Biochem* **23**:437-43

Diamandis, EP and Christopoulos, TK (1991). The biotin-(strept)avidin system: principles and applications in biotechnology. *Clin Chem* **37**:625-36

Diehl, F, Grahlmann, S, Beier, M and Hoheisel, JD (2001). Manufacturing DNA microarrays of high spot homogeneity and reduced background signal. *Nucleic Acids Res* **29**:E38

Dussault, JH, Parlow, A, Letarte, J, Guyda, H and Laberge, C (1976). TSH measurements from blood spots on filter paper: a confirmatory screening test for neonatal hypothyroidism. *J Pediatr* **89**:550-2

Ekins, R and Chu, F (1992). Multianalyte microspot immunoassay. The microanalytical 'compact disk' of the future. *Ann Biol Clin (Paris)* **50**:337-53

Ekins, R, Chu, F and Biggart, E (1990). Multispot, multianalyte, immunoassay. *Ann Biol Clin (Paris)* **48**:655-66

Ekins, R and Edwards, P (1997). On the meaning of "sensitivity". *Clin Chem* **43**:1824-31

Ekins, RP (1989). Multi-analyte immunoassay. *J Pharm Biomed Anal* **7**:155-68

Ekins, RP and Chu, FW (1991). Multianalyte microspot immunoassay--microanalytical "compact disk" of the future. *Clin Chem* **37**:1955-67

Ellman, GL (1959). Tissue sulfhydryl groups. *Arch Biochem Biophys* **82**:70-7

Elsner, H and Mouritsen, S (1989). A method for modifying the surface of a polymer. EP 0319957

Engvall, E and Perlmann, P (1972). Enzyme-linked immunosorbent assay, Elisa. 3. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *J Immunol* **109**:129-35

Esser, P (1990). Detergents in polystyrene ELISA. *Nunc Bulletin No. 8.* NUNC A/S, Roskilde, Denmark.

Esser, P (1992a). Covalent binding of DNA to CovaLink™ NH - methods and applications. *Nunc Bulletin No. 10.* NUNC A/S, Roskilde, Denmark.

Esser, P (1992b). The surface/volume ratio in solid phase assays. *Nunc Bulletin No. 10.* NUNC A/S, Roskilde, Denmark.

Esser, P (1997). Principles in adsorption to polystyrene. *Nunc Bulletin No. 6*. Nunc A/S, Roskilde, Denmark.

Fenton, JJ, Brunstetter, S, Gordon, WC, Rippe, DF and Bell, ML (1984). Diagnostic efficacy of a new enzyme immunoassay for creatine kinase MB isoenzyme. *Clin Chem* **30**:1399-401

Fowler, JM, Stuart, MC and Wong, DK (2007). Self-assembled layer of thiolated protein G as an immunosensor scaffold. *Anal Chem* **79**:350-4

Gering, JP, Quaroni, L and Chumanov, G (2002). Immobilization of antibodies on glass surfaces through sugar residues. *J Colloid Interface Sci* **252**:50-6

Gitlin, G, Bayer, EA and Wilchek, M (1988). Studies on the biotin-binding site of streptavidin. Tryptophan residues involved in the active site. *Biochem J* **256**:279-82

Glökler, J and Angenendt, P (2003). Protein and antibody microarray technology. *J Chromatogr B Analyt Technol Biomed Life Sci* **797**:229-40

Gobi, KV, Matsumoto, K, Toko, K, Ikezaki, H and Miura, N (2007). Enhanced sensitivity of self-assembled-monolayer-based SPR immunosensor for detection of benzaldehyde using a single-step multi-sandwich immunoassay. *Anal Bioanal Chem* **387**:2727-35

Goding, JW (1978). Use of staphylococcal protein A as an immunological reagent. *J Immunol Methods* **20**:241-53

Green, NM (1990). Avidin and streptavidin. *Methods Enzymol* **184**:51-67

Green, NM, Konieczny, L, Toms, EJ and Valentine, RC (1971). The use of bifunctional biotinyl compounds to determine the arrangement of subunits in avidin. *Biochem J* **125**:781-91

Haab, BB, Dunham, MJ and Brown, PO (2001). Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. *Genome Biol* **2**:

Hackler, L, Jr., Dorman, G, Kele, Z, Urge, L, Darvas, F and Puskas, LG (2003). Development of chemically modified glass surfaces for nucleic acid, protein and small molecule microarrays. *Mol Divers* **7**:25-36

Hemmilä, I (1985). Fluoroimmunoassays and immunofluorometric assays. *Clin Chem* **31**:359-70

Hemmilä, I, Dakubu, S, Mukkala, VM, Siitari, H and Lövgren, T (1984). Europium as a label in time-resolved immunofluorometric assays. *Anal Biochem* **137**:335-43

Hermanson, GT (1996a). Antibody modification and conjugation. *Bioconjugate Techniques*. Academic Press Inc., San Diego. pp. 456-493

Hermanson, GT (1996b). Functional targets. *Bioconjugate Techniques*. Academic Press Inc., San Diego. pp. 3-136

Hermanson, GT (1996c). Tags and Probes. *Bioconjugate Techniques*. Academic Press Inc., San Diego. 297-416

Hochuli, E, Dobeli, H and Schacher, A (1987). New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *J Chromatogr* **411**:177-84

Holmberg, K, Jönsson, B, Kronberg, B and Lindman, B (2003). Introduction to surfactants. *Surfactants and Polymers in Aqueous Solution*. John Wiley & Sons, Ltd., 1-37

Howell, EE, Nasser, J and Schray, KJ (1981). Coated tube enzyme immunoassay: factors affecting sensitivity and effects of reversible protein binding to polystyrene. *J Immunoassay* **2**:205-25

Höök, F, Rodahl, M, Kasemo, B and Brzezinski, P (1998). Structural changes in hemoglobin during adsorption to solid surfaces: effects of pH, ionic strength, and ligand binding. *Proc Natl Acad Sci U S A* **95**:12271-6

Ikeda, T, Hata, Y, Ninomiya, KI, Ikura, Y, Takeguchi, K, Aoyagi, S, Hirota, R and Kuroda, A (2008). Oriented immobilization of antibodies on a silicon wafer using Si-tagged protein A. *Anal Biochem*

Jackson, TM and Ekins, RP (1986). Theoretical limitations on immunoassay sensitivity. Current practice and potential advantages of fluorescent Eu3+ chelates as non-radioisotopic tracers. *J Immunol Methods* **87**:13-20

Johannsson, A, Ellis, DH, Bates, DL, Plumb, AM and Stanley, CJ (1986). Enzyme amplification for immunoassays. Detection limit of one hundredth of an attomole. *J Immunol Methods* **87**:7-11

Kang, IK, Lee, DW, Lee, SK and Akaike, T (2003). Grafting of lactose-carrying styrene onto polystrene dishes using plasma glow discharge and their interaction with hepatocytes. *J Mater Sci Mater Med* **14**:611-6

Kanno, S, Yanagida, Y, Haruyama, T, Kobatake, E and Aizawa, M (2000). Assembling of engineered IgG-binding protein on gold surface for highly oriented antibody immobilization. *J Biotechnol* **76**:207-14

Kaplan, MM (1999). Clinical perspectives in the diagnosis of thyroid disease. *Clin Chem* **45**:1377-83

Karir, T, Hassan, PA, Kulshreshtha, SK, Samuel, G, Sivaprasad, N and Meera, V (2006). Surface modification of polystyrene using polyaniline nanostructures for biomolecule adhesion in radioimmunoassays. *Anal Chem* **78**:3577-82

Karyakin, AA, Presnova, GV, Rubtsova, MY and Egorov, AM (2000). Oriented immobilization of antibodies onto the gold surfaces via their native thiol groups. *Anal Chem* **72**:3805-11

Katrukha, AG, Bereznikova, AV, Esakova, TV, Pettersson, K, Lovgren, T, Severina, ME, Pulkki, K, Vuopio-Pulkki, LM and Gusev, NB (1997). Troponin I is released in bloodstream of patients with acute myocardial infarction not in free form but as complex. *Clin Chem* **43**:1379-85

Kersten, B, Wanker, EE, Hoheisel, JD and Angenendt, P (2005). Multiplex approaches in protein microarray technology. *Expert Rev Proteomics* **2**:499-510

Khatri, OP, Adachi, K, Murase, K, Okazaki, K, Torimoto, T, Tanaka, N, Kuwabata, S and Sugimura, H (2008). Self-assembly of ionic liquid (BMI-PF6)-stabilized gold nanoparticles on a silicon surface: chemical and structural aspects. *Langmuir* **24**:7785-92

Klajnert, B and Bryszewska, M (2001). Dendrimers: properties and applications. *Acta Biochim Pol* **48**:199-208

Kobayashi, A, Goto, M, Sekine, T, Masumoto, A, Yamamoto, N, Kobayashi, K and Akaike, T (1992). Regulation of differentiation and proliferation of rat hepatocytes by lactose-carrying polystyrene. *Artif Organs* **16**:564-7

Kochwa, S, Brownell, M, Rosenfield, RE and Wasserman, LR (1967). Adsorption of proteins by polystyrene particles. I. Molecular unfolding and acquired immunogenicity of IgG. *J Immunol* **99**:981-6

Kokko, L, Sandberg, K, Lövgren, T and Soukka, T (2004). Europium(III) chelate-dyed nanoparticles as donors in a homogeneous proximity-based immunoassay for estradiol. *Anal Chim Acta* **503**:155-162

Koutsoukos, PG, Mumme-Young, CA, Norde, W and Lyklema, J (1982). Effect of the nature of the substrate on the adsorption of human plasma albumin. *Colloids and Surfaces* **5**:93-104

Krebber, A, Bornhauser, S, Burmester, J, Honegger, A, Willuda, J, Bosshard, HR and Pluckthun, A (1997). Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. *J Immunol Methods* **201**:35-55

Kurkela, R, Vuolas, L and Vihko, P (1988). Preparation of F(ab')2 fragments from monoclonal mouse IgG1 suitable for use in radioimaging. *J Immunol Methods* **110**:229-36

Kusnezow, W and Hoheisel, JD (2003). Solid supports for microarray immunoassays. *J Mol Recognit* **16**:165-76

Kusnezow, W, Syagailo, YV, Goychuk, I, Hoheisel, JD and Wild, DG (2006). Antibody microarrays: the crucial impact of mass transport on assay kinetics and sensitivity. *Expert Rev Mol Diagn* **6**:111-24

König, T and Skerra, A (1998). Use of an albumin-binding domain for the selective immobilisation of recombinant capture antibody fragments on ELISA plates. *J Immunol Methods* **218**:73-83

Ladam, G, Schaaf, P, Decher, G, Voegel, J and Cuisinier, FJ (2002). Protein adsorption onto auto-assembled polyelectrolyte films. *Biomol Eng* **19**:273-80

Lee, JM, Park, HK, Jung, Y, Kim, JK, Jung, SO and Chung, BH (2007). Direct immobilization of protein G variants with various numbers of cysteine residues on a gold surface. *Anal Chem* **79**:2680-7

Lee, M and Walt, DR (2000). A fiber-optic microarray biosensor using aptamers as receptors. *Anal Biochem* **282**:142-6

LePage, J, Peters, S and Lehotay, DC (2004). Assay of neonatal TSH from blood spots using the Architect i2000, an immunoassay analyzer. *Clin Biochem* **37**:415-7

Linder, M, Szilvay, GR, Nakari-Setala, T, Soderlund, H and Penttila, M (2002). Surface adhesion of fusion proteins containing the hydrophobins HFBI and HFBII from Trichoderma reesei. *Protein Sci* **11**:2257-66

Linder, MB, Szilvay, GR, Nakari-Setala, T and Penttila, ME (2005). Hydrophobins: the protein-amphiphiles of filamentous fungi. *FEMS Microbiol Rev* **29**:877-96

Lubarsky, GV, Browne, MM, Mitchell, SA, Davidson, MR and Bradley, RH (2005). The influence of electrostatic forces on protein adsorption. *Colloids Surf B Biointerfaces* **44**:56-63

Lövgren, T, Meriö, L, Mitrunen, K, Mäkinen, ML, Mäkela, M, Blomberg, K, Palenius, T and Pettersson, K (1996). One-step all-in-one dry reagent immunoassays with fluorescent europium chelate label and time-resolved fluorometry. *Clin Chem* **42**:1196-201

Lövgren, T and Pettersson, K (1990). Time resolved fluoroimmunoassays, advantages and limitations. *Luminescence immunoassay and molecular applicaitons*. CRC Press, Boston, USA. 234-253

MacBeath, G and Schreiber, SL (2000). Printing proteins as microarrays for high-throughput function determination. *Science* **289**:1760-3

Mahajan, S, Vaijayanthi, B, Rembhotkar, G, Chand Gupta, K and Kumar, P (2007). Choice of polymer matrix, its functionalization and estimation of functional group density for preparation of biochips. *Methods Mol Biol* **381**:165-87

Malan Borel, I, Gentile, T, Angelucci, J, Margni, RA and Binaghi, RA (1990). Asymmetric Fab glycosylation in guinea-pig IgG1 and IgG2. *Immunology* **70**:281-3

Mariani, M, Camagna, M, Tarditi, L and Seccamani, E (1991). A new enzymatic method to obtain high-yield F(ab)2 suitable for clinical use from mouse IgGl. *Mol Immunol* **28**:69-77

Marquette, CA, Bouteille, F, Corgier, BP, Degiuli, A and Blum, LJ (2008). Disposable screen-printed chemiluminescent biochips for the simultaneous determination of four point-of-care relevant proteins. *Anal Bioanal Chem*

Matson, RS, Rampal, JB and Coassin, PJ (1994). Biopolymer synthesis on polypropylene supports. I. Oligonucleotides. *Anal Biochem* **217**:306-10

Matthews, OA, Shipway, AN and Stoddart, JF (1998). Dendrimers - branching out from curiosities into new technologies. *Prog Polym Sci* **23**:1-56

McNaught, AD and Wilkinson, A (1997). IUPAC Compendium of Chemical Terminology - The Gold Book. Blackwell Scientific Publication, Oxford.

Mendoza, LG, McQuary, P, Mongan, A, Gangadharan, R, Brignac, S and Eggers, M (1999). High-throughput microarray-based enzyme-linked immunosorbent assay (ELISA). *Biotechniques* **27**:778-80, 782-6, 788.

Miller, GA (1998). Method for nucleotide sequence amplification. US Patent 5846716

Min, DH and Mrksich, M (2004). Peptide arrays: towards routine implementation. *Curr Opin Chem Biol* **8**:554-8

Mukkala, V-M, Hänninen, E and Hemmilä, I (1993). Synthesis of two biotin isothiocyanate derivatives. Eighth European Symposium on Organic Chemistry. Sitges, Barcelona, Spain, 86

Mukkala, VM, Mikola, H and Hemmila, I (1989). The synthesis and use of activated N-benzyl derivatives of diethylenetriaminetetraacetic acids: alternative reagents for labeling of antibodies with metal ions. *Anal Biochem* **176**:319-25

Niveleau, A, Sage, D, Reynaud, C, Bruno, C, Legastelois, S, Thomas, V and Dante, R (1993). Covalent linking of haptens, proteins and nucleic acids to a modified polystyrene support. *J Immunol Methods* **159**:177-87

Nomellini, JF, Duncan, G, Dorocicz, IR and Smit, J (2007). S-layer-mediated display of the immunoglobulin G-binding domain of streptococcal protein G on the surface of Caulobacter crescentus: development of an immunoactive reagent. *Appl Environ Microbiol* **73**:3245-53

Norde, W (1986). Adsorption of proteins from solution at the solid-liquid interface. *Adv Colloid Interface Sci* **25**:267-340

Oreskes, I and Singer, JM (1961). The mechanism of particulate carrier reactions. I. Adsorption of human gamma-globulin to polystyrene latex particles. *J Immunol* **86**:338-44

O'Shannessy, DJ and Hoffman, WL (1987). Site-directed immobilization of glycoproteins on hydrazide-containing solid supports. *Biotechnol Appl Biochem* **9**:488-96

Page, JD, Derango, R and Huang, AE (1998). Chemical modification of polystyrene's surface and its effect on immobilized antibodies. *Colloids Surf A Physicochem Eng Asp* **132**:193-201(9)

Peluso, P, Wilson, DS, Do, D, Tran, H, Venkatasubbaiah, M, Quincy, D, Heidecker, B, Poindexter, K, Tolani, N, Phelan, M, Witte, K, Jung, LS, Wagner, P and Nock, S (2003). Optimizing antibody immobilization strategies for the construction of protein microarrays. *Anal Biochem* **312**:113-24

Penney, CL, Gauldie, J, Evelegh, M, Penney, MT, Chong, D and Horsewood, P (1989). Polycarbonate membranes: a novel surface for solid-phase determinations with utility in field format serological assays. *J Immunol Methods* **123**:185-92

Pesce, AJ, Ford, DJ, Gaizutis, M and Pollak, VE (1977). Binding of protein to polystyrene in solid-phase immunoassays. *Biochim Biophys Acta* **492**:399-407

Piironen, T, Villoutreix, BO, Becker, C, Hollingsworth, K, Vihinen, M, Bridon, D, Qiu, X, Rapp, J, Dowell, B, Lovgren, T, Pettersson, K and Lilja, H (1998). Determination and analysis of antigenic epitopes of prostate specific antigen (PSA) and human glandular kallikrein 2 (hK2) using synthetic peptides and computer modeling. *Protein Sci* **7**:259-69

Prime, K and Whitesides, G (1991). Self-assembled organic monolayers: model systems for studying adsorption of proteins at surfaces. *Science* **252**:1164-1167

Rasmussen, S (1990). Covalent immobilization of biomolecules onto polystyrene MicroWells for use in biospecific assays. *Ann Biol Clin (Paris)* **48**:647-50

Renberg, B, Shiroyama, I, Engfeldt, T, Nygren, PK and Karlstrom, AE (2005). Affibody protein capture microarrays: synthesis and evaluation of random and directed immobilization of affibody molecules. *Anal Biochem* **341**:334-43

Riener, CK, Kada, G and Gruber, HJ (2002). Quick measurement of protein sulfhydryls with Ellman's reagent and with 4,4'-dithiodipyridine. *Anal Bioanal Chem* **373**:266-76

Sanger, F (1959). Chemistry of insulin; determination of the structure of insulin opens the way to greater understanding of life processes. *Science* **129**:1340-4

Schetters, H (1999). Avidin and streptavidin in clinical diagnostics. *Biomolecular Engineering* **16**:73-78

Schreiber, F (2000). Structure and growth of self-assembling monolayers. *Prog Surf Sci* **65**:151-256

Shervedani, RK, Mehrjardi, AH and Zamiri, N (2006). A novel method for glucose determination based on electrochemical impedance spectroscopy using glucose oxidase self-assembled biosensor. *Bioelectrochemistry* **69**:201-8

Sigal, GB, Bamdad, C, Barberis, A, Strominger, J and Whitesides, GM (1996). A self-assembled monolayer for the binding and study of histidine-tagged proteins by surface plasmon resonance. *Anal Chem* **68**:490-7

Singh, P, Moll, F, 3rd, Lin, SH and Ferzli, C (1996). Starburst Dendrimers: A Novel Matrix for Multifunctional Reagents in Immunoassays. *Clin Chem* **42**:1567-8

Singh, P, Moll, F, 3rd, Lin, SH, Ferzli, C, Yu, KS, Koski, RK, Saul, RG and Cronin, P (1994). Starburst dendrimers: enhanced performance and flexibility for immunoassays. *Clin Chem* **40**:1845-9.

Soltys-Robitaille, CE, Ammon, DM, Jr., Valint, PL, Jr. and Grobe, GL, 3rd (2001). The relationship between contact lens surface charge and in-vitro protein deposition levels. *Biomaterials* **22**:3257-60

Spencer, CA, Schwarzbein, D, Guttler, RB, LoPresti, JS and Nicoloff, JT (1993). Thyrotropin (TSH)-releasing hormone stimulation test responses employing third and fourth generation TSH assays. *J Clin Endocrinol Metab* **76**:494-8

Sun, X, Liu, J and Lee, ML (2008). Surface modification of glycidyl-containing poly(methyl methacrylate) microchips using surface-initiated atom-transfer radical polymerization. *Anal Chem* **80**:856-63

Suzuki, N, Quesenberry, MS, Wang, JK, Lee, RT, Kobayashi, K and Lee, YC (1997). Efficient immobilization of proteins by modification of plate surface with polystyrene derivatives. *Anal Biochem* **247**:412-6

Takalo, H, Mukkala, VM, Mikola, H, Liitti, P and Hemmila, I (1994). Synthesis of europium(III) chelates suitable for labeling of bioactive molecules. *Bioconjug Chem* **5**:278-82

Tischer, W, Maier, J and Deeg, R (1991). Process for preparing a carrier useful in immunoassays by deposition of a complex of a specifically binding substance with hydrophobic protein, and the resulting carrier. US Patent 5061640

Turkova, J (1999). Oriented immobilization of biologically active proteins as a tool for revealing protein interactions and function. *J Chromatogr B Biomed Sci Appl* **722**:11-31

Wacker, R, Schroder, H and Niemeyer, CM (2004). Performance of antibody microarrays fabricated by either DNA-directed immobilization, direct spotting, or streptavidin-biotin attachment: a comparative study. *Anal Biochem* **330**:281-7

Walter, JG, Kokpinar, O, Friehs, K, Stahl, F and Scheper, T (2008). Systematic investigation of optimal aptamer immobilization for protein-microarray applications. *Anal Chem* **80**:7372-8

van Erp, R, Linders, YE, van Sommeren, AP and Gribnau, TC (1992). Characterization of monoclonal antibodies physically adsorbed onto polystyrene latex particles. *J Immunol Methods* **152**:191-9

Varga, JM, Klein, GF and Fritsch, P (1990). Binding of a mouse monoclonal IgE (anti-DNP) antibody to radio-derivatized polystyrene-DNP complexes. *Faseb J* **4**:2678-83

Warren, DJ, Bjerner, J, Paus, E, Bormer, OP and Nustad, K (2005). Use of an in vivo biotinylated single-chain antibody as capture reagent in an immunometric assay to decrease the incidence of interference from heterophilic antibodies. *Clin Chem* **51**:830-8

von Lode, P, Hagren, V, Palenius, T and Lovgren, T (2003a). One-step quantitative thyrotropin assay for the detection of hypothyroidism in point-of-care conditions. *Clin Biochem* **36**:121-8

von Lode, P, Rosenberg, J, Pettersson, K and Takalo, H (2003b). A europium chelate for quantitative point-of-care immunoassays using direct surface measurement. *Anal Chem* **75**:3193-201

Wu, AH, Feng, YJ, Moore, R, Apple, FS, McPherson, PH, Buechler, KF and Bodor, G (1998). Characterization of cardiac troponin subunit release into serum after acute myocardial infarction and comparison of assays for troponin T and I. American Association for Clinical Chemistry Subcommittee on cTnI Standardization. *Clin Chem* **44**:1198-208

Väisänen, V, Peltola, MT, Lilja, H, Nurmi, M and Pettersson, K (2006). Intact free prostate-specific antigen and free and total human glandular kallikrein 2. Elimination of assay interference by enzymatic digestion of antibodies to F(ab')2 fragments. *Anal Chem* **78**:7809-15

Välimaa, L, Pettersson, K, Rosenberg, J, Karp, M and Lövgren, T (2004). Quantification of streptavidin adsorption in microtitration wells. *Anal Biochem* **331**:376-84

Välimaa, L, Pettersson, K, Vehniainen, M, Karp, M and Lovgren, T (2003). A high-capacity streptavidin-coated microtitration plate. *Bioconjug Chem* **14**:103-11

Välimaa, L, Ylikotila, J, Kojola, H, Soukka, T, Takalo, H and Pettersson, K (2008). Streptavidin-coated spot surfaces for sensitive immunoassays using fluorescence surface readout. *Anal Bioanal Chem* **391**:2135-44

Yalow, RS and Berson, SA (1960). Immunoassay of endogenous plasma insulin in man. *J Clin Invest* **39**:1157-75

Yang, H, Fung, SY, Pritzker, M and Chen, P (2007). Modification of hydrophilic and hydrophobic surfaces using an ionic-complementary peptide. *PLoS ONE* **2**:e1325

Young, DB, Fohn, MJ and Buchanan, TM (1985). Use of a polysulfone membrane support for immunochemical analysis of a glycolipid from Mycobacterium leprae. *J Immunol Methods* **79**:205-11

Zammatteo, N, Girardeaux, C, Delforge, D, Pireaux, JJ and Remacle, J (1996). Amination of polystyrene microwells: application to the covalent grafting of DNA probes for hybridization assays. *Anal Biochem* **236**:85-94

Zhang, H and Meyerhoff, ME (2006). Gold-coated magnetic particles for solid-phase immunoassays: enhancing immobilized antibody binding efficiency and analytical performance. *Anal Chem* **78**:609-16

Zhang, S, Holmes, T, Lockshin, C and Rich, A (1993). Spontaneous assembly of a self-complementary oligopeptide to form a stable macroscopic membrane. *Proc Natl Acad Sci U S A* **90**:3334-8

Zhang, S, Lockshin, C, Cook, R and Rich, A (1994). Unusually stable beta-sheet formation in an ionic self-complementary oligopeptide. *Biopolymers* **34**:663-72

Zhang, S, Wang, N, Yu, H, Niu, Y and Sun, C (2005). Covalent attachment of glucose oxidase to an Au electrode modified with gold nanoparticles for use as glucose biosensor. *Bioelectrochemistry* **67**:15-22