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DISSERTATION

Analytical Protein Microarrays: Advancements towards Clinical Applications

ausgeführt zum Zwecke der Erlangung des akademischen Grades einer Doktorin der Naturwissenschaften unter der Leitung von

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Kurzfassung der Dissertation

Protein Mikroarrays stellen eine leistungsstarke Technologie mit einem breiten Spektrum an Einsatzmöglichkeiten dar. Medizinische Diagnostik, Pharmaforschung, Lebensmittelsicherheit oder Umweltmonitoring - zahlreiche Anwendungsgebiete können von dieser Technologie profitieren. Bisher kamen Protein Mikroarrays vor allem in der Grundlagenforschung zur Anwendung, während ihr Potential in klinischen und industriellen Bereichen noch nicht voll ausgeschöpft ist.

Die wichtigsten Charakteristika analytischer Protein-Mikroarrays sind hoher Durchsatz und relativ niedrige Kosten aufgrund des minimalen Verbrauchs an Reagenzien; Multiplexing, kurze Analysenzeiten, und die Möglichkeit der funktionellen Integration in kompakten Messinstrumenten oder Lab-on-Chip Systemen. Noch wird die Methode in erster Linie in Forschungslabors verwendet. Damit die Technologie an Attraktivität für den diagnostischen Markt gewinnt, müssen weitere Verbesserungen hinsichtlich Messempfindlichkeit, Reproduzierbarkeit und Analysezeiten erreicht werden. Weiters muss die Automatisierung und Entwicklung von Point-of-Care Systemen vorangetrieben werden, und die Kosten der erforderlichen Instrumente für die Chipherstellung und Auslese reduziert werden.

Die wichtigste Ergebnisse der vorliegenden Arbeit waren: die Einführung eines kombinierten Assay-Formats, das einerseits für die gleichzeitige Messung von Analyten in sehr hohen (µg/mL) und sehr niedrigen (pg/mL) Konzentrationsbereichen geeignet ist und zweitens in einem automatisierten System einsetzbar ist. Weiters Protokolle für verkürzte Analysenzeiten; die Optimierung der Assays in komplexen biologischen Flüssigkeiten, um eine hohe Empfindlichkeit zu erreichen; die Auswertung und Charakterisierung von Oberflächenmodifizierungen für Mikroarrays auf Glas, Metall und polymeren Trägern; und die Herstellung, Charakterisierung und Integration von Nanostrukturen für plasmonische Biochips als Mittel zur Verstärkung von Fluoreszenzsignalen. Mit den erzielten Ergebnissen konnten somit Verbesserungen bezüglich Sensitivität und Analysenzeit erreicht werden und die Basis für weitere Chipentwicklungen im diagnostischen Bereich gelegt werden.

Abstract

Protein microarrays represent a powerful technology with the potential to serve as tools for the detection of a broad range of analytes in numerous applications such as diagnostics, drug development, food safety, and environmental monitoring. So far, especially fundamental studies in molecular and cell biology have been conducted using protein microarrays, while the potential for clinical and industrial applications is not yet fully exploited.

Key features of analytical protein microarrays include high throughput and relatively low costs due to minimal reagent consumption, multiplexing, fast kinetics and hence measurements, and the possibility of functional integration. Still, to date the technology is primarily used in research laboratories due to some technical hurdles and a lack of approved standards. Issues that need significant improvement to make the technology more attractive for the diagnostic market are for instance: too low sensitivity and deficiency in reproducibility, inadequate analysis time, lack of automation and portable instruments, and cost of instruments necessary for chip production and read-out.

The scope of the thesis at hand was to solve some of these problems. Main achievements reported herein are: the introduction of a combined assay format for the simultaneous measurement of high and low abundant analytes applicable for automated measurements; protocols for reduced assay times; the optimization of assays in complex biological fluids achieving high sensitivity; the evaluation and characterization of surface chemistries on glass, metal and polymeric supports for the integration into a biochip; and the characterization and integration of nanostructures manufactured by nanoimprint lithography for application in plasmon enhanced fluorescence read-out. Based on the improvements in sensitivity and analysis time achieved herein further chip developments in the diagnostic field will be pursued.

Acknowledgements

First I would like to express my gratitude to my supervisor Prof. Martina Marchetti-Deschmann for her support and for stepping in unhesitatingly.

Sincere thanks are given to Dr. Claudia Preininger for her continuous support and advice. Claudia, all I know about biosensors, I have learned from you.

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The Austrian Research Promotion Agency (FFG) financially supported NILbiochip (no. 815810:1304BI) and NILplasmonics (no. 830271), projects within the research cluster NILaustria I and NILaustria II.

Above all I would like to thank my spouse Robert Hany-Schmatzberger and my whole family, in particular my parents Inge und Hans Sauer, for supporting me at all times.

Dedicated to the memory of Prof. Georg Haberhauer.

Contents

1 Aim of the thesis

In a review article from 2012, Zhu and Qian from John Hopkins University School of Medicine claim, "As a powerful technology platform, it would not be surprising if protein microarrays will become one of the leading technologies in proteomic and diagnostic fields in the next decade" [Zhu and Qian 2012].

Already in the past protein microarrays have been hyped as future key technologies in a wide field of applications, from basic research to industrial applications. In some respects the promises materialized, as for instance protein microarrays are now widely used in basic research and drug screening. On the other hand microarrays are still far from being used routinely in clinical practice. The question arises what features have to be implemented and what improvements have to be made in order to fully exploit the technology. In the past we have identified various obstacles that have to be overcome in order to promote protein microarray technology in the diagnostic field.

Challenges:

- Low reproducibility

The accurate quantification of a signal following a binding event on the chip relies on low intra- and inter- spot variability in the first line and results from a highly controlled probe dispensing. Optimization of the experimental set-up including quality of chip substrate and coating technique, print buffers, immobilization strategy, dispensing (pins), probe concentration, blocking and assay design, as well as image and data analysis is crucial in order to decrease variability [Preininger and Sauer 2003a, b, Sauer et al. 2005a].

- Low sensitivity

For proteins there is no efficient signal amplification method available such as PCR for DNA microarrays. Crucial factors for high sensitivity are the intrinsic affinity of the biorecognition element (BRE) as well as its immobilization rendering biological activity and accessibility. Further high quantum yield labels and efficient detection techniques promote low detection limits [Preininger et al. 2005a, b].

- Assay time

Quick diagnosis and immediate treatment is what a clinician expects from a bioanalytical method [Kost et al. 1999]. Not only reaction kinetics is a parameter for the time a test takes. Assay design in terms of incubation- and washing steps necessary, read out and even more data analysis can significantly increase the time a test takes from sample input to answer output.

- Miniaturization and system integration

Standard microarray instruments are big and heavy, but also most of the competing techniques are not portable, e.g. chromatographic techniques such as high performance liquid chromatography (HPLC), mass spectrometry (MS), and flow cytometry, depend on big instruments in central labs. Making instruments for microarrays portable will be a major driver for market entry [Tsafarti Bar-Ad et al. 2011].

- Production cost

Even the best diagnostic tool will only succeed in the health market if it is cheap enough to be accepted by health insurances for reimbursement. The cost factor has to be considered when designing the assay, choosing the biorecognition elements, substrates, labels and surface chemistries. Integration of nanotechnology for signal enhancement for instance may increase production cost tremendously, while miniaturization can reduce reagent and sample consumption.

The scope of the thesis at hand was to work on some of these challenges:

Immobilization of the capture molecules in an optimal way is the unconditional basis for **sensitive and reproducible assays**. In Chapter 3 the effect of probe immobilization on **stability, accessibility, sensitivity and reproducibility** is discussed. Immobilization chemistry, substrate coating, and surface blocking had been one of our research focuses in the past, which is also reflected in a number of publications (see p5).

Multiplexed measurements, **assay time** and **sensitivity** are addressed in Chapter 4. The combination of biomarkers relevant for the diagnosis of sepsis is a demanding task. Proteins of **different quaternary structure and size** such as a monomer (IL-6) and a pentamer (CRP) but also neopterin, a pteridin of low molecular mass have to be detected. Further, **high and low abundant proteins** have to be quantified in parallel.

Chapter 5 takes the diagnostic chip to the next level by dealing with reliable point-of-care **analyte testing in human biological fluids serum, plasma and saliva**. Main obstacles for working in body fluids are interferences from matrix proteins such as fibrinogen, IgG and lysozyme as well as crossreactivity of antibodies. The aim of this work was to establish a test that does not rely on a prior purification step for the clinical samples.

The outcome of this endeavour was published in one peer reviewed book chapter and two peer reviewed journals. A review article was submitted.

U. Sauer [2011] **Impact of substrates for probe immobilization**. Chapter in: Protein Microarrays: Methods and Protocols. U. Korf (ed.) Methods in Molecular Biology, vol 785, pp 363-378, Springer Science and Business Media, NY.
The book chapter describes fundamental requirements for immobilization strategies in protein microarray production and reviews popular surface chemistries. (Chapter 3)

U. Sauer, P. Domnanich, C. Preininger [2011] **Protein chip for the parallel quantification of high and low abundant biomarkers for sepsis.** Anal. Biochem. 419, 46-52. The paper deals with the challenge of multiplexed measurements of biomarkers for early sepsis diagnosis and describes assay designs varying in time and sensitivity. (Chapter 4)

U. Sauer, J. Pultar, C. Preininger [2012] **Critical role of the sample matrix in a point-of-care protein chip for sepsis.** J. Immunol. Methods, 378, 44–50.
An important step towards real life applications is to deal with the challenges of measuring in body fluids without costly and time consuming sample preparation. (Chapter 5)

Parts of Chapter 2, General Introduction, have been submitted as a review article with the title **Analytical Protein Microarrays: Advancements towards Clinical Applications** to Sensors, MDPI AG, Basel, Switzerland.

Finally, Chapter 6 includes four conference presentations dealing mainly with the integration of nanotechnology for improved probe immobilization and signal enhancement.

U. Sauer, J. Pultar, C. Preininger. **A biochip for the detection of CRP, PCT and IL-6, the major biomarkers for inflammation.** Advances in Microarray Technologies, 7-8 May 2008, Barcelona, Spain. The poster presentation introduces the concept for a biochip for inflammation. For the purpose of signal enhancement several strategies of 3D-immoblization were examined, such as hydrogels, nanowells, particles, and enzymatic crosslinking of probes.

U. Sauer, C. Preininger, M. Chouiki, R. Schöftner. **Fabrication of nanostructures for protein chips: effect of wettability on immobilization and assay performance.** Fifth International Conference on Advanced Materials and Nanotechnology (AMN-5), February 7-11 2011, Wellington, New Zealand. Poster. For fabrication of nanostructured chips we use epoxy-functional materials compatible with the NIL process and able to bind proteins. The effect of nanostructuring on both the immobilization capacity and chip performance was studied.

U. Sauer, A. Solar, C. Preininger. **Gold discs produced by residue-free UV-NIL and subsequent lift-off for integration in biosensors.** 12th International Conference on Nanoimprint and Nanoprint Technology 2013, 21-23 October 2013, Barcelona, Spain. Poster presentation on the development of a lab-scale process for residue free nanoimprinting.

U. Sauer, C. Preininger, J. Dostalek, K. Gier, S. Gogalic, S. Hageneder, M. Bauch, A. Solar. **Making protein biochips more attractive for real-life applications.** 11th BBMEC, International Biosensor Conference, Regensburg, Germany, September 26 -30, 2015. Oral presentation proposing new developments for protein biochips, such as integration of plasmonic structures, and combining biological and artificial biorecognition elements.

Some selected earlier publications are listed in the following. They shall demonstrate preliminary work in the field of functional surfaces, signal enhancement and data analysis, which was essential to come up with the mature biomarker detection platform.

U. Sauer, L. Bodrossy, C. Preininger (2009) **Evaluation of substrate performance for a microbial diagnostic microarray using a 4 parameter ranking.** Analytica Chimica Acta 632/2, 240-246.

J. Pultar, U. Sauer. P. Domnanich, C. Preininger (2009) **Aptamer-antibody on-chip sandwich immunoassay for detection of CRP in spiked serum.** Biosensors & Bioelectronics 24, 1456–1461.

P. Domnanich, U. Sauer, J. Pultar, C. Preininger (2009) **Protein microarray for the analysis of human melanoma biomarkers.** Sensors & Actuators B 139, 2-8.

K. Derwinska, U. Sauer, C. Preininger (2008) **Adsorption versus covalent, statistically oriented and covalent, site-specific IgG immobilization on poly(vinyl alcohol)-based surfaces.** Talanta 77, 52-658.

K. Derwinska, L. A. Gheber, U. Sauer, L. Schorn, C. Preininger (2007) **Effect of surface parameters on the performance of protein-arrayed hydrogel chips: a comprehensive study.** Langmuir 23, 10551-10558.

U. Sauer, C. Preininger and Hany-Schmatzberger (2005) **Quick & Simple: Quality Control of Microarray Data.** Bioinformatics 21, 1572-1578.

U. Sauer, P. Preininger, G. Krumpel, N. Stelzer, W. Kern (2005) **Signal enhancement of protein chips.** Sensors and Actuators B 107/1, 178-183.

C. Preininger, U. Sauer, W. Kern and J. Dayteg (2004) **Photoactivatable copolymers of vinylbenzylthiocyanate as immobilization matrix for biochips.** Anal Chem.76/20, 6130-6.

Current follow – up of this work shortly described in the following shall demonstrate the continuing research endeavours, mainly dealing with real-life applications such as neonatal sepsis and bladder cancer; miniaturization and automatization, and implementation of signal enhancement strategies.

Another paper dealing with measurements in a complex matrix, namely urine, was published 2015: S. Gogalic, U. Sauer, S. Doppler, C. Preininger (2015) **Bladder cancer microarray to detect aberrant levels of proteins in urine.** Analyst 140 (3):724-35.

In P. Buchegger, U. Sauer, H. Toth-Székély, C. Preininger, 2012. **Miniaturized Protein Microarray with Internal Calibration as Point-of-Care Device for Diagnosis of Neonatal Sepsis.** Sensors 12(2), 1494-1508. we established a miniaturized protein microarray platform. Consuming only 4 µl of patient serum it is an excellent tool for diagnosis in newborns.

Further miniaturization on probe level was achieved in I. Tsarfati-BarAd, U. Sauer, C. Preininger, L. A. Gheber, 2011. **Miniaturized protein arrays: model and experiment.** Biosensors & Bioelectronics 26, 3774-3781. The paper describes the theoretical basis for spot miniaturization and discusses the impact of surface chemistry.

The full integration of the multiparameter sepsis chip into a point-of-care instrument developed at Fraunhofer IPM in Freiburg was reported in M. Kemmler, U. Sauer, E. Schleicher, C. Preininger, A. Brandenburg, 2014. **Biochip point-of-care device for sepsis diagnostics.** Sensors and Actuators B, 192, 205-215. The instrument combines fluidic handling for all assay steps, a special biochip, a detection system based on Total Internal Reflection Fluorescence and software for image analysis and data processing.

Studies on the integration of micro-nanostructures were reported in C. Preininger, U. Sauer, M. Chouiki, R. Schöftner, 2011. **Nanostructures in protein chips: effect of print buffer additive and wettability on immobilization and assay performance.** Microelectronic Eng. 88, 1856-1859.

The paper S. Gogalic, S. Hageneder, C. Ctortecka, M. Bauch, I. Khan, C. Preininger, U. Sauer, J. Dostalek, 2015. **Plasmonically Amplified Fluorescence Bioassay with Microarray Format.** Proc. SPIE 9506, Optical Sensors 2015, 95060N (May 5, 2015) reports on the integration of crossed relief gratings for plasmonically amplified fluorescence read-out in a standard protein microarray platform.

2 General Introduction

The concept of DNA microarray technology of the early 1990s was quickly followed by the development of immunoanalytical microarrays. With the first reports of Ekins and co-workers fluorescence immunoassays started to replace methods using radioisotopic labels dominating medicine and other biologically-related fields at that time. But the authors not only aimed at a non-isotopic immunoassay method, they also suggested the concept of "microspot immunoassays" on solid supports allowing multianalyte measurements [Ekins et al. 1990, Ekins and Chu 1991]. Nowadays the general concept of a protein microarray comprises arraying of capture probes to discrete positions onto a solid support, sample incubation, and optical detection of the analyte binding.

In some respects protein microarrays outperform conventional chromatographic techniques such as GC-MS or HPLC-MS. Key features are high throughput and low cost due to minimal reagent consumption, multiplexing, fast kinetics and hence measurements, and the possibility of functional integration. Miniaturization is one of the pre-requisites for the latter one. Nowadays microarray technology is embracing nanotechnolgy as well; as for instance by integration of functional elements in the nanoscale serving as labels, separation and support material for biorecognition elements, and for signal enhancement [Nam et al. 2004; Chen et al. 2010; Preininger et al. 2011].

Depending on the application Zhu and Snyder [2001] define two types of protein microarrays: analytical and functional [Zhu and Qian 2012]. Functional protein microarrays are developed for the study and elucidation of the function and interaction of various biological molecules, while analytical protein microarrays aim at the quantitative detection of analytes in various samples.

Many fundamental studies have been conducted in a large variety of molecular and cell biology areas, using protein microarrays. Functional protein microarrays have been developed for instance for analysis of enzyme kinetics [Arenkov et al. 2000], analysis of expression profiles [Templin et al. 2002, Haab et al. 2001], and understanding disease at molecular level [Guthy and Voshol 2015].

Analytical protein microarrays are often implemented using antibodies as recognition and detection elements and are thus referred to as "on-chip immunoassays" herein. On-chip immunoassays have been shown to detect a vast range of analytes in numerous applications, such as clinical diagnosis [Kemmler et al. 2014] and patient stratification [Domnanich et al. 2009], drug development [Sereni et al. 2013], environmental monitoring [Tschmelak et al. 2005], and food safety [Sapsford et al.2006].

On-chip immunoassays (representing analytical microarrays) are the main focus of this thesis at hand since they constitute the type of microarray that can lead to powerful tools in clinical practice and industrial applications.

Table 1. lists a number of examples, to demonstrate the broad interest in the technology and wide range of research topics that may be addressed.

Each spot in a microarray can be seen as a reaction chamber for a biosensor. A biosensor is defined as analytical device incorporating a biological or biologically derived sensitive 'recognition' element and a transducer that converts a biological response into a digital electronic signal [Turner 2000]. Microarrays (or the equivalent term "biochips") are sometimes referred to as arrayed biosensors. In contrast to a classical biosensor microarrays are often not regenerable (with a few exceptions e.g. [Kloth et al. 2009]), a disposable chip is preferred, and online measurements (*in situ* monitoring, real-time measurements) are still rare and depend on the integration of microfluidics. After molecular recognition of the target molecule microarrays need a) an additional detection step (e.g. adding labelled detection antibodies) and often b) a separation step (washing off unbound material.

Figure 1 depicts important components of a protein chip experiment, which will be discussed in the following chapters.

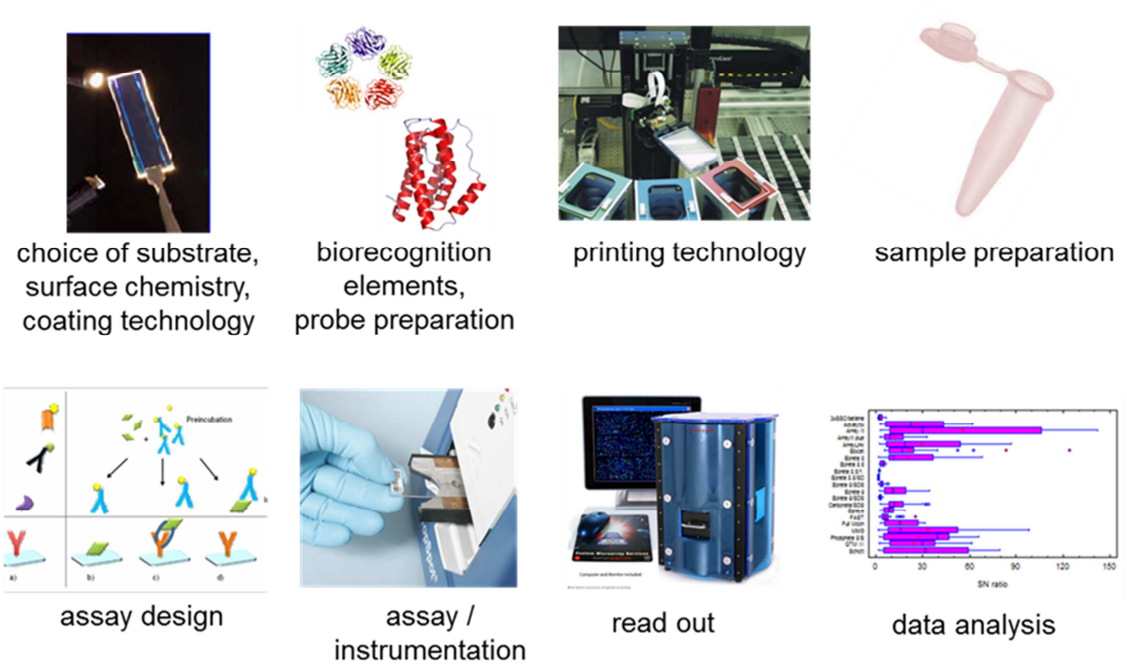


Figure 1. Components of a protein chip experiment. The individual parts are discussed in the following chapters.

Table 1. Examples for applications of protein microarrays.

Targets	assay format	matrix	detection system	Reference
Plant viruses	competitive/sandwich	leaves	colorimetric	Abdullahi and Rott 2009
Antibiotics	competitive assay	milk	chemiluminescence	Kloth et al. 2009
Food allergens	sandwich assay	pasta extract	planar waveguide/ fluorescence	Shriver-Lake et al. 2004
Foodborne pathogenic bacteria	sandwich assay	ground beef filtrate	fluorescence	Gehring et al. 2008
Escherichia coli* O157:H7, *Salmonella typhimurium*, and *Legionella pneumophila	sandwich assay	buffer	chemiluminescence	Karsunke et al. 2009
Pesticides: atrazine; 2,6-dichlorobenzamide	competitive assay	water environment	fluorescence	Belleville et al. 2004
IgGs to Epstein–Barr virus, cytomegalovirus, *Toxoplasma gondii*, and hepatitis C virus	sandwich assay	human serum	fluorescence	Feron et al. 2013
Red blood cells AB0	direct assay	blood	SPR	Charriere et al. 2015
Surface molecules on cancer cells	proteome profiling	surgical gastric samples	fluorescence	Ellmark et al. 2006
Secreted cellular products	sandwich assay	cell culture medium	fluorescence	Jones et al. 2008

In microarray technology multiplexing is achieved by positional encoding. Each position in the array (i.e. each spot) represents a specific capture probe and hence a single label ("color") is usually sufficient for read out of hundreds of different analytes. To overcome some problems with slow diffusion and low sensitivity of big arrays on solid supports, so called "solution arrays" were introduced. Here encoding is accomplished either by fluorescently labelled microbeads or by barcoded particles [Nicewarner-Pena et al. 2001]. But this approach has the disadvantage of a limited number of analytes.

2.1 Supports and immobilization strategies

A review on protein immobilization onto solid supports is given in Chapter 3 discussing covalent binding, physical and electrostatic adsorption, and affinity binding in two and three dimensional regimes. Microarrays require surfaces to be biocompatible and rich in binding sites. The choice of the surface chemistry is governed by some technology inherent requirements such as low background (for fluorescence read out that means low autofluorescence at the excitation wavelength), low unspecific binding (often referred to as antifouling properties); providing good stability, and accessibility of the probes. Last but not least, it depends on the solid support which is often glass, plastic, silicon dioxide or gold.

Hydrogels [Derwinska et al. 2007a, b], gel pads [Guschin et al. 1997], and nitrocellulose [Stillman and Tonkinson 2000] are popular 3D substrates for protein microarrays. BREs can also be entrapped in transparent porous sol-gels, protecting and stabilizing them, given that the sol-gel preparation is modified to be biocompatible (i.e. reduced alcohol content and introduction of appropriate buffers). Sol–gel immobilized biomolecules are reported to retain their structural integrity and biological activity [Jeronimo et al. 2007].

Commonly used planar surfaces provide reactive aldehyde-, epoxy-, isothiocyanate, amino-, or mercapto-groups (see Figure 2 a)). Some latest developments and controversial views on immobilization are highlighted in the following.

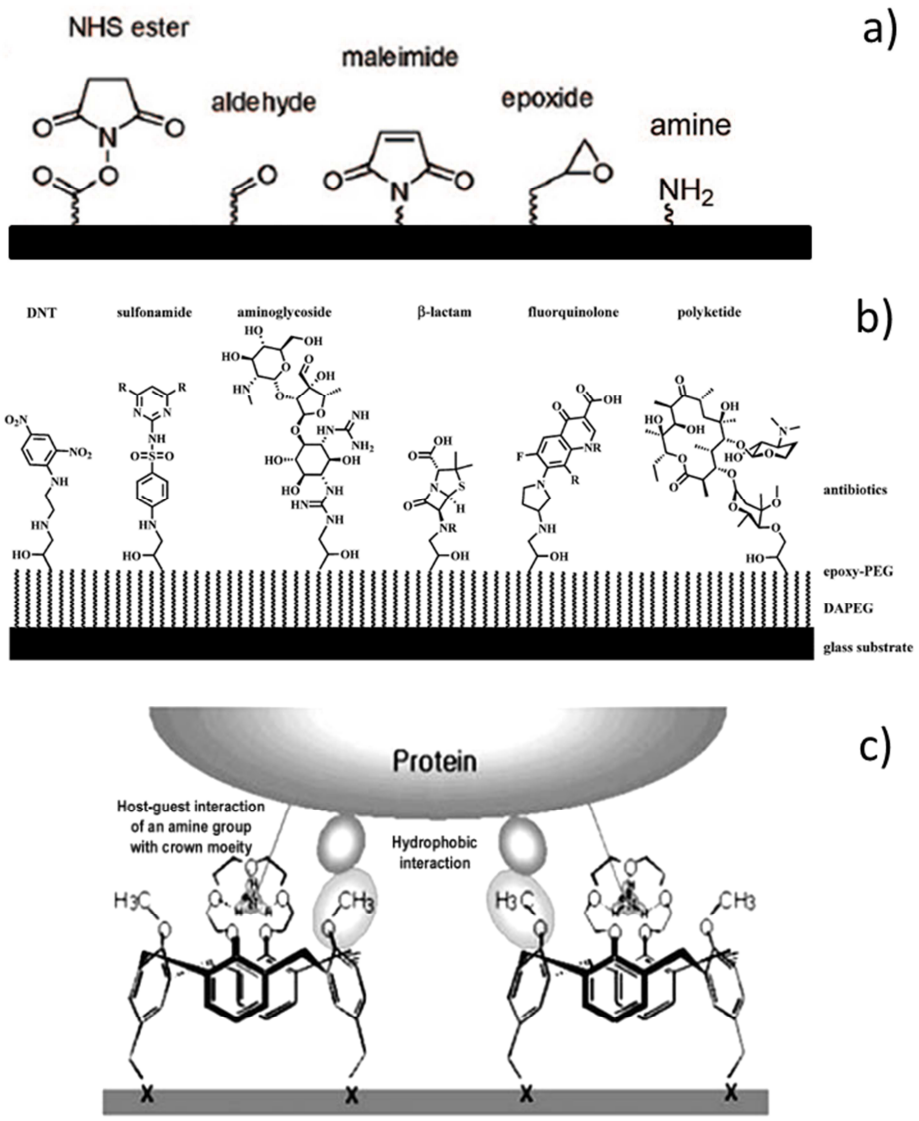


Figure 2. Immobilization strategies – examples from literature: a) commonly used reactive surface groups for covalent protein immobilization: NHS esters and aldehyde groups are amine reactive, maleiimide reacts with thiol groups, epoxides bind both, amine and thiol, and surfaces with exposed amino groups may bind to EDC/NHS activated carboxy groups [modified from Wong et al. 2009] b) possibilities to bind antibiotics covalently to a PEG surface modified with epoxy groups [Kloth et al. 2009] c) Protein binding to Calixcrown molecules: the protonated amine groups of the protein bind to the crown moiety of the linker by ionic interaction [Oh et al. 2005].

2.1.1. Pros and cons of oriented immobilization

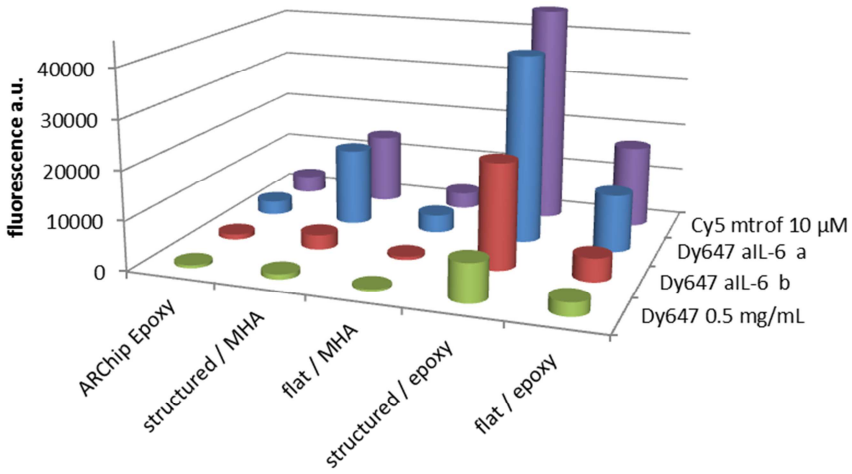
In contrast to randomly immobilized antibodies (e.g. via adsorption) and statistically oriented antibodies (i.e. covalent binding to certain accessible amino acids), where preferred binding of functional moieties to a surface is assumed, oriented antibody immobilization aims at optimal presentation of the Fab regions for most efficient antigen

capturing. Pros and cons of orientation of the capture molecules have been discussed for a long time. While for “micro”arrays orientation of antibodies may not have such a big impact, for “nano” spots high binding densities of oriented capture molecules may become crucial in order to generate a signal [Tsafarti BarAd et al. 2011]. Clearly, orientation is also very important for label free detection methods and for methods where distance is a crucial parameter such as SPR and Total Internal Reflection Fluorescence (TIRF). Orientation may go along with chemical modification of antibodies and the question whether the immobilization strategy ends up with a higher number of biologically active antibodies often remains open. Protein A and Protein G, naturally occurring proteins from *Staphylococcus aureus* and *Streptococcus sp.*, exhibit a strong affinity to the Fc part of IgGs of some species as for instance of human IgG, Rat IgG2C and Mouse IgG2A (for Protein A). In principle this end-on attachment allows control of antibody orientation presenting the Fab regions toward the sample solution and does not require modification of the antibodies. The dissociation of the Abs at higher pH on the other hand can be used for regeneration of chips. Covalent binding of antibodies using amine reactive surfaces will result in random orientation, but an intermediate layer of protein G is not oriented either when using amine coupling and hence first and foremost orientation of the protein G itself should be pursued in order to really control antibody orientation [Song et al. 2012]. This may be obtained with thiolated protein G self assembling on gold [Fowler et al. 2007]. The 3 times higher mass of protein G mediated antibody immobilization compared to covalent binding via NHS ester may also be attributed to low efficiency of the esters formed.

In [Soler et al. 2014] the authors compare covalent binding of antibodies to surface plasmon resonance (SPR) gold chips via alkanethiol self assembled monolayers (SAMs) to protein G mediated binding and to an oriented calixarene-based immobilization (ProLinker^{TM}). The protein is captured by the calix crown derivative by a host-guest interaction of the ionized amine groups and the crown moieties [Lee et al. 2003]. The ProLinker strategy showed significantly lower LOD and wider measurement range compared to the two other immobilization strategies and worked also in pure urine and diluted serum. The covalent attachment employing an alkanethiol SAM in combination with carbodiimide/N-hydroxysuccinimide (EDC/NHS) chemistry yielded very low amount of antibody on the surface, the authors assume a very low density of NHS-esters formed [Soler et al. 2014]. This is in accordance with our own unpublished results. A thin layer of epoxy resin outperformed conventional alkanethiol chemistry (Figure 3.) modified with EDC on flat and nanostructured gold substrates for covalent binding of labelled biomolecules. Another approach for oriented immobilization on gold using recombinant

bispecific antibodies is reported in Watanabe et al. [2011]. An antigold antibody was combined with anti-lysozyme antibody via a rigid linker. The lysozyme binding capacity of this immobilized construct was calculated as 82% from the amounts of immobilized antibody and antigen compared to 59% using conventional EDC/NHS chemistry.

a)



b)

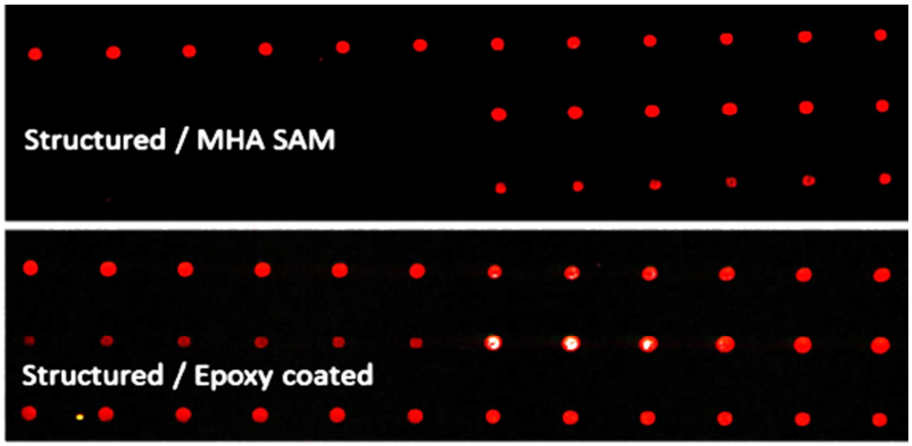


Figure 3. a) Comparison of the fluorescence signal originating from spotted biomolecules labelled with a dye as stated in the graph on ARChip Epoxy (glass) and flat and structured gold substrates coated with mercaptohexadecanoic acid / carbodiimide (MHA/EDC) or epoxy resin Epikote 157. b) Example of microarray image scan comparing the two covalent binding chemistries on a nanostructured gold chip.

2.1.2. Surface chemistries for small molecules

The detection of small molecules is particularly challenging. The size of the molecules hampers sandwich immunoassays, only binding inhibition or competitive assays are possible (see 2.5.2). As a consequence immobilization of target molecules is necessary in a way providing good accessibility for the detection antibodies. This task has been

accomplished by coating conjugates of small molecules with big proteins, e.g. BSA, HSA, HRP, KLH etc. [Poller et al. 2015, Sauer et al. 2011]. Another way to present molecules on a surface is to either coat them onto microparticles which are then arrayed onto the chip [Preininger et al. 2005b], entrap them in sol-gels [Jeronimo et al. 2007] or other porous materials, immobilization onto dendrimers [Soler et al. 2015, Souto et al. 2015] or polymer brushes [Barbey et al. 2010, Liu et al. 2011].

2.1.3. Coating of substrates

A uniform coating for chip functionalization is a pre-requisite for reproducible and stable binding of biorecognition elements. In Figure 4 some coating instruments and techniques are depicted. The choice of a suitable coating technique depends on substrate, chemistry of the functional material and desired film thickness. Dip coating of 1% SU8 (v=100 mm/min) onto glass for instance yielded about 20 nm film thickness, determined by scanning a scratch in the coating with AFM [Levi A. Gheber, personal communication]. Spincoating of the same material, on the other hand, depending on the spin coating parameters resulted in 26 nm (10 s @ 1800rpm, 30 s @ 300 rpm; acc= 500 rpm/s) and 19 nm layers (40 s @ 4000 rpm, acc = 1300 rpm/s), determined via a TM angular reflectivity spectrum [Jakub Dostalek, personal communication]. Using a manual film applicator the wet layer thickness can be chosen as e.g. 15 µm, 30 µm, 60 µm, and 100 µm. The film applicator is used for solutions with high vapour pressure. Metallic coatings can be produced by physical or chemical vapour deposition.

Silanization of chips is usually accomplished by liquid phase deposition of the silane in an organic solvent [Cras et al. 1999; Jo and Park 2000] or gas phase deposition [Pallandre et al. 2004].

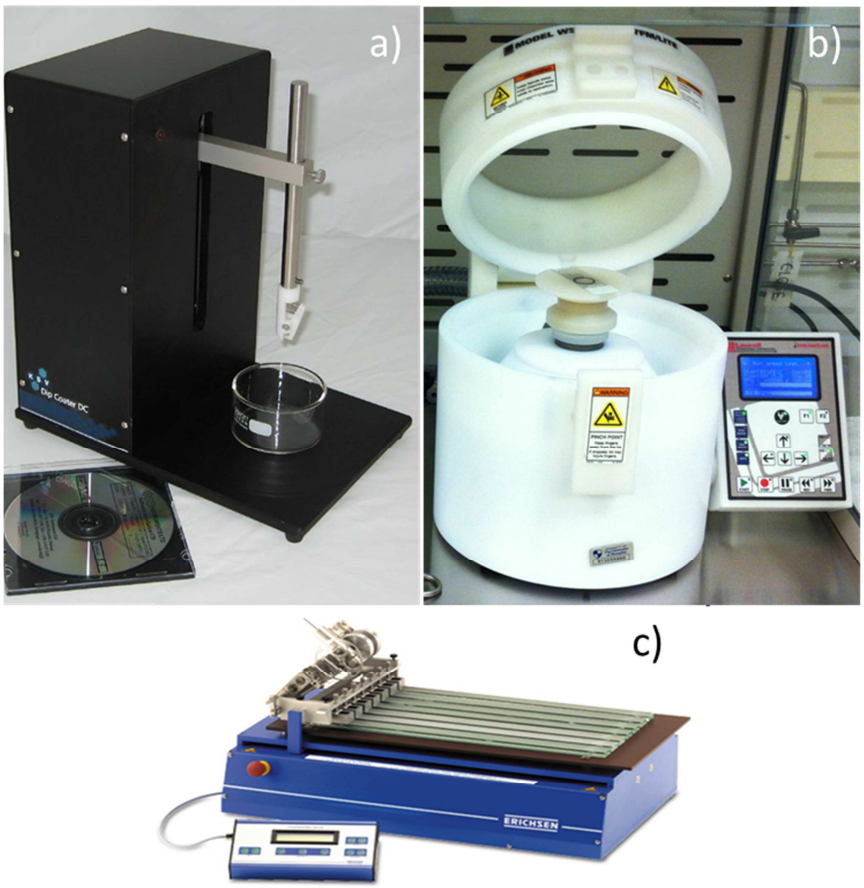


Figure 4. Coating instruments: a) single vessel dipcoater KSV Instruments (www.ksvnima.com); b) spin coater from Laurell Technologies Corporation (www.laurell.com); c) film applicator from Erichsen (www.erichsen.de).

2.1.4. Characterization of surfaces and immobilized biomolecules:

Important criteria in surface characterization are:

- Optical properties
- Topography, roughness, layer thickness
- Wetting behaviour
- Chemistry
- Functionality / biological activity

For optical characterization we used a laser scanner to determine the autofluorescence at the excitation wavelengths of commonly used fluorescence dyes, namely λ = 635 nm and λ = 532 nm [Preininger and Sauer 2003]. Topography, roughness parameters and layer thickness can be determined by ellipsometry or AFM (see Figure 5). In order to assess the roughness of blank and coated support materials, AFM images were recorded and root-mean-square (rms), arithmetic average height (R_a), and peak-to-valley

roughness data calculated using JPK data processing software. The R_a, defined as the average deviation of the center line, is a commonly used roughness parameter. The root mean square roughness is the standard deviation of the distribution of surface heights and more sensitive to large deviations from the mean line. Roughness data of some common substrates (glass (Melvin Brand), Histobond™ and gold slides (Thermo Scientific™ BioGold™)) and coated substrates are summarized in Table 2. Surface roughness is known to influence the existing wetting behaviour in a way that a hydrophobic surface gets even more hydrophobic and hydrophilic ones get more hydrophilic.

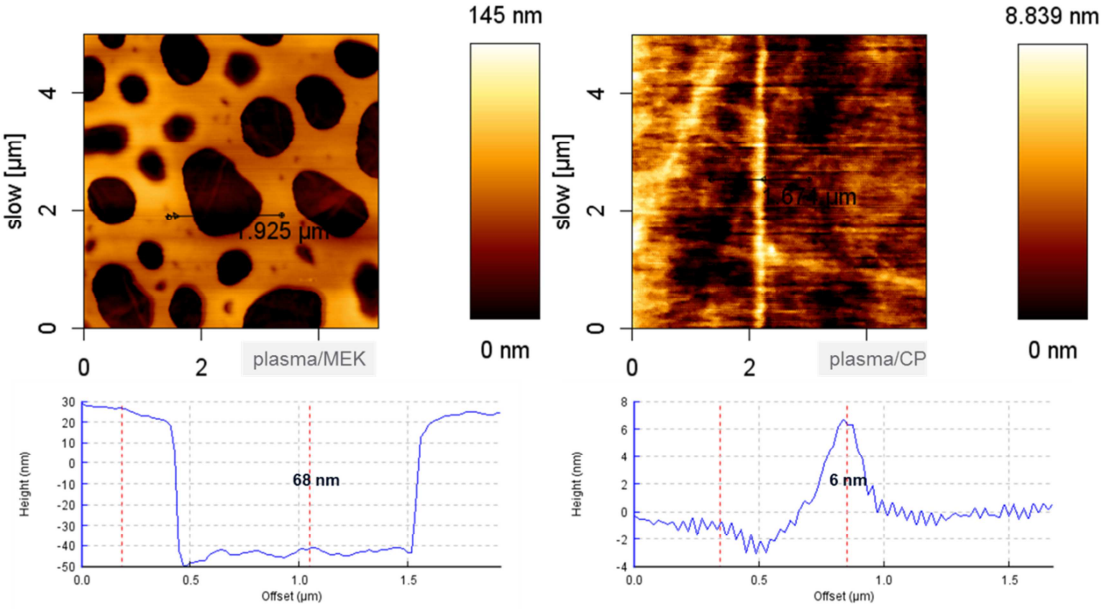


Figure 5. AFM scans of the epoxy resin Epikote 157 (SU-8) spin coated onto a Zeonex chip. Left image: chip was treated with oxygen plasma before coating epoxy resin in methyl ethyl ketone. The coating shows holes of a diameter up to 1 µm, the thickness of the coating at the edges of the holes is 80 nm. Right image: using a different solvent, namely cyclopentanone changes the coating characteristics tremendously. A smooth thin layer with some scratches from the support outline through the layer.

Table 2. Roughness data of substrates and coated substrates (3% (3-Aminopropyl)triethoxysilane - APTES; 3% (γ-mercapto-propyl)trimethoxysilane - MPTES; 1% Epikote 157 – SU-8; and 2% Vinylbenzyl Thiocyanate – VBT)

	RMS [pm]	Ra [pm]	peak-to -valley [nm]
Glass	3018	1533	163.9
Histobond	778	586	44.9
Gold	1748	1107	60.4
APTES/Glass	1496	789	26.6
MPTES/Glass	5026	3778	127.3
SU-8/Glass	4679	1753	48.6
VBT/Histobond	7129	4331	53.8

Contact angle measurements provide information on the wetting behaviour of a surface, which is governed by the interfacial tension between surface, liquid and air. Wettability plays not only a role in coating a substrate with a reactive surface but also influences spreading of spotting solutions and hence spot size and protein distribution within a spot. The composition of the spotting buffer for a specific surface and biomolecule is devised also with regards to an optimal wetting, i.e. forming a small spot, still letting all capture molecules reach the surface.

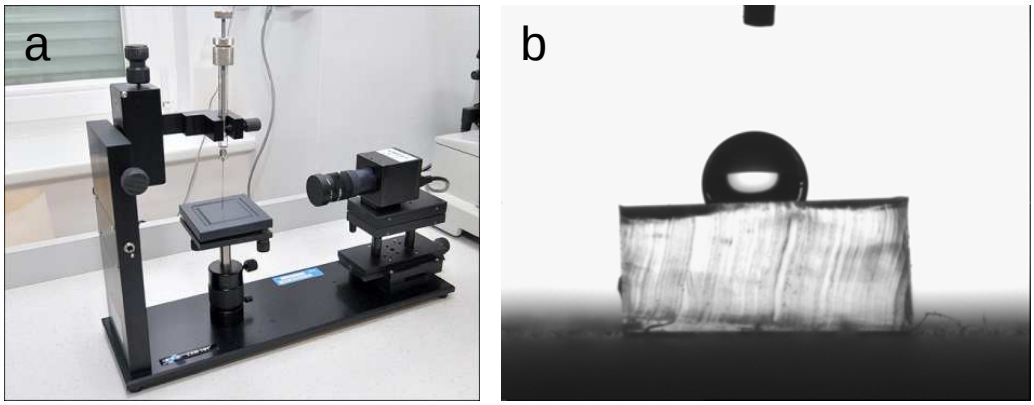


Figure 6. Contact angle measurement: a) a simple tensiometer arrangement with stage, camera and a syringe for droplet deposition (KSV Instruments) and b) camera image of a drop of water on a PDMS stamp.

A description of the surface chemistry can be accomplished by for instance X-ray photoelectron spectroscopy (XPS) and time of flight secondary ion mass spectroscopy (TOF SIMS), methods which are particularly surface sensitive. Time-of-flight secondary mass spectrometry was apt to distinguish if antibodies presented their Fc or their Fab region because of characteristic ions from amino acids enriched differentially in those two fragments [Liu et al. 2010]. Atomic Force Microscopy (AFM) and neutron reflectivity (NR) investigations showed that antibodies adsorbed to silicon oxide adopted a predominantly flat orientation, meaning that the Fc and two Fab parts were lying flat on the surface [Xu et al. 2006]. Oh et al. 2005 demonstrated the oriented immobilization of IgG via a Calixcrown linker by probing the spots with labelled protein A. Where the Calixcrown captured the Fc region of the IgG no signal was generated as the Protein A could not bind. Also SPR was apt to distinguish “lying” and “standing” antibodies immobilized onto a surface. However, in order to show if IgGs were immobilized “end-on” or “head-on”, additional probing with protein G was necessary: when no shift in the SPR angle occurs, an “end-on” immobilization is indicated [Chen et al. 2010].

2.2 Biological and biomimetic recognition elements (BREs) in immunoanalytical microarrays

Biorecognition elements briefly described in the following differ in affinity to the target molecules (Kd values), dynamic range, specificity, size and hence density on a substrate, stability under harsh conditions, shelf life and last but not least production cost. Originally BREs were isolated from living systems such as antibodies, enzymes, receptors, even whole cells may be used. Now a growing number of artificial biorecognition elements are employed for sensing.

2.2.1. Antibodies

Antibodies (immunoglobulins) are glycoprotein receptors of vertebrates serving the immune system for identifying and neutralizing foreign substances. Analytical applications make use of the natural immunoreaction where an antibody is recognizing an analyte with high specificity and sensitivity. The Y shaped antibody molecules feature dimensions of about 14 nm in height, 8.5 nm in width, and 4 nm thickness and are formed by two identical light chains and two identical heavy chains, linked by disulphide bridges and non covalent bonds (see Figure 7). Antibodies can only be produced for targets which elicit an immunogenic response on one hand and are not killing the host animal on the other hand. Polyclonal antibodies are produced in a number of mammal species such as mouse, rat, rabbit, goat, donkey, and llama. They are actually a mixture

of antibodies produced in different B-cells and target different epitopes. The quality of polyclonal antibodies may differ from batch to batch. Monoclonal antibodies on the other hand are produced by only one cell line and hence target only one specific epitope. For sandwich assays two antibodies specific for the analyte are necessary, often a mix of e.g. a monoclonal capture antibody and a polyclonal labelled detection antibody is used. Instead of a labelled antibody, detection may be accomplished by a third, species specific secondary antibody with a label.

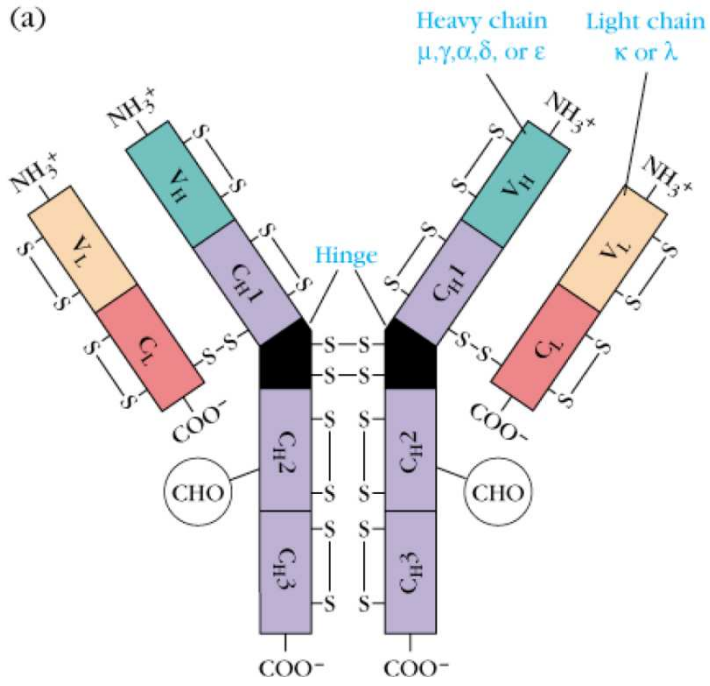


Figure 7. Antibody structure. From Kubi Immunology [Kindt et al. 2007]

Multiplexed quantification of high numbers of analytes such as pesticides, drugs and their metabolites, endocrine-disrupting compounds or other contaminants of food, feed and environment, asks for as many specific antibodies or even antibody pairs. Specificity, cross reactivities, problems with toxic compounds and not to forget very high development costs are limiting the applicability of antibody based systems.

2.2.2. Peptides

Peptides, short polymers of amino acids linked by peptide bonds, have been used as biorecognition elements for proteins, antibodies, DNA, and metallic ions. Peptides with high affinity to targeted analytes can be either chosen by screening peptide libraries or are known natural ligands to the target molecule. The production of the specific sequences is accomplished by solid-phase synthesis, and modifications for

immobilization and labelling may be included in the process. Peptides are especially useful in combination with environment-sensitive fluorophores, fluorescent resonance energy transfer (FRET) or as part of an excimer, a dimer with a longer emission wavelength than the monomer [Liu et al. 2015].

2.2.3. Aptamers

Aptamers are artificial nucleic acid ligands which were selected to show a high affinity to a certain target. The sequence of the DNA or RNA oligonucleotides is determined by the SELEX process (Systematic Evolution of Ligands by Exponential enrichment), in which big libraries of artificial oligonucleotides undergo an iterative process of adsorption, recovery and amplification [Mairal et al. 2008]. A specific aptamer shows affinity to a peptide, a protein, a cell or a small organic or inorganic molecule comparable to antibodies but with a number of advantages compared to those. First, there is no immunogenic response of an animal needed and hence also very small or toxic targets are possible. Aptamers may be readily manufactured with a linker to bind to a sensor surface and a label for detection without altering the affinity to the target. Further they can be produced and will function under conditions where antibodies fail to work, such as in organic solvents or extreme pH. On the other hand, aptamers and especially RNA aptamers have to be protected against the ubiquitous nucleases. This is accomplished by chemical modification or by using mirror-image nucleotides, so called Spiegelmers ® (http://www.noxxon.com).

Aptamers have been used in various assay formats (direct, competitive, binding inhibition, sandwich assays) alone or in combination with antibodies. In Pultar et al. [2009] an aptamer specific to C- reactive protein is used in multiplexed on-chip immunoassays. The lower affinity of the aptamer shifts the working range of the chip to the desired high serum concentrations of this biomarker for inflammation.

2.2.4. Molecularly imprinted polymers (MIPs)

MIPs are cross-linked polymers designed to specifically and selectively interact with target molecules [Uzun and Turner 2016]. Monomers displaying functional groups are polymerized together with cross-linkers in the presence of the template molecules. When the template is removed, cavities complementary in size, shape and functionality to the target are created [Haupt and Mosbach 1998]. MIPs are stable and more robust than natural BREs, they work also in extreme environments, such as in the presence of acids or bases, in organic solvents, or at high temperatures and pressures [Haupt and Mosbach, 2000].

MIPs have been applied for separation and purification mainly; now they are entering the field of drug delivery and detection of molecules as well as whole cells [Dickert et al. 2003, Cohen et al. 2010] and even viruses [Bolisay et al. 2006]. In [Buchegger et al. 2014] a ready to use epoxy resist was used for hot embossing of lipopolysaccharide and lipoteichoic acid, surface markers specific for Gram-negative and Gram-positive bacteria. The affinity of bacteria imprinted sol-gel films towards their target organisms was found to be governed by the morphology of the cavity and residual surface components entrapped in the imprint surface [Cohen et al. 2010].

2.3 Up-to-date patterning of BREs

For protein microarrays there are two standard methods for arraying pre-synthesized capture probes, namely contact and contactless printing. Other techniques such as micro-contact printing (µCP) and nanobiolithography are only of minor commercial importance yet but are gaining more and more interest in the context of miniaturization and system integration. *In-situ* synthesis using photolithography can be applied for DNA directed protein immobilization. Figure 8 depicts various tools for delivering probes to a surface.

2.3.1. Non contact printing

Inkjet printing is based on the ejection of drops from a nozzle, shot onto a surface. The generation of the drop is accomplished by piezoelectric micropumps, a continuous stream controlled by valves, or thermal inkjet technology, being the first one the most common jetting technique [McWilliam et al. 2011]. Non contact printing can be applied to practically all substrates but is especially apt for damageable surfaces. The piezo voltage has to be optimized for different printing solutions which makes non contact printing less flexible compared to contact printing. The probe volume needed to fill the syringes including a dead volume is relatively high compared to the one for a contact printer pin, thus non contact printing is suitable for printing high number of spots with one printing solution. The high speed a non contact printer of today can reach is another advantage of the technique.

Commercially available systems include the TopSpot® from Biofluidix, Germany [http://www.biofluidix.com/en-products-topspot-topspottechnology.html], Marathon from ArrayJet, GB [http://www.arrayjet.co.uk] and the NanoPlotter from Gesim, Germany [http://gesim-bioinstruments-microfluidics.com/category/liquid-handling-en/nanoplotter-en/basic-features-en/]. The TopSpot with a printhead containing 24 reservoirs for different spotting solutions prints all probes in parallel and contactless to the substrate by

a piezo actuated print mechanism. Several thousand dots can be printed without refill. Microarray printers from Arrayjet reach a very high throughput what concerns both, slides (up to 1000) and probes.

2.3.2. Contact printing

Contact spotters use pins to deliver the probe to the surface by physical contact. Contact printing is the more technically simple and robust technique compared to non contact printing. The method is very flexible what concerns both, substrate type and hydrophobicity, and probe composition and viscosity. Required sample volumes are usually very low (e.g. 10 µl in a well of a source plate) and remaining sample in the source plate can be frozen and reused. Split pins carry the sample in a capillary and deposit a small amount of it onto the surface by tapping. Solid pins, on the other hand, are less delicate than the split pins, but have to revisit the source plate after probe deposition. Material consumption is minimized using solid pins.

Print heads with up to 192 pins are available (e.g. ArrayIt, USA) [http://www.arrayit.com/]. Stealth pins with various capillary dimensions can be employed for a wide range of probes including cells, beads and macromolecules. ArrayIt e.g. offers pin with tip diameters from 37 µm up to 375 µm.

2.3.3. µ-contact printing (µCP)

Stamps with a bas-relief made of elastomer are used to transfer ink to a surface. The application areas are manifold, depending on the transferred pattern and the ink, which can be e.g. gold, solvents, polymers, self assembled monolayers, but also biomolecules and cells. Usually stamps are made of polydimethylsiloxane (PDMS), a material that can easily be moulded from a master stamp with a resolution down to about 50 nm. It allows precise transfer of ink to a substrate due to its flexible nature. On the other hand, the deforming of PDMS does not allow transferring high aspect ratios, or patterns with low features in a wide distance. In the latter case hybrid stamps with a rigid backbone may add stability [Odom et al. 2002]. Another condition for efficient transfer is that the ink exhibits more affinity to the substrate than to the stamp [Alom Ruiz and Chen 2007]. For good printing results relative hydrophobicities of substrate and stamp [Tan et al.2002], ink concentration, contact time, temperature, and humidity need to be optimized.

An alternative approach to directly patterning proteins by µCP is creating hydrophilic and hydrophobic regions or regions which are resistant to protein adsorption by patterning of

SAMs. While oligo(ethylene glycol) terminated alkanethiols are blocking the surface, methyl groups at the SAM's tail promote protein adsorption [Alom Ruiz and Chen 2007].

µCP of proteins was employed to functionalize a two channel SPR chip [Lu et al. 2001]. PDMS sheets were equilibrated with the protein solution for half an hour, washed with PBS and water removing excess material and leaving a monolayer of protein on the stamp. The loaded stamp was dried under a stream of nitrogen and placed on the chip. Transfer of the protein was accomplished in 1s solely using the force of the PDMS stamp's weight and interfacial adhesion.

Automated microcontact printing for microarray applications was lately introduced by Gesim, Germany and by Biosoft Technolgies, France [http://biosoftlab.com/index.php/biosoft-technologies/soft-lithography] [Cau et al. 2013].

2.3.4. Nanobiolithography

Several groups have developed methods for the printing of nanoarrays, all of them involving atomic force microscopy (AFM). Taha et al [2003] describe the writing of proteins onto aldehyde coated glass slides using a nano fountain pen (NFP), a cantilevered nanopipette controlled by an NSOM- SPM system. With the NFP it is possible to print dots and lines of biomolecules, but also etching of protein surfaces by patterning an enzyme was demonstrated [Gheber 2008].

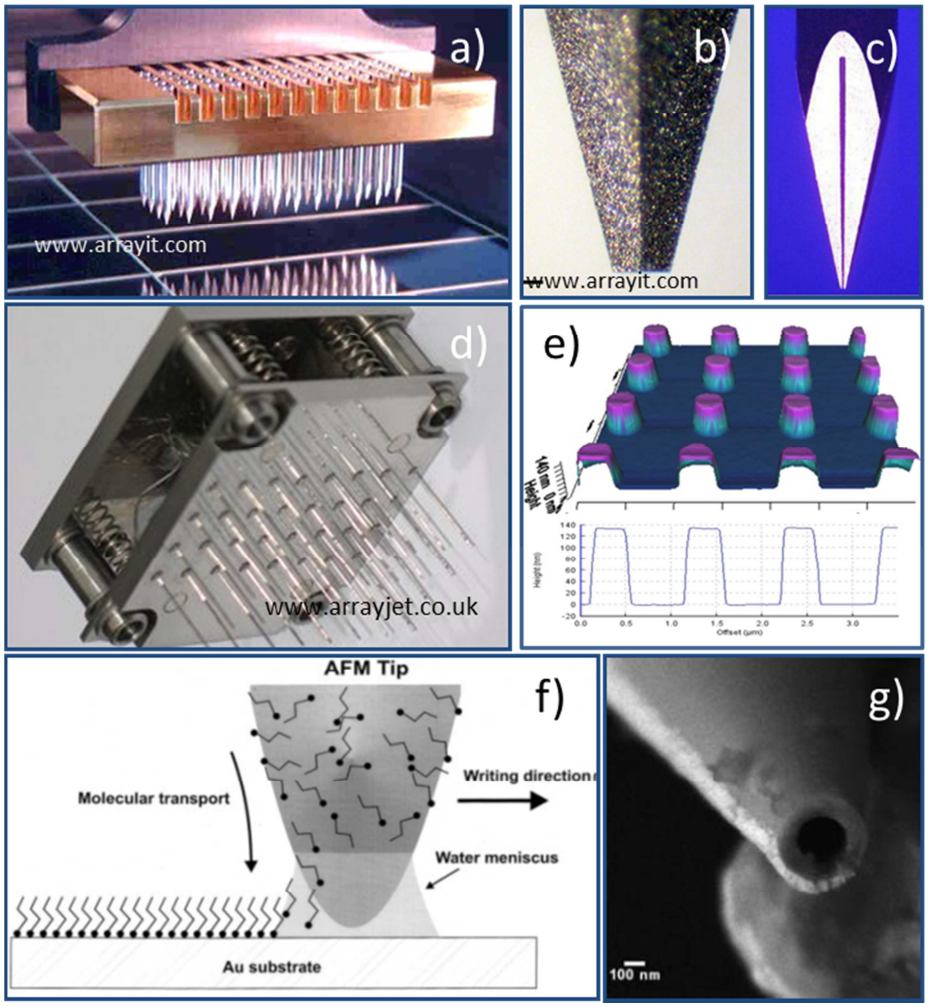


Figure 8. Tools for patterning of biorecognition elements: a) microarray printhead with pins for contact printing from ArrayIt Corporation b) solid pin (SNS6, tip width 150 µm) and c) split pin (SMP3, capillary width 75 µm) for contact printing from ArrayIt Corporation d) jetSpyder inkjet printhead for non-contact printing from Arrayjet e) AFM image of a PDMS stamp for µ CP f) the principle of DPN [Piner et al. 1999] g) NFP – aperture of a nanopipette [Gheber 2008].

Chad Mirkins group at Northwestern University invented the so-called Dip-Pen Nanolithography (DPN), where an AFM probe is delivering an “ink” to a substrate as shown in Figure 8. Patterning of proteins was accomplished by adsorption to DPN fabricated MHA (16-mercaptohexadecanoic acid) dots or grids [Lee et al. 2002]. Direct writing of his-tagged proteins has been achieved on nickel oxide surfaces with reasonable diffusion time [Nam et al. 2004].

Ellmark et al. [2009] and Petersson et al. [2014] report on the printing of antibodies into attovials, small (diameter = 500 nm- 4 µm) containers made with e-beam lithography, using nanoscale dispensing (NADIS). NADIS also uses an atomic force microscope probe with hollow cantilever and tip which deposits the probe to the surface upon contact, spots produced are in the order of 1 µm.

2.4 Sample preparation

For many applications sample preparation can be avoided in protein microarray technology or reduced to dilution of a sample matrix with assay buffer. We have developed sensitive biomarker assays in complex matrices such as saliva, serum, plasma, urine and cell culture supernatant, without relying on sample preparation other than dilution (see Chapter 5). Dilution with assay buffer is done for various purposes. First it stabilizes proteins; pH and ionic strength are adjusted to optimal conditions for the (bio-)activity of assay reagents. Second it dilutes interfering substances in the matrix. And thirdly, assay reagents such as antibodies or labelled target molecules for competitive or binding inhibition formats can be introduced. On the other hand, also low abundant analytes are diluted. Hence, an optimal dilution factor has to be found. We have been working with sample concentrations of 10% to 90% and were able to detect for instance cytokines in the pg/mL range.

Where analytes are present in the matrix at a too low concentration several enrichment procedures have been proposed, e.g. desalting; size exclusion; ion exchange [Gogalic et al. 2015]; filter enrichment as for instance ultrafiltration and monolithic filtration [Kunze et al. 2015]; magnetic particles; and MIPs.

2.5 On-chip Immunoassays

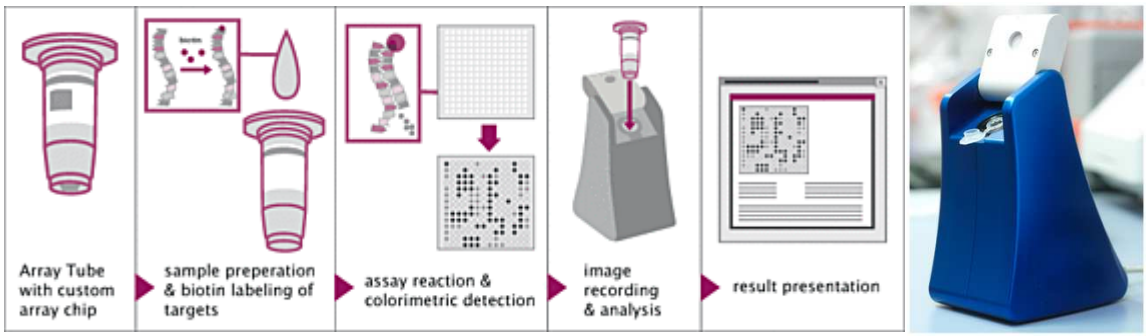
2.5.1 Platforms: slides, micro- or nano well chips/plates

The choice of platforms for the immobilization of biorecognition elements is wide, not only what concerns materials (plastic, glass, metal) but also two- or three- dimensional forms and sizes: from glass microarray slides (75 mm x 25 mm, Figure 10) to plastic chips [Carion et al. 2007], micro- or nanotiter plates; membranes; tubes (Figure 9) [Liu et al. 2006; www.alere-technolgies.com]; microchannels [Baldini et al. 2008] and more.

Two dimensional platforms need a gasket or frame to form the reaction chambers or channels for calibration standards and samples (see Figure 10). Other than a wide range of commercial products such as EMS SecureSeal™, Corning® hybridization chambers, ArrayIt ® hybridization frames or FastFrames™ from Whatman, self-made PDMS frames may be a cheap and flexible alternative.

In systems without microfluidics, implementation of shaking or stirring can improve assay performance considerably. An orbital shaker for this purpose, equipped with a water bath for heating, is shown in Figure 10. Stirring with magnetic particles for instance was improving assay sensitivity by a factor two in binding inhibition assays and a factor 4 in sandwich assays [Buchegger et al. 2012].

a)



b)

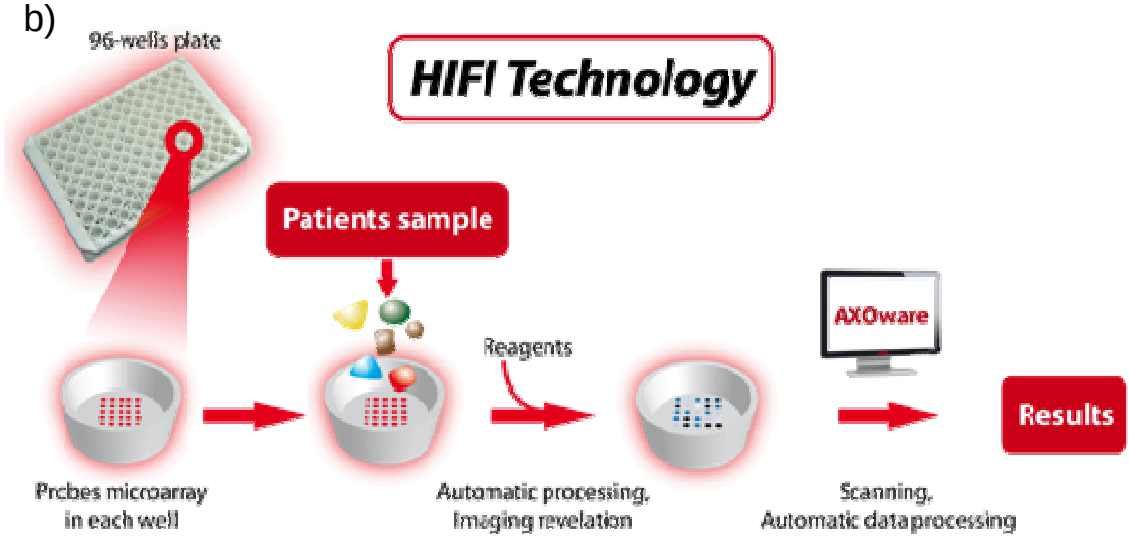


Figure 9. a) Array tube platform and reader from Alere www.alere-technologies.com b) microarray in a 96-well plate format from AXOScience: www.axoscience.com

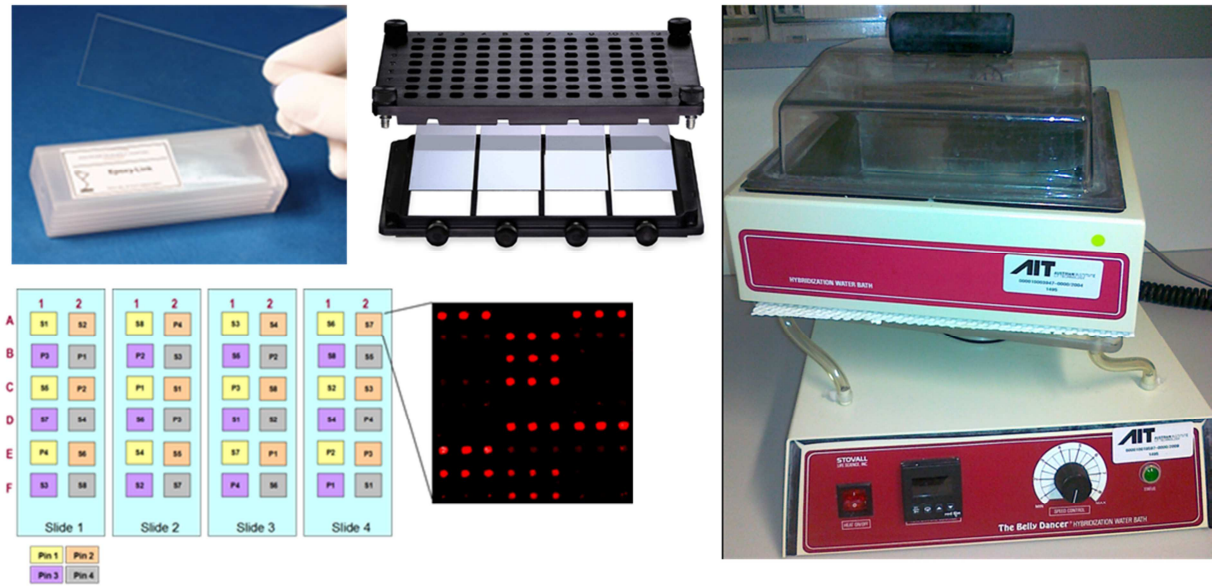


Figure 10. Protein microarray in a standard glass slide format, using hybridization frames (ArrayIt, USA) to create up to 14 single incubation wells per slide. A four-slide set can be used for a 9 - point standard curve and nine patient samples in one experiment. On the right hand side an orbital shaker with water bath is shown (Stovall Life Science Inc).

2.5.2 Assay formats

In contrast to homogenous immunoassays, with all assay components being in the liquid phase, on-chip immunoassays are heterogeneous, meaning that one component is immobilized and a separation (washing) step is usually necessary. The washing step can be omitted only if the detection scheme is able to distinguish surface bound molecules or labels from molecules/labels in solution.

Forward phase protein arrays describe formats where a target molecule is captured by an immobilized biorecognition element and comprise direct assays (the captured biomolecule is labelled) and sandwich assays (the binding of the captured molecule is detected by a second labelled affinity reagent). The signal rises with increasing abundance of the target molecules. Binding inhibition and competitive assays are applied when only one antibody is available or the analyte is too small for providing two epitopes. In binding inhibition assays, the analyte is immobilized and competes with targeted molecules in the sample for binding of the labelled antibody. Competitive formats use labelled target molecules competing with the analyte in the sample for binding to immobilized antibodies. For both formats, the signal decreases with increasing analyte concentrations. Figure 2 in Chapter 4 (p.68) depicts schemes for a sandwich assay and three different approaches to carry out a binding inhibition assay.

Reversed phase assays rely on immobilization of the target molecules and subsequent binding of a labelled antibody. Spotting of the samples (e.g. cell lysate) is followed by

incubation with (labelled) specific antibodies. Here, only one Ab species per label can be detected. Reversed phase assays are mainly applied in drug discovery or screening for molecular markers.

2.5.3 Assay protocol for ARChip Epoxy

ARChip Epoxy, a solution of SU8 in an organic solvent, is a proprietary surface chemistry developed at AIT and used as a gold standard in all applications described herein. ARChip Epoxy binds covalently to amino- and thiol- groups in a ring opening process. The thin coatings produced by dip- or spin-coating, are characterized by low roughness (see Section 2.1.3, Table 2 and Figure 5) and feature a high contact angle (75° for 1x phosphate buffered saline (PBS)). Further, ARChip Epoxy has a shelf life at ambient conditions of at least one year. Instead of coating the surface chemistry onto substrates the material can also be spotted for site specific immobilization of probes.

After arraying of biorecognition elements, slides are stored at 4°C for a minimum of three days to ensure complete probe binding.

Immediately before performing on-chip assays, slides have to be blocked in order to remove unbound material and deactivate residual functional groups. Slides are placed in a tray with 1x PBS (pH7.2) / 0.1% Tween 20 and incubated for 30 – 60 minutes while slowly shaking or stirring. Blocking solution is discarded and slides are rinsed with 1x PBS twice. After a final rinsing step in A.d., slides are dried with compressed air or in a centrifuge @ 900 rpm for 3 minutes.

Slides are mounted in a frame (see Figure 10) in order to create incubation chambers. Samples or calibration standards are applied in an appropriate dilution and assay buffer, e.g. {1x PBS (pH 7.2) / 0.1% Tween-20}; or {0.1M Tris (pH 7.4), 10 mM $CaCl_2$, 100 mM NaCl, 0.1% Tween-20} [Domnanich et al. 2009] and incubated for 2.5 hours on an orbital shaker.

Depending on the assay format, one to three incubation steps are necessary, in between washing of the slides with blocking buffer can be done directly in the frames using a multipipette. Slides should never be allowed to dry between the incubation and washing steps. After the final incubation, slides are washed with 1x PBS/ 0.1% Tween-20; 1x PBS; and distilled water, before drying with compressed air or by centrifugation.

During incubation steps with fluorescence dyes and after completion of the assays, slides are always protected from light until read-out.

2.5.4 Immuno-PCR

In contrast to DNA targets, to date proteins cannot be amplified directly. Too low sensitivity poses a serious problem for protein microarrays, since often very low concentrations (e.g. pg/mL; fMol /mL) have to be detected in complex matrices. When proteins involved in assays are labelled with oligonucleotides (e.g. a detection antibody is labelled with a specific DNA), those can be amplified via PCR and the DNA produced can be detected. Schweitzer and co-workers describe an adaptation of a rolling circle amplification for sensitive detection of IgE, with a limit of detection of 0.1 ng/mL, that is two orders of magnitude lower than in a conventional ELISA [Schweitzer et al. 2000].

2.6 Signal transduction and readout

The binding event of a ligand to an immobilized biorecognition element needs to be converted into a readable signal by a transducer. Optical signal transduction is clearly dominating microarray applications, as it is a sensitive method, apt for multiplexing and not troubled by electromagnetic noise [Vikesland et al. 2010]. Optical detection schemes of multi-analyte affinity-based systems range from fluorescence excited by lasers or LEDs, total internal fluorescence reflectance (TIRF) [Tschmelak et al. 2004, Sapsford et al. 2006], Förster resonance energy transfer (FRET) [Nagl et al. 2008], absorbance [Wen et al. 2012], chemiluminescence [Weller et al. 1999] to label free techniques (e.g. interferometry, resonant mirrors and surface plasmon resonance) [Homola et al. 2005].

In chemiluminescence light is produced by a chemical reaction. The detection probe is labelled with e.g. horse radish peroxidase (HRP), upon addition of the substrate an excited state product is generated locally which decays to a lower energy state by emitting light [Mirasoli et al.2012]. The advantage of chemiluminescence, a technique which has been typically used in Western Blotting and ELISA, is that there is no need for an expensive excitation light source or additional optics [Kloth et al. 2009]. Enzymes generating colored products, often HRP or Alkaline Phosphatase, are conjugated to detection antibodies in colorimetric assays. Colorimetric results can be viewed by eye, but for quantification a device is needed. Portable readers, office scanners, (video) cameras, and even smartphones have been used for imaging. For the latter two changing ambient light conditions have to be compensated, for instance by conversion of RGB values into the International Commission of Illumination (CIE) 1931 color space terms [Shen et al. 2012].

Bio-layer Interferometry uses the interaction of two light waves, namely the interference pattern of the light reflected from the optical layer and the one reflected from the bio-

layer. Upon binding of analytes to the biolayer, this interference pattern changes in a concentration dependent way (www.fortebio.com).

With SPR technology biomolecular interactions can be observed in real-time. The technology has been commercialized by several companies (www.biacore.com; www.reichertspr.com/; www.bio-rad.com; www.biosensingusa.com). Label free techniques such as surface plasmon resonance (SPR), and interferometry often suffer from too low detection limits and unspecific binding [Weller et al. 1999]. Fluorescent labels on the other hand may increase unspecific background, and the labelling process may alter protein function and adds to total test costs.

In the present work we solely employed antibodies labelled with a fluorescent dye or with a biotin label for subsequent binding of dye conjugated streptavidin and a non-confocal laser scanner for read out. The labelling itself is usually done with the active ester method. In contrast to site specific labelling of antibodies targeting the FC portion or the carbohydrate moieties, amine reactive dyes (N-hydroxysuccinimidester, sulfo-NHS) are attached covalently to the antibody. Purification is accomplished on a size exclusion spin column optimized for ≥40 kDa proteins.

Commercial microarray scanners usually work with helium-neon and argon lasers for excitation at λ=635 nm, λ=532 nm and λ=488 nm, a stage for x-y movements and a photomultiplier tube as detector [Schäferling and Nagl 2006]. Signals in arrays are detected pixel by pixel and pixel size may be chosen between 1 and 20 µm.

2.6.1 Signal enhancement

High sensitivity is one of the most important factors of success for competitive protein microarrays.

Strategies for signal enhancement include

- High density and accessibility of probes (i.e. immobilized biorecognition elements)
- High density of labels per binding event
- Enhanced intensity per fluorophore

3D immobilization matrices provide higher binding capacities compared to 2D surfaces but often suffer from high intrinsic background (e.g. nitrocellulose), higher unspecific binding, reduced stability and reproducibility [Derwinska et al. 2007]. As an alternative to hydrogels and membranes, polymer brushes with functional groups on their side chains have been developed. They can be prepared in a highly controlled way by surface

initiated polymerization [Barbey et al. 2010]. In Liu et al. [2011] both, probe immobilization and reporter immobilization were accomplished using polymer brushes which results in high density of probes and high density of labels. A glycidyl methacrylate poly(ethylene glycol) methacrylate (GMA-co-PEGMA) copolymer was synthesized on PMMA for antibody immobilization, combining the antifouling properties of PEGMA and covalent antibody binding via the epoxy groups of GMA. The same GMA-co-PEGMA brushes were synthesized on silica nanoparticles for detection antibody binding. The synergistic amplification strategy yielded enhanced sensitivity in a sandwich immunoassay for carcinoembryonic antigen by two orders of magnitude.

The integration of nanomaterials is a promising field of research in biosensor and biochip technologies. Nanomaterials may serve as carrier or immobilization matrix for the biorecognition element [Lee et al. 2004, Preininger et al. 2011], as labels [Nam et al. 2003] or energy donors [Zhou et al.2015], and often they are closely related to signal transduction and signal enhancement. Polymer nanocomposites can be functionalized with a high number of labels (as for example gold nanoparticles (AuNP), quantum dots [Dong et al. 2010, Resch-Genger et al. 2008], and organic dyes) for signal amplification.

Another approach is the development of novel biochips that exploit plasmon-enhanced fluorescence. The plasmonic structures can for instance be implemented by using cost-effective NIL-based technologies and the resulting chip is compatible with established microarray-based fluorescence methods.

The fluorescent labels are probed by the confined field of surface plasmons that originate from collective oscillations of charge density at a surface of metallic films or metallic nanoparticles. The excitation of surface plasmons is accompanied with strongly increased intensity of electromagnetic field which couples with fluorophores [Dostalek 2015] Through plasmon-enhanced fluorescence, the sensitivity of currently used assays can be enhanced by combining three effects: a) increasing the excitation rate and decreasing background by the strongly enhanced and localized surface plasmon field intensity, b) improving photo-stability owing to the shorter decay time of the fluorophore and c) enhancing the efficiency of fluorescence light collecting via surface plasmon-coupled emission [Bauch et al. 2014].

2.7 Miniaturization

Standard microarray formats of 25 x 75 mm usually harbour spots of about 50-100 µm in diameter with a spot to spot distance of 300 to 500 µm. There has been a lot of progress regarding size reduction of microarrays lately. The most important implications are

- Higher spot density and consequently higher number of BREs on a given chip size;

- Faster reaction kinetics and lower assay times;

- Reduced consumption of reagents and most important of (patient) samples;

- Small arrays avoid scanning and hence reduce size and costs of read-out instruments;

- Reduced chip size is needed for integration into (portable) instruments.

For analytical protein chips the number of probes is often low and spot/array size may not be a crucial factor when working with standard microarray scanners in central microarray facilities. For global proteome analysis, however, more than 10,000 analytes may be targeted asking for high density arrays [Wingren and Borrebaeck 2007]. Zhu et al. [2001] for instance printed 13,000 protein samples in duplicates onto the area of a standard microscope slide for a yeast proteome microarray in order to test for protein-protein and protein-lipid interactions. Chip size reduction on the other hand is crucial for firstly reducing sample consumption and secondly for making instrumentation portable and hence independent from big laboratories. In our group we developed a test for 9 biomarkers of neonatal sepsis working with only 4 µl patient sample. Streptavidin magnetic particles allow detection of binding of biotinylated antibodies and at the same time serve as micro-stirring components [Buchegger et al. 2012].

In order to accommodate the more than 20.000 probes necessary for global proteome analysis, attovial antibody arrays have been developed in the group of Carl Borrebaeck at Lund University [Ellmark et al. 2009]. The attovials were made by structuring 200 nm polymethyl-methacrylate (PMMA) layers on glass slides with electron beam lithography. Probes were deposited using the nanoscale dispenser NADIA (see 2.3 for a description of the technology). The authors discuss the limits of miniaturization, such as the number of proteins that can be captured in one spot and the maximum resolution of optical imaging. Comparing vials of 0.5 up to 4 µm, they achieved highest sensitivity and dynamic range with the bigger vials. Tsarfati-BarAd et al. [2011] however point out the role of the immobilization chemistry, the binding site density, its homogeneity, and intrinsic non-binding area dimensions of a particular surface. As the intensity of the signal resulting from a spot is proportional to the binding area only, not to the spot area, the diameter of the spot has to be large compared to the non-binding area (see Figure 10 taken from Tsafarti-BarAd et al. 2011).

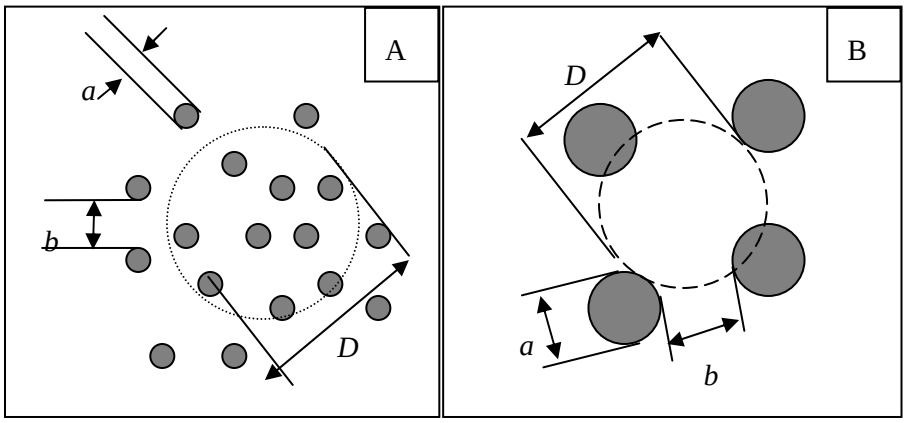


Figure 10. (A) An illustration of an immobilization substrate. The grey circles describe patches of dense binding sites, with diameter a. The typical distance between edges of binding patches is b. The area outside the binding patches does not bind any molecule. The dashed-line circle represents a drop of molecules deposited on the substrate, with diameter D. Here D is large compared with (a+b). (B) A zoomed view of (A), and a drop with a diameter D comparable to the dimensions of b. Very few molecules contained in the drop will bind to the surface in such a case. [Tsafarti-Bar-Ad et al. 2011]

For the detection of Human Immunodeficiency Virus Type 1 p24 antigen (HIV -1 p24) in plasma Lee et al. [2004] fabricated nanoarrays on gold using DPN (see 2.3 for a description of the technology). Arrays with antibodies to HIV -1 p24 antigen were produced by patterning MHA in 60 nm dots, passivating the areas around the nanodots with 11-mercaptoundecyl-tri(ethylene glycol), adsorbing the antibodies to the deprotonated MHA and blocking the arrays with 10% BSA in 10 mM PBS. The sandwich immunoassay with antigen and gold nanoparticles functionalized with polyclonal p24 antibodies was evaluated by AFM height images reaching far better sensitivity compared to a conventional ELISA in only 1 µL of patient sample. Site specific immobilization of Cowpea Mosaic Virus was achieved by writing a mixture of two dialkyl disulfides, one PEG terminated and one with a maleiimide group. The density of the functional maleiimide groups can be tuned to efficiently capture mutant Mosaic Virus bearing cysteine groups [Smith et al. 2003].

2.9 Automated platforms

Especially but not exclusively, for application in medical diagnostics, microarray systems have to be fully automated in order to keep errors by users as small as possible, make measurement procedures safe for users and patients, and enhance reproducibility of read outs. Portable systems are preferred, since they enable bed-side measurements, application in outpatient care, and in doctor's offices. Point-of-Care testing (POC), i.e. diagnostic tests at or near the patient [Kost et.al 1999], represents an especially

promising field for (arrayed) biosensors. POC features several advantages such as rapid real-time analysis, no transport of patient samples, no danger of confusing samples, and no sample preparation is needed. POC testing may improve the mutual trust of clinician and patient and reduce time for therapeutic decisions [Kost et al. 1999].

The Array Biosensor developed at the Naval Research Laboratory in Washington represents a semi-automated device. It consists of a microscope slide holding the array of BREs, a PDMS flow cell, which is addressed by a syringe needle connecting to a fluid reservoir from where the assay solutions are pumped through the flow cells. After the assay, the PDMS flow cell is removed and the microarray imaged with a CCD camera for fluorescence read-out [Rowe et al. 1999, Shriver-Lake et al. 2004, Sapsford et al. 2006]

One of the first automated platforms was the parallel affinity sensor array (PASA) with chemiluminescence detection [Weller et al. 1999]. The instrument consists of auto sampler, flow cell onto which the chip is mounted, CCD detector and computers for control and data evaluation. The prototype was used for detection of triazine herbicides contaminating water. Determination of antibiotics in milk was demonstrated with the Munich Chip Reader (MCR), a follow up of PASA [Kloth et al. 2009].

The Gauglitz group at the University of Tübingen has published numerous papers on fully automated biosensor arrays for water analysis using the optical immunosensor River Analyzer (RIANA) and the AWACSS system [Tschmelak et al. 2004, Tschmelak et al. 2005].

With a POC device for the diagnosis of sepsis, incorporating a Total Internal Reflection Fluorescence (TIRF) detection system and a fluidic unit, the parallel detection of C-reactive protein, Interleukin-6, procalcitonin and neopterin was achieved in only 10-75 µl human plasma or serum within 25 minutes [Kemmler et al. 2014].

Integration of microfluidics results in a number of advantages, such as automation of the sample processing steps, integration of mixing, reduction of assay times, and integration of read-out instruments. Microfluidic devices are fabricated from silicon, glass, or polymers. Production of plastic devices is cheaper and less time consuming, while the advantages of glass and silicon are well defined surfaces, chemical resistance, thermal stability, and excellent optical properties [Situma et al. 2006].

In contrast to static incubation, using a flow system can significantly improve assay times and detection limits, as slow diffusion kinetics hinder efficient analyte binding. Gehring et al. pointed out that sensitivity improvement of at least two logs for the detection of bacteria could be achieved. Cells flowing over the capture antibodies were more efficiently recognized than in a static system. Bacterial cells feature essentially the same density as water and therefore efficiency of capturing cells at planar surfaces is very poor [Gehring et al. 2008, Delehanty et al. 2002].

2.10 Data analysis

The images produced by instruments for read-out have to undergo image processing for spot detection and extraction of signal intensities from raw data (i. e. the image). A number of algorithms (such as fixed circle-, adaptive circle-, seeded region growing-, or edge detection- methods) is available to classify pixels either as foreground or background pixel, a process called image segmentation. A regularly spaced mask, the so called grid, is aligned with the spots and a matrix with spot ID and coordinates, signal intensities, background intensities, and a number of quality measures (for instance spot size, morphology, saturation level) are issued by the image analysis software. The guide dots, defining the grid position on the chip, are located at defined positions and contain labelled material (Figure 9.). Gridding and alignment usually allow or even ask for a certain degree of intervention by the user. Fully automated image processing on the other hand can increase throughput and may reduce error [Gierahn et al. 2014]. The spot quality measures can be used to filter data in order to reduce error estimates [Sauer et al. 2005]. A high quality spot is characterized by a high signal-to-noise ratio or signal – background value, respectively, stable spot size and regular shape. Intensity variations within a spot and spot homogeneity can be checked with the standard deviation of the mean pixel intensities and visualized by 3D views showing the intensity values of each pixel [Preininger and Sauer 2003] and Figure 9. We usually measure nine to twelve replicate spots and calculate arithmetic mean and standard deviation for each sample.

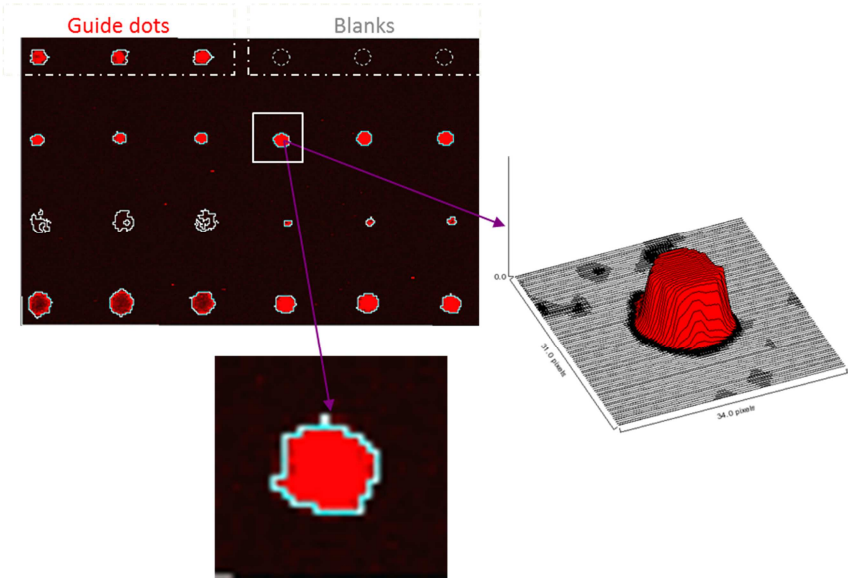


Figure 9. Detail of a scan image after gridding and spot segmentation. In the upper left corner three guide dots help positioning the grid and in the upper right corner three blanks (i.e. buffer spots) are used as a control for carry over during the spotting process. Spots are segmented on foreground and background pixel. The 3D graph shows a spot spreading in x and y axes, while z shows the signal intensity.

Quantification of analytes by protein microarrays is usually accomplished by calibration with multiplexed standards including a zero standard (i.e. pooled serum from healthy patients or another biological matrix, e.g. pooled analytical urine, saliva, cell culture supernatant, etc.); each analyte has to be represented in the mix. The standard mixes of a number of concentrations (ideally 6 or more) are assayed in separated compartments or chips, covering the application relevant measuring range. In instruments with flow channels either a number of channels are used for calibration and patient samples [Baldini et al. 2009] or calibration and measurement takes places sequentially in a single channel given the chip is regenerable [Seidel & Niessner 2008]. Calibration curves are set up using an appropriate model such as the 4-parameter logistic fit (see Figure 10). In analytical protein microarrays usually foreground – background intensities are fed to a model rather than signal to noise ratios. Important key parameters of a fit and their definitions are listed in Table 3. For bioanalytical methods validation there are certain acceptance criteria defined by e.g. FDA. General acceptance criteria are e.g. 85-115% accuracy (= recovery of a spiked analyte) and a precision of more than 85% (meaning a coefficient of variation (CV) of less than 15%). At the lower limit of quantification (LLOQ) acceptance criteria are less strict (20% CV and 80% recovery) [FDA 2001].

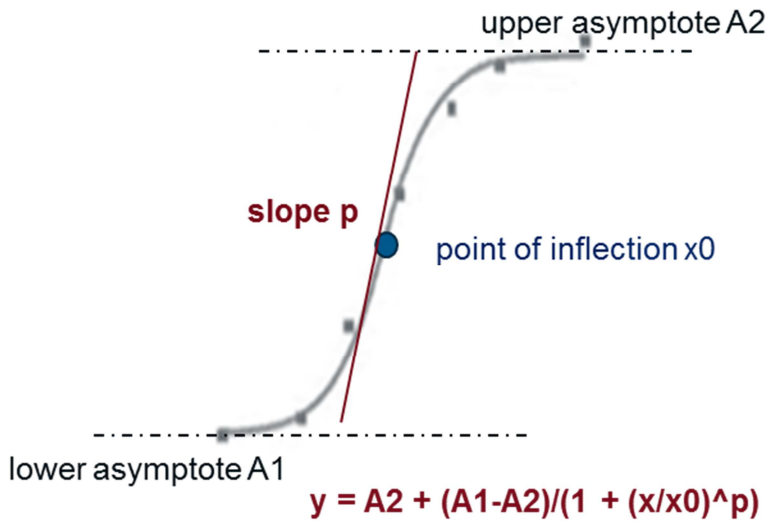


Figure 10. Four parameter logistic fit.

Table 3. Key parameters used for describing a calibration curve fitted with the 4-parameter logistic model.

Limit of detection - LOD	lowest detectable concentration; $y = y_0 + 3\ SDy_0$
Lower limit of quantification - LLOQ	lowest quantifiable concentration; $y = y_0 + 10\ SDy_0$
Upper limit of quantification - ULOQ	highest quantifiable concentration; A2−SDy
Working range	ULOQ -LLOQ
Coefficient of variation - CV	measure for precision; CV% = SD / mean × 100
Coefficient of determination – R^2	how well do the data fit the model; $R^2 = 1- (SS_{res} / SS_{tot})$
Point of inflection x0	measure for sensitivity
Slope of the tangent at x0 – p; IC50	measure for sensitivity
Recovery	measure for accuracy; % rec = calculated x / spiked x ×100

2.11 Detection of disease related biomarkers

Protein microarrays require only small volumes of sample material, which makes them attractive for measuring patient samples in clinical applications. Protein biomarkers are signals of specific states of a patient, indicating for instance the presence or stage of a disease [Rifai et al. 2006]. It is commonly accepted now that rather than determining a single marker a multiplexed analysis will result in better diagnosis, prognosis, and prediction of response to therapy [Xiao et al. 2005; Humpel 2011]. Measuring biomarker profiles and combining this with conventional pathological classification would yield more precise classifiers, and enhance clinical development of personalized therapy [Matsumoto et al. 2015]. For quite a while now efforts have been undertaken in order to find disease specific markers for personalized medicine. Novel biomarkers may be found by methods, such as microarrays and mass spectrometry and interpreted by bioinformatics [Humpel 2011]. Also systematic literature searches may be helpful to set up a disease related panel or biomarker sets for patient stratification [Gogalic et al. 2015]. Further, Omics profiles leading to delineation of molecular disease maps can be used for selecting biomarkers. A list of more than 1000 proteins, believed to be differentially expressed in human cancers, has been compiled, but only nine have gained final FDA approval as tumour-associated antigens [Biophoenix 2009].

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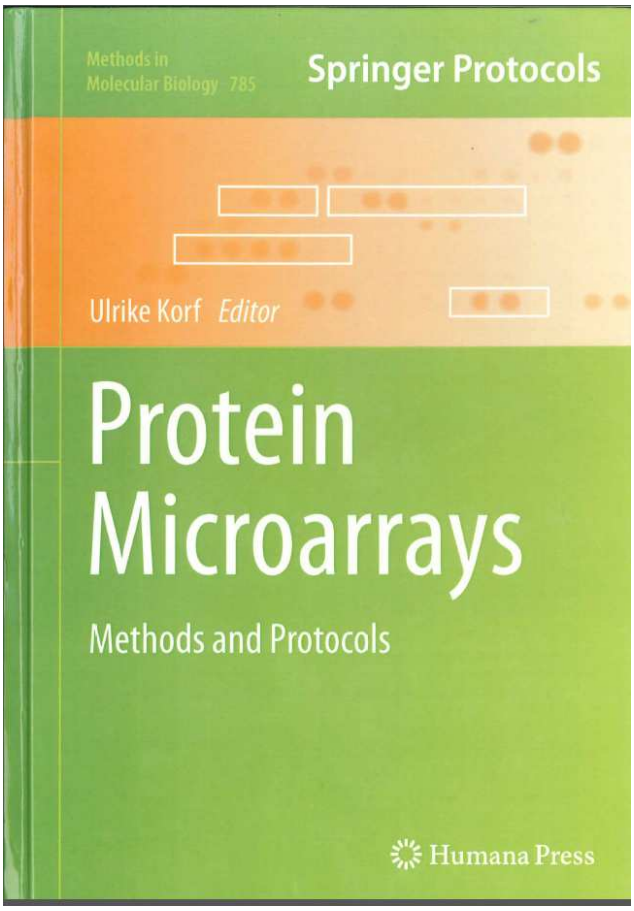
3 Impact of substrates for probe immobilization



Chapter 24

Impact of Substrates for Probe Immobilization

Ursula Sauer

Abstract

Protein chips are becoming a key technology in proteomic research and medical diagnostics. Surface chemistry for immobilization of proteins forms the basis for assay design and determines the properties of protein microarrays. Optimal substrates provide a homogeneous environment for probes, preventing loss of biological activity and unspecific adsorption. Numerous immobilization approaches, based on covalent binding, affinity, or adsorption, have been proposed thus far, and these represent the toolbox for choosing optimized strategies for each individual application.

Key words: Protein patterning, Coating, Surface chemistry, Affinity binding, Adsorption, Covalent binding, Hydrogel

1. Introduction

Solid supports and immobilization strategies for probe attachment play a central role in the development of protein biochips by determining sensitivity, specificity, and reproducibility. The immobilization of proteins to solid phase surfaces has been of interest since immunological techniques emerged. With the emergence of biosensor arrays (also referred to as protein microarrays or protein biochips), the demand for suitable immobilization strategies has grown. In contrast to immobilization of proteins for immunosensors in general, parallelization as a main feature of microarrays requires a patterning of probes as opposed to simple coating. Nonetheless, when applicable for patterning, knowledge about immobilization derived from ELISA techniques, affinity chromatography or biosensors can often be employed. This "patterning" of protein chips, providing regions of specific binding of ligands and nonadhesive regions, is primarily done by robotic printing,

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Abstract

Protein chips are becoming a key technology in proteomic research and medical diagnostics. Surface chemistry for immobilization of proteins forms the basis for assay design and determines the properties of protein microarrays. Optimal substrates provide a homogeneous environment for probes, preventing loss of biological activity and unspecific adsorption. Numerous immobilization approaches based on covalent binding, affinity or adsorption have been proposed thus far, and these represent the toolbox for choosing optimized strategies for each individual application.

1. Introduction

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The second resource for potential protein biochip substrates is DNA microarray technology, in which consideration must be given to the differing chemical and physical properties of proteins and nucleic acids. While DNA, being negatively charged, provides a uniform chemistry, proteins exhibit a vast chemical and structural diversity; they differ in size, charge and reactive groups on the surface. Protein purification is complicated, and the lack of an amplification method such as PCR causes sensitivity problems. Furthermore, proteins are less stable than DNA and more prone to lose biological activity when immobilized. The role of substrates in the dynamics of rapidly drying protein spots after printing is not yet fully elucidated.

Immobilization matrices for protein microarrays can be classified according to their coupling chemistry (adsorption, affinity binding, covalent binding) or their dimensionality, namely one-dimensional (monolayers), 2-dimensional (2D), more or less planar surfaces or 3-dimensional (3D) surfaces, e.g. membranes or hydrogels (Figure 1).

Reports on characterization and in-depth comparisons of the performance of such substrates are rather sparse, while little has been published about the underlying biophysical mechanisms of protein binding to surfaces (1 -10).

No general recommendation or recipe for an immobilization method can be given here; an optimal protocol will in fact need to be chosen for each application.

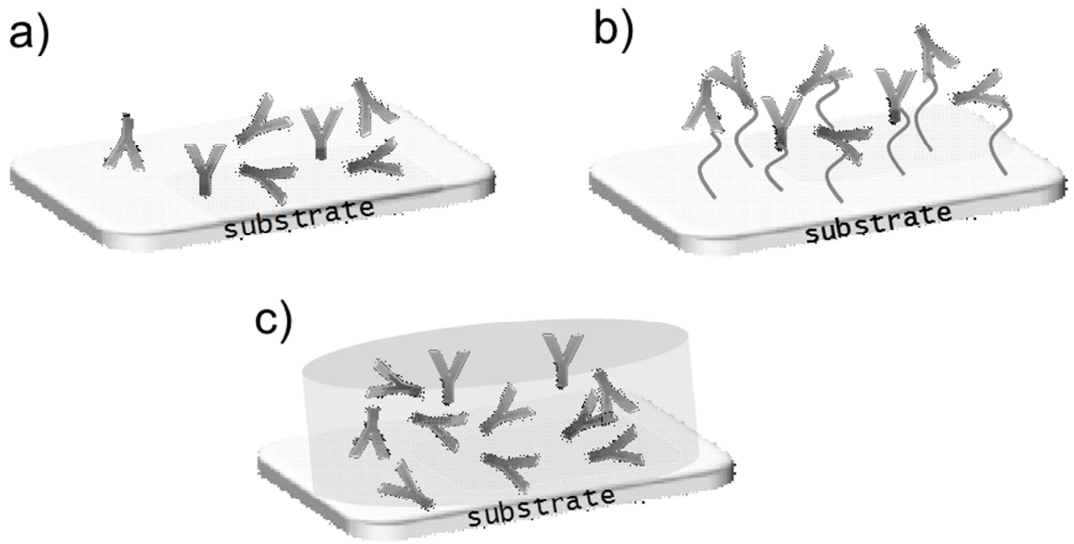

Figure 1. Scheme of randomly immobilized antibodies in (a) one-dimensional (monolayer) (b) 2-D (silanes, crosslinkers) and (c) 3-D (hydrogels, membranes, dendrimers) coatings

Decision criteria for an immobilization method
Solid support
Required sensitivity
Assay format/ targeted analytes
Stability of the probes
Detection system
Possible costs/economic mass production

The most common sources of solid support for microarray systems are fused silica and glass substrate materials due to their good optical properties (low autofluorescence at excitation wavelengths), mechanical and thermal stability and chemical inertness. For the micro-fabrication of lab-on chip systems, alternative materials had to be introduced, e.g. cycloolefin copolymers (COC), poly(methyl methacrylate) (PMMA) and polycarbonate; these substances are suitable for high throughput processing such as molding, hot embossing or laser welding. Either immobilization matrices are coated onto these

substrates or the substrates themselves are chemically modified in order to create appropriate binding sites for biomolecules.

Choice of surface chemistry is also driven by the detection system. Planar waveguide-based detection only works for coatings with a layer thickness of less than 100 nm and surface plasmon resonance (SPR) requires immobilization onto gold surfaces, while fluorescence-based approaches call for substrates of low autofluorescence at the excitation wavelengths.

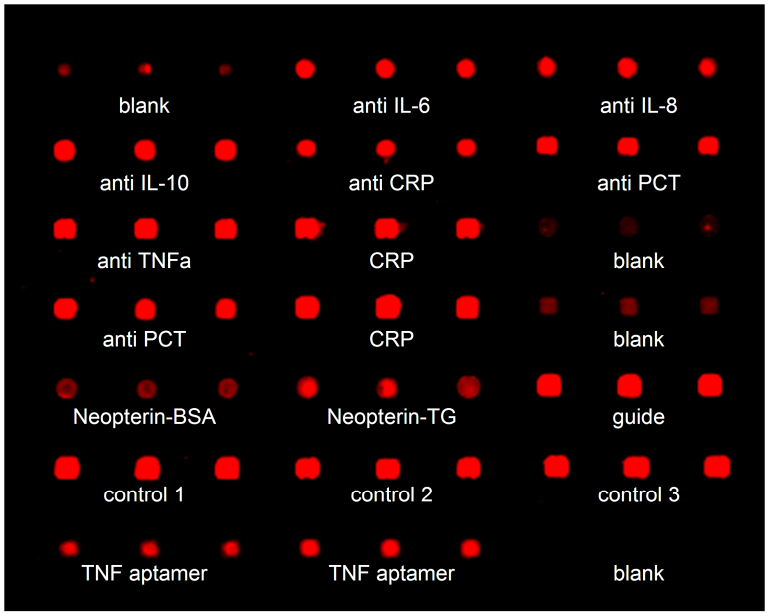

Figure 2. Array of capture antibodies, antigens and antigen conjugates printed onto ARChip Epoxy. Detail of a chip for the quantification of biomarkers after an assay with a mixture of spiked proteins and fluorescently labelled antibodies.

2. General requirements for immobilization matrices

An important criterion of biomolecule immobilization is the high functionality of the chip surface. A proper density of binding sites consistent over the entire slide surface is a prerequisite for effective biosensing. Increasing the solid phase concentration of antibodies results in increased sensitivity and extended working range (11, 12). Otherwise, when capture molecules are bound too densely, steric hindrance and decreased target binding efficiency may result. Furthermore, attached molecules have to be presented in such a way that epitopes/binding sites are lifted away from the surface and hence well accessible for the target.

One of the major issues in microarray development is fabricating a surface that, in addition to excellent signals, results in as little background noise as possible. Figure 2 shows a typical high quality scan image of a biomarker chip using an epoxy resin as immobilization matrix. One source of noise is unspecific protein adsorption, which is controlled by the choice of surface chemistry and blocking protocols. For optical read-out based on fluorescence, the intrinsic autofluorescence of the surface can be a major contributor to noise, especially for nitrocellulose. Fluorescence background is usually computed for each individual spot based on a local background subtraction technique.

Kusnezow et al. point out the importance of antibody microspot kinetics, namely for the analyte to migrate in solution as well as across the immobilization surface (13). Protein receptors in solution display homogeneous binding affinities and kinetics for their ligand, while upon immobilization they display heterogeneous binding characteristics. Vijayendran and co-workers evaluated this heterogeneity of five different immobilization strategies (14). The most homogeneous behavior was found with antibodies immobilized oriented via their carbohydrate moiety: the amount of heterogeneity with respect to affinity to the ligand was closely related to heterogeneity in analyte-antibody kinetics.

High quality spots with uniform pixel intensities are a key requirement for meaningful data analysis (15). Spots must be of the same shape and size throughout a slide and from one slide to another. This is achieved by the optimized interplay of surface chemistry, probe, printing technique and print buffer. Hydrophobic surfaces tend to produce small but inhomogeneous spots, whereas most hydrophilic surfaces yield homogeneous spots, which, however, are often irregular in shape (9). High reproducibility is even more important for quantitative analyses of biomarker detection, since the standard deviation of replicate spots is part of sensitivity measures such as the calculation of limit of detection (LOD) and limit of quantification (LOQ).

In order to be applicable as routine analytical tools, microarray substrates have to be affordable, suitable for mass production, easy to handle and provide reasonable shelf life.

Requirements for immobilization matrices
Availability of protein binding sites
Accessibility of the binding sites for capturing the target molecules
Maintained capability of specifically capturing antigen, non-denaturing conditions
Reproducibility
Low non specific binding of the surface (background)
Excellent spot morphology for reproducible image analysis
Reasonable shelf life

3. Sensing molecules to be immobilized (probes)

For sandwich type and competitive on-chip immunoassays, as well as for protein expression profiling, antibodies or their fragments have to be attached to a solid support, while proteins, recombinant proteins or peptides are used as probes for binding inhibition assays. In the first case, probes are more or less of similar nature, namely monoclonal or polyclonal antibodies; consequently the requirements for the immobilization chemistry are comparable. In the second case, proper probe immobilization may be more sophisticated, as molecules of different chemistry, size, quaternary structure and loading have to be immobilized on one common solid support, without biological activity being negatively affected by denaturation and conformational changes.

Further designs posing high demands on surface chemistries include antigen arrays for studying autoimmune diseases [http://proteomics.stanford.edu/robinson/antigen.html], gylcan-microarrays for the interrogation of glycan – protein interactions to study cell communication (16) (e.g. see the Consortium for Functional Glyomics; http://www.functionalglycomics.org/static/index.shtml), protein kinase assays (17), ATP and GTP binding assays, and studies of protein-protein interactions.

4. Physical adsorption

A simple and affordable immobilization method is the adsorption of proteins via intermolecular forces as employed previously in microtiter ELISAs. Adsorption is based on the non-specific electrostatic, hydrophobic or Van der Waals forces. Local dipoles in the participating molecules are stationary, forming strong hydrogen bonds, or alternating dipoles in non-polar regions of the reagents, forming weaker hydrophobic interactions (18). Proteins are oriented randomly upon adsorption. Drawbacks are desorption of the proteins during assays, structural deformation and the denaturation of biomolecules commonly observed. High bulk concentrations lead to less contact with the surface per

molecule and hence to less unfolding. Butler et al. (19) report on high losses of protein function upon adsorption on polystyrene. Only 5-10% of polyclonal antibodies were capable of capturing antigen, while a streptavidin-mediated immobilization of biotinylated IgG resulted in up to 70% preservation of the antigen binding sites.

Widely used materials for protein adsorption are polystyrene (www.nuncbrand.com) (20), poly-L-lysine, aminosilane and nitrocellulose (21- 23). Figure 3 shows the microporous 3D structure, responsible for the high binding capacity of nitrocellulose, imaged by means of scanning electron microscopy (www.whatman.com).

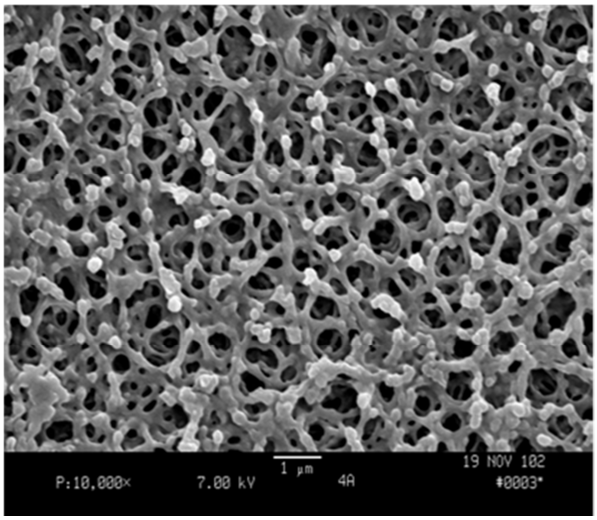

Figure 3. 3D structure of Whatman nitrocellulose, scanning electron microscopy, magnification x 10,000 (http://www.whatman.com: the FAST Guide to Protein Microarrays).

5. Hydrogels

Hydrogels are considered especially suitable for protein immobilization, providing a controlled nano-environment that can keep the protein hydrated and stabilize the structure. Binding principles on hydrogels are adsorption or, where reactive groups are available, covalent attachment, e.g. the hydrophilic polymer of Nexterion Slide H (www.schott.com) is activated with N-Hydroxysuccinimide (NHS)-ester, which reacts with primary amino groups of proteins covalently (Figure 4).

Widely used as 3D-immobilization matrices are agarose (24, 25), poly(acrylamide) (26), polyurethane (27), poly(vinyl alcohol) (28), dextran (29, 30) and polyethyleneglycol (31). Hydrogels can be tuned to a certain extent in order to mimic the biological environment of proteins. For example, Moorthy et al. (32) found the binding interactions between IgG and Protein A enhanced as the pore size of polyacrylamide decreased.

Hydrogel coatings are produced by spin-coating, dip-coating onto solid supports or by covalent binding of the gel on silanized substrates. Gels are not only produced as coatings but directly co-spotted with probes. Rubina et al. (26) used a polymerization-mediated immobilization method to produce hydrogel protein chips for the detection of biotoxins, in which case the polymethacrylamide hydrogel containing the proteins is spotted onto the slides. Dominguez et al. (33) fabricated antibody-entrapped hydrogel chambers by arraying solutions of both tetra- or octa-amine functionalized peptide-based branch macromolecules and IgG on aldehyde glass slides. These methods single-step, rapid and keep the antibody hydrated and in its original conformation, since no modification of the antibody is necessary.

Clearly, diffusion coefficients for proteins should be lower in gels, slowing down assay times. Kinetic curves for binding Cy3-labelled ricin with immobilized antibodies in acrylamide gel-pads reached equilibrium only after 15 hours (26). Several methods for accelerating diffusion in microarray experiments have been suggested, such as peristaltic pumps (34), creating a constant flow, and ultrasonic mixing.

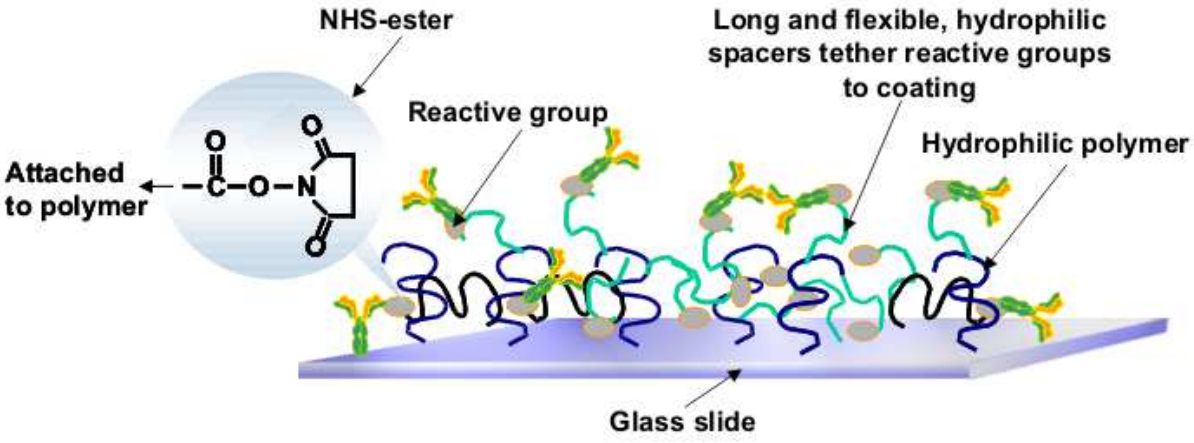

Figure. 4 Scheme of immobilization chemistry of Nexterion Slide H (http://www.schott.com)

6. Covalent binding of probes

Functional groups of amino acids exposed to the protein surface can be employed for direct covalent attachment, which results in “statistically oriented” immobilization. Lysines are numerous on a protein surface for example, while cysteines are less abundant.

Commonly used 2D surfaces for covalent immobilization provide aldehyde, epoxy, amino, mercapto or isothiocyanate groups (35) or N-hydroxysuccinimide (NHS) ester. Proteins are coupled to amine reactive surfaces on the formation of a Schiff’s base linkage, primary amines may be provided by lysines on the protein surface. Epoxy

functionalities bind via nucleophilic substitution to highly abundant groups on protein surfaces as amino, thiol and hydroxyl groups. McBeath et al. (36) attached proteins covalently to aldehyde-derivated glass slides as well as to BSA-N-hydroxysuccinimide slides for three applications: screening for protein-protein interactions, identifying substrates of protein kinases and finding protein targets of small molecules. Other chemical functionalities of proteins used are: -SH (cysteine), -COOH (aspartic acid, glutamic acid), -OH (serine). Carbohydrate or carboxyl groups can be activated with (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) EDC and bind to an amine reactive surface (1), while thiol groups may be used for covalent coupling to epoxy- and maleimide surfaces. Numerous coupling strategies have been developed for immobilizing antibodies on different solid surfaces through the formation of defined linkages in which glutaraldehyde, carbodiimide and other reagents such as succinimide ester, maleinimide and periodate are employed. However, problems can be seen in many cases, associated with the loss of the native functional state upon immobilization of antibodies (37). Covalent attachment is less denaturing than adsorption, while nonetheless only a certain proportion of the antibodies stay biologically active and accessible.

Seong (38) compared IgG immobilization on commercial silylated slides and epoxy slides, both of which are amine-reactive, and reported a superior binding capacity on epoxy-coated slides (www.xenopore.com). According to the findings of Olle (39) as well, epoxysilane (www.eriesci.com) was superior to HydrogelTM (Perkin Elmer) and SuperAldehyde (Telechem) in IgG binding with respect to signal intensity and low background.

The covalent immobilization of histone proteins onto N-Hydroxysuccinimide ester and agminated surface modified with maleic anhydride-alt-methyl vinyl ether (MAMVE) copolymer was investigated (40). The immunoassay on MAMVE-functionalized surfaces displayed a limit of detection 50 times lower than that of the ELISA assay in polystyrene plates.

The accessibility of antibodies immobilized via a long and flexible spacer such as poly(ethylene glycol) was investigated by AFM (41). An AFM tip was coated with E. coli in order to analyze by means of force-distance curves the interaction between bacteria and the specific antibody and evaluate optimal surface coverage and spacer length.

Dendrimers are highly branched macromolecules that form a 3-dimensional structure with a variety of possible chemical functionalities, maximizing the density of binding sites (for examples, see www.dendritech.com; www.dendrimercenter.org; www.sigmaaldrich.com). High density protein chips were prepared by activation of Si or

glass wafers and poly(propyleneimine) dendrimers modified with a sulfosuccinimide ester, providing a fixed number of functionalities for covalent protein binding (42). Yam et al. (43) prepared poly(amidoamine) dendrimers (PAMAM) functionalized with biotinylated oligo(ethylene glycol) (OEG) derivatives consisting of self-assembled monolayers (SAMs) of 11-mercaptoundecanoic acid (MUA) on gold substrates, which minimized non-specific protein adsorption and at the same time provided a high density of avidin-binding sites.

7. Affinity binding of probes

Immobilization via biochemical affinity ideally results in the oriented attachment of probes. The avidin/biotin system is especially widely used since it offers several advantages, particularly the strong affinity and specificity of the interaction (44). Biotinylated proteins are attached to streptavidin-coated surfaces (Figure 5); several are commercially available (e.g. www.xenopore.com; www.arrayit.com). Bathia et al. describe silanization and treatment with succinimide ester for subsequent coating with Neutravidin, ready for binding biotinylated probes (45). Provided that biotinylation takes places in a non-binding region of the protein, this approach is more likely to maintain the native function of a protein. For biotinylation, amino groups of the proteins are often used, resulting in random attachment of biotin and consequently random immobilization of the biotinylated probes. Site-directed biotinylation at the hinge region of F(ab')2, on the other hand, was demonstrated to allow controlled oriented antibody immobilization with detection capabilities up to 20 times greater compared to random biotinylation (46). Peluso et al. (47) studied the effect of four different methods of binding biotinylated antibodies or fragments onto streptavidin surfaces with respect to surface density and binding activity. The study involved comparing random biotinylation of monoclonal IgG and Fab' fragments to biotinylation with the biotin-aminooxy compound ARP after oxidzing the glycosylation site at the Fc portion. Oriented immobilization outperformed random coupling, with up to a ten-fold increase in analyte-binding capacity.

Metal complexes had been employed for affinity binding of proteins in affinity chromatography prior to being used in microarray technology. Histidine -tagged recombinant proteins are captured with high affinity by metal ions, retaining the native conformation (www.xenopore.com). Nitriloacetic acid (NTA) forms a tetradentate chelate with the Ni2+ ion, although other transition metal ions with a coordination number of six can be used (e.g., Co2+, Cu2+, Zn2+). In order to overcome the drawback of low affinity,

which reduces the yield of immobilized protein on Ni-NTA surfaces, double his-tags were introduced by Khan and coworkers (48).

A screening of libraries of polymer chelating surfaces containing different metal ions for efficient antibody binding was done by Muir et al. (49), and the secondary amine, the metal counter ion and chelating ligand were identified as main variables.

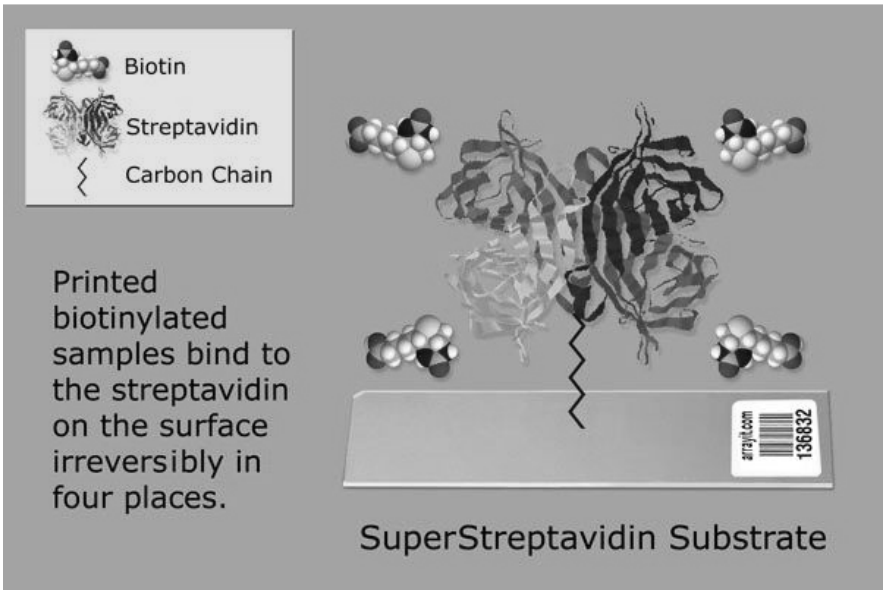

Figure 5. ArrayIt Super Streptavidin Substrate. Image provided by https://www.arrayit.com. Copyright 2010, ArrayIt Corporation. All Rights Reserved World Wide.

8. Oriented versus random immobilization

Non-oriented immobilization of antibodies does not discriminate binding sites in or near the Fab fragment of an antibody, therefore the antigen binding sites may be not accessible, which either entirely blocks or at least hinders the ability to specifically bind antigen. In order to avoid such problems, several strategies for oriented antibody immobilization have been developed (37). The advantages of the oriented immobilization of proteins are a good steric accessibility of the active binding site and increased stability. Danczyk et al (1) found improved antigen capture capabilities of antibodies attached using protein A, although the amount of immobilized antibodies is smaller than the number directly bound or adsorbed, suggesting that a higher proportion stays functional.

Oriented antibody immobilization
Via antibody receptors
Chemical or enzymatic oxidation
Disulfide bond reduction
Site specific biotinylation
Recombinant antibodies with tags
DNA-directed

Orientation is achieved by coupling via antibody receptors such as protein A, protein G or recombinant protein A/G which bind in the Fc region of the antibody. Coupling via antibody receptors may cause problems with IgG from serum that may also bind to the receptors if they are not saturated with capture antibodies or if the blocking of remaining receptors is insufficient. Affinity of protein A for IgG subclasses differs, and the same is true for protein G; an alternative product combining binding sites from protein A and G is the recombinant protein A/G (www.arrayit.com).

Chemical or enzymatic oxidation of the carbohydrate moiety located in the Fc fragment to aldehyde groups goes without significantly impairing the active sites of the antibody. The oxidized antibodies can then be immobilized to hydrazide-activated supports by forming covalent hydrazone bonds. Periodate oxidized antibodies were also first used for immunoaffinity gels. The method was successful with polyclonal antibodies, while for monoclonal antibodies milder oxidation conditions have to be employed. Another approach uses the sulfhydryl group of the Fab region between the light and heavy chain to create an oriented antibody fragment.

Self-assembled monolayers consist of a single layer of molecules on a substrate. In self-assembly techniques, thiols and disulfides are mostly used on metal substrates such as gold and silver, while silanes are used on non-metallic surfaces such as SiO2 and TiO2 (8).

Surface preparations of self-assembled monolayers composed of ssDNA thiols and oligo(ethylene glycol) terminated thiols were introduced for DNA directed protein immobilization (50). The mixed SAM allows rational control over the DNA probe surface density. Antibodies conjugated to ssDNA with a sequence complementary to the surface-bound ssDNA are hybridized on the biosensor and convert the DNA surface into a protein surface in a single step. The surface can be completely regenerated with NaOH to dehybridize the DNA. Alternatively, DNA-streptavidin conjugates were used to immobilize biotinylated antibodies onto DNA surfaces (51). DNA-directed immobilization was compared to direct spotting on activated glass and strepatividin-biotin attachment

with regard to signal intensity, assay sensitivity and reproducibility (52). All three methods allowed the detection of 150 pg/mL IgG in a sandwich immunoassay, while DNA-directed immobilization was superior with regard to very low antibody consumption, spot homogeneity and reproducibility.

Shriver-Lake et al. (53) compared nine heterobifunctional crosslinkers as to their ability to bind antibodies, and tested the immunological activity with a fiber-optic biosensor. One approach used thiol-terminal silanes and heterobifunctional crosslinker with a succinimide moiety, reacting with the primary amines of the antibody (non-oriented). The other group were crosslinkers containing hydrazide, reacting with the carbohydrate moiety in the Fc region of the antibody and therefore providing orientation. Immobilization via the carbohydrate region resulted in higher packing density and higher levels of antigen-binding capacity (over 30% of the antibodies being active), which is explained by the distance between crosslinker reaction site and antigen binding site. Disadvantages were high loss of antibody (up to 50%) in the multi-step immobilization procedure and reports of decreased antibody activity after periodate treatment.

The methods for oriented immobilization listed above require chemical treatments of the probes, which may result in a significant loss of material and hence neutralize the positive effect of oriented immobilization. Kusnezow et al. reported a loss of up to 40% of antibodies as a result of activation and purification, leading to similar signal/noise ratios as for non-activated probes (54).

9. Advanced Materials

Protein biochips have to detect low target concentrations. In addition to optical techniques such as planar waveguide, evanescent resonator platforms, integration of micro optical elements, mirror slides and optical interference coatings, strategies for enhancing the sensitivity of protein microarrays include substrates with increased surface area, allowing high density of probes in a highly ordered manner.

Brush polymeric coating based on a copolymer of N, N-dimethylacrylamide (DMA) and N, N-acryloyloxysuccinimide (NAS) were produced for the detection of allergen-specific immunoglobulins (55). Since IgE-binding epitopes are mostly conformational, it is imperative to maintain the native conformation of the immobilized allergens.

Nijdam et al. (56) used modified silicon as a substrate for reverse phase protein microarrays (RPMA), yielding protein binding comparable to nitrocellulose. Mixtures of proteins from cellular lysate were directly spotted onto silicon that was roughened by

reactive ion etching and chemically functionalized using 3-aminopropyltriethoxysilane (APTES) and mercaptopropyltrimethoxysilane (MPTMS).

Coatings with calixarene derivatives for amine glass or gold were demonstrated to bind proteins in an oriented manner, yielding excellent sensitivity as low as 1-10 fg/mL of analyte (57). The authors proposed that the calixarenes, bifunctional affinity linkers, form a self-assembled monolayer which binds antibodies in the Fc region, stretching the antigen binding sites to the solution phase. Oh and coworkers (58) found a Calixcrown chip to be 10- to 100-times as sensitive than aldehyde and carboxyl chip in a sandwich immunoassay for PSA.

Zhu et al. (17) manufactured microwells from poly(dimethylsiloxane) (PDMS) with an acrylic mold. Rectangular arrays of 18 x 28 mm, optimized for a protein kinase assay, consisted of 10x14 wells with a volume of 300 nl; but arrays of smaller dimensions, for high-throughput screening, could be produced using the most recent molding techniques. Resulting elastomer sheets were placed on microscope slides for handling purposes. For protein immobilization PDMS was modified with 5M H2SO4, 10M NaOH, hydogen peroxide or 3-glycidoxypropyltrimethoxysilane (GPTS), the latter resulting in the greatest protein adsorption, namely up to 8x10-9 µg/µm² HRP anti-mouse Ig.

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4 Protein Chip for the parallel quantification of high and low abundant biomarkers for sepsis.

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Protein chip for the parallel quantification of high and low abundant biomarkers for sepsis

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ABSTRACT

We present herein a protein chip for diagnosis of sepsis that combines both a sandwich and a binding inhibition format in order to quantify high (CRP) and low abundant proteins (cytokines, PCT, neopterin) in parallel. Using the combined assay format the lowest detectable concentrations for CRP, IL-6, IL-8, IL-10, TNFα, PCT, and neopterin are 3 ng/L, 15 ng/L, 26 ng/L, 65 ng/L, 40 ng/L, 78 ng/L, and 0.46 μg/L. Four different combined assay formats are tested, using separate or joint incubation steps of analytes and detection antibodies. Yet, low limit of detection (LOD) and short processing time are contradictory: while the combined assay performed in a multistep protocol is extremely sensitive (e.g., the LOD for IL-6 is 15 ng/L), but more time-consuming (4 h), the all-in-one protocol takes only 2.5 h, but suffers from lower sensitivity compared with the multistep protocol (e.g., the LOD for IL-6 is up to 40 times enhanced). Reproducibility is good in both cases (CV 5–20%).

Cytokines, such as interleukin-6 (IL-6),[1] IL-8, IL-10, TNFα, and acute phase proteins, such as C-reactive protein (CRP) are biomarkers reported for infections, inflammatory processes, and sepsis. The combination of CRP, procalcitonin (PCT), and neopterin is especially valuable for the differentiation of infectious versus noninfectious inflammation [1] and for distinguishing bacterial from viral infections [2]. Rapid diagnosis of infection or sepsis is of particularly high priority for neonates as it is a leading cause of neonatal mortality [3]. Taking blood samples and other invasive procedures on neonates is kept to a minimum; thus, reducing sample volumes is an utmost concern.

The analytical range of the biomarkers to be detected is quite different: while CRP occurs in gram per liter concentrations, cytokines and PCT develop in the nanogram per liter range, and neopterin in the microgram per liter range. Krämer et al. [4] demonstrated lately a proof of concept for determining concurrently TNFα, PCT, and CRP via sandwich assays using a TIRF system.

In order to address the various clinically relevant concentration ranges, different assay principles and detection schemes are applied: for example, Watkin et al. [5] evaluated the serum levels and diagnostic value of IL-6, TNFα, IL-1β, procalcitonin, LBP, and CRP in patients with infective endocarditis employing automated latex enhanced immunoturbidimetry (CRP), immunochromatography (PCT), and enzyme-linked immunosorbent assay (IL-6, TNFα, IL-1β; separately LBP). Balci et al. [6] investigated PCT in comparison with CRP, IL-2, IL-6, IL-8, and TNFα as biomarkers to differentiate between systemic inflammatory response syndrome (SIRS) and sepsis. As in the work by Watkin et al. the respective parameters were measured sequentially using different assays: turbidimetry assay for CRP; chemiluminescence kits for IL-2, IL-6, IL-8, and TNFα; and immunolumiometric assay for PCT. Procalcitonin (~13 kDa), a precursor of the hormone calcitonin, may increase to 1000 μg/L in sepsis [7] and is measured by enzyme-linked fluorescent assay, time-resolved amplified cryptate emission, or luminescence immunoassays (BRAHMS, Hennigsdorf, Germany).

By contrast, the protein biomarker chip presented herein measures simultaneously CRP, IL-6, IL-8, IL-10, TNFα, PCT, and neopterin in clinically relevant analytical ranges employing sandwich and binding inhibition assay formats (see Table 2). The chip works with microliter sample volumes and uses fluorescence detection. The measurement scheme combines sandwich and binding inhibition formats and therefore is also apt for detecting small and large molecules in multiparametric immunoassays. The system provides the advantage of reduction of sample volume and assay time, as well as an increase in the number of analytes that can be measured simultaneously. Moreover it has the potential for extension, in the sense that new analytes can be added without difficulty.

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[1] *Abbreviations used:* BSA, bovine serum albumin; CRP, C-reactive protein; IL, interleukin; PBS, phosphate-buffered saline; PCT, procalcitonin; TG, thyroglobulin.

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Abstract

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1 Introduction

Cytokines, such as IL-6, IL-8, IL-10, TNFα, and acute phase proteins, such as C-reactive protein (CRP) are biomarkers reported for infections, inflammatory processes and sepsis. The combination of CRP, PCT and neopterin is especially valuable for the differentiation of infectious versus non infectious inflammation [1] and for distinguishing bacterial from viral infections [2]. Rapid diagnosis of infection or sepsis is of particular high priority for neonates as it is a leading cause of neonatal mortality [3]. Taking blood samples and other invasive procedures on neonates is kept to a minimum, thus reducing sample volumes is an utmost concern.

The analytical range of the biomarkers to be detected is quite different: while CRP occurs in g/ L concentrations, cytokines and procalcitonin (PCT) develop in the ng/ L range, and neopterin in the µg/ L range. Krämer et al. [4] demonstrated lately a proof of concept for determining concurrently TNFα, PCT and CRP via sandwich assays using a TIRF system.

In order to address the various clinically relevant concentration ranges, different assay principles and detection schemes are applied: for example, R.W. Watkin et al. [5] evaluated the serum levels and diagnostic value of IL-6, TNFα, IL-1α, procalcitonin, LBP and CRP in patients with infective endocarditis employing automated latex enhanced immunoturbidimetry (CRP), immunochromatography (PCT), and enzyme-linked immunosorbent assay (IL-6, TNFα, IL-1α; separately LBP). C. Balcı et al. [6] investigated

PCT in comparison with CRP, IL-2, IL-6, IL-8, and TNFα as biomarkers to differentiate between systemic inflammatory response syndrome (SIRS) and sepsis. As in the work by Watkin et al. the respective parameters were measured sequentially using different assays: turbidimetry assay for CRP, chemiluminescence kits for IL-2, IL-6, IL-8, and TNFα, and immunolumiometric assay for PCT. Procalcitonin (~13 kDa), a precursor of the hormone calcitonin, may rise up to 1000 µg/L in sepsis [7] and is measured by Enzyme Linked Fluorescent Assay, Time Resolved Amplified Cryptate Emission or luminescence immunoassays (BRAHMS, Hennigsdorf, Germany).

By contrast, the protein biomarker chip presented herein measures simultaneously CRP, IL-6, IL-8, IL-10, TNFα, PCT and neopterin in clinically relevant analytical ranges employing sandwich and binding inhibition assay formats (see Table 2.) The chip works with µL sample volumes and uses fluorescence detection. The measurement scheme combines sandwich and binding inhibition format and therefore is also apt for detecting small and large molecules in multiparametric immunoassays. The system provides the advantage of reduction of sample volume and assay time, as well as an increase in the number of analytes that can be measured simultaneously. Moreover it has the potential for extension, in the sense that new analytes can be added without difficulty.

2 Materials & Methods

2.1. Reagents

Proprietary ARChip Epoxy (EP 02799374; US 10/490543) was used as chip platform. CRP, anti-CRP (clone 5), and Dy647-labelled anti-CRP (clone 5 and 7), anti-human TNFα (B-F7) and recombinant human TNFα were purchased from Exbio (Czech Republic). Human procalcitonin (#HOR-295) was ordered from ProSpec-Tany TechnoGene Ltd (Israel). Anti-procalcitonin (16B6), biotinylated anti-procalcitonin (MAb42), biotinylated anti-human TNFα (F6C5) and CRP-free serum for spiking experiments were purchased from HyTest (Finland). Neopterin conjugates with bovine serum albumin and thyroglobulin, and antibodies mAb 3E2 were kindly provided by Milan Franek, Veterinary Research Institute (Czech Republic) [8] and labelled with Dy633 by Exbio (Czech Republic). Anti-IL-8 antibody (H8A5), recombinant human IL-8 and biotinylated anti-IL-8 antibody (E8N1) were from Biolegend (USA). Recombinant human IL-6, IL-10, biotin-conjugated anti-human IL-6 (MQ2-39C3) and anti-IL-10 (JES3-12G8) proteins as well as anti-human IL-6 (MQ2-13A5) and anti-human IL-10 (JES3-9D7) antibodies were from eBioscience (USA). Bis[sulfosuccinimidyl]suberate (BS3) was from Thermo Scientific (USA), and Dy647-Streptavidin from Dyomics (Germany). Phosphate buffered saline (PBS) (10x) (pH 7.2) was from Gibco/Invitrogen (Austria); Sodium

deoxycholate (minimum 97%), CHAPS (>98.0% TLC) and polyoxyethylene-sorbitan monolaurate (Tween-20) (Molecular biology tested) were purchased from Sigma (Austria).

2.2. Microarray fabrication and processing

2.2.1. Microarray printing

Probes were arrayed onto ARChip Epoxy using the OmniGrid contact spotter from GeneMachines (pin SMP3; 0.6 nL/spot). 1x PBS (pH 7.2)/0.01% sodium-deoxycholate and 1x PBS (pH 7.2)/0.005% CHAPS/0.01% BSA were used as print buffers. The spot-to-spot distance was 370 µm. All probes were spotted in triplicate, and 12 identical arrays were printed per chip.

2.2.2. Postarraying & blocking

After arraying, the slides were stored at 4 °C until use, at least for 3 days and up to 6 months. For blocking slides were incubated in 1x PBS (pH 7.2)/0.1% Tween-20 for 30 min, washed two times with 1x PBS and dried in a centrifuge (900 rpm, 1 min).

2.2.3. Immunoassay

For calibration, matrix (CRP-free Hytest serum, diluted 1:10 with assay buffer [0.1 M Tris (pH7.4), 10 mM $CaCl_2$, 100 mM NaCl, 0.1% Tween-20]) was spiked with serial dilutions of antigen standards. The assay comprises a preincubation of samples and detection antibodies for CRP and neopterin (RT, 10 min), an on-chip incubation of samples and detection antibodies (RT, 120 min), an incubation with biotinylated antibodies for the cytokines and PCT (1 mg/ L each) (RT, 45 min), and with 2 mg/ L Dy647-streptavidin (RT, 45 min). Variations of the standard protocol are described in the text. All incubation steps are performed in FAST frame™ multiwell chambers (Whatman Ltd, GB) on a shaker (Stovall, USA). The FAST frame™ is a multi-slide plate holding four slides which are assembled with disposable incubation chambers providing 16 wells per slide. The total volume used for each incubation chamber is 50 µl. Assuming 4 replicate wells as described in 3.1. and a 1:10 dilution of samples, the volume of patient sample needed was 20 µL. Each incubation step was followed by washing the slides 3 times with PBS (pH 7.2)/0.1% Tween-20. Finally chips were washed 2 times in PBS and dried in a centrifuge (2 minutes, 900 rpm).

2.2.4. Fluorescence detection and data analysis

Fluorescence measurements were taken using a Genepix™ 4000B non-confocal scanner by Axon Instruments. Optimal PMT values were chosen for each probe, avoiding more than 10% saturated pixel per spot. All fluorescence (a.u.) data is background-corrected. Non valid spots were either those given a flag “not found” by the image analysis software (Genepix 6.0), or were excluded by testing for outliers as described in [9]. The initial number of replicates for each reading point was 12, the average number of valid spots used for calibration and quantification after the outlier test was 9. Calibration curves were fitted using the four-parameter logistic model contained in the stats package of the statistic Program R. The limit of detection (LOD) was calculated as the concentration achieving a signal equal to the mean signal of the zero sample + 3 standard deviations (SD). Moreover, the inflection point of the curve (IC50), the slope of the tangent at IC50 (k), the amplitude (between upper and lower asymptote) and the coefficient of determination R2 were used as key parameters. Upper limit of quantification (ULOQ) was the highest standard concentration that could be discriminated from the next lower one (confidence interval, $\alpha = 0.05$). The coefficient of variation (CV% = SD / mean x 100) was a measure for data reproducibility.

3 Results and discussion

3.1. Design and workflow of the sepsis chip

Biomarker chips were printed using a contact printer with 4 pins in 3 subsequent runs, each pin spotting identical arrays (12 in total), an assembly of a number of capture elements, blanks, and guide dots. Mounted in a FAST frame™, each of these arrays can be assayed separately with a sample, either a standard of the calibration curve or a patient sample, in the course of an experiment. The variation of replicate spots caused by production parameters was tested by comparing signals of an assay with a single target concentration for all 48 arrays of a 4 slide-set by means of analysis of variance (ANOVA; data not shown). The main source of variation are the print pins, they may be blocked or fail to deliver a reproducible amount of probe. Three runs of spotting are performed to create 12 arrays on each slide which is a source of variation as well, while little variation is introduced by the slides. We concluded that measures have to be taken to improve reproducibility of microarrays in order to meet the demands of sensitive analytical methods for clinical applications. First, we set the number of replicate spots per measurement to twelve and arranged those replicates taking into account the results from the ANOVA, namely the variation between pins, slides and the spatial variation within a slide. Each sample was assayed in 4 replicate wells, produced by 4 pins, arranged on 4 slides instead of using 1 slide for a standard curve and one patient

sample. Figure 1 depicts an example of the sample distribution representing an 8 point standard curve and 4 patient samples on a set of 4 slides. Secondly, signals of 12 replicates underwent outlier testing before entering downstream analysis. As a result, the average CV of a standard curve was emended to 5 - 20 %. Variability caused by printing may be further minimized in a fully automated routine production process.

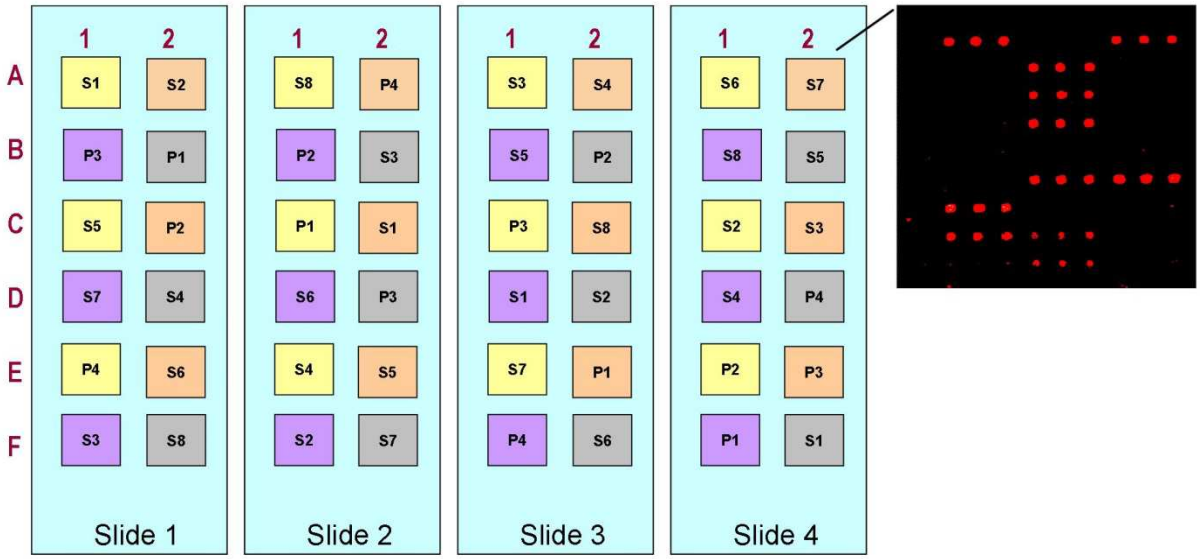

Fig. 1 Experimental set-up. Distribution of calibration samples (S1-S8) and spiked samples ("patient samples" P1-P4) in a 4-slides-set. The insert shows a typical fluorescence scan of one array.

3.2. Assay development for single biomarkers in a multiplexed format

3.2.1. Assay formats

On-chip sandwich immunoassays were developed for IL-6, IL-8, IL-10, TNFα, and PCT, while binding inhibition assays were implemented for the detection of CRP and neopterin. As shown in Figure 2a the sandwich immunoassay comprises a maximum of three incubation steps (addition of sample, biotinylated detection antibody, streptavidin-dye conjugate), whereas the competitive immunoassay essentially compasses one step (incubation of chip with mixture of sample and tracer antibodies). Sandwich assays were given priority due to their enhanced sensitivity compared to binding inhibition assays. Neopterin, a pteridin of low molecular mass (253 g/mol), is too small for providing two epitopes, therefore a binding inhibition format was used rather than a sandwich immunoassay. In case of CRP the binding inhibition format was primarily selected to reduce assay sensitivity. This will be explained in detail in the following section.

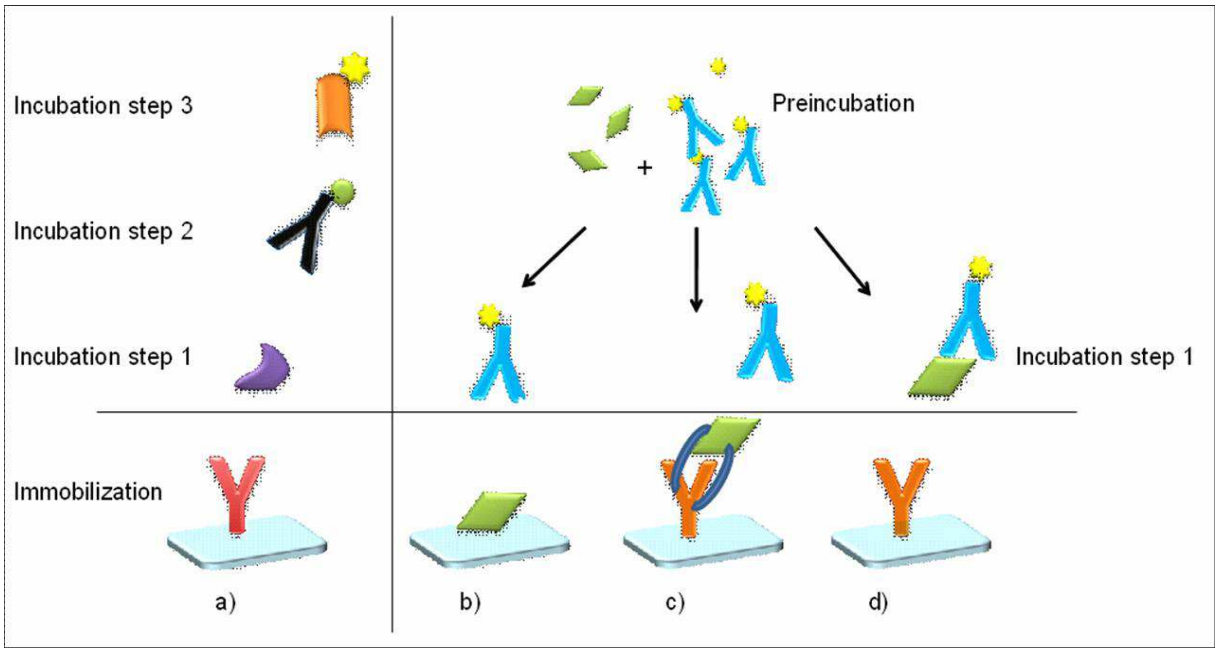

Fig. 2 Assay types evaluated for on-chip combination: a) Sandwich immunoassay b)-d) binding inhibition assays which immobilize as probe b) the analyte molecule or an analyte-conjugate, c) an analyte-specific antibody to which the analyte is cross-linked for oriented immobilization or d) an analyte specific antibody.

3.2.2. Binding inhibition assay for CRP

We recently reported on a sandwich immunoassay for CRP [10] which features a working range of 0.45 µg/ L – 0.16 mg/ L CRP (Figure 2a). At first sight very attractive the high sensitivity shapes up as drawback in combination with other sepsis-relevant biomarkers: as elevated CRP levels in serum are in the mg/ L range. Diluting the sample in order to meet the range of the CRP sandwich assay will decrease the cytokine concentrations under the system's detection limit. In order to shift the working range of the CRP assay to higher concentrations without diluting, the binding inhibition format was chosen, as it shows less sensitivity. In fact, as reported in [11] a direct comparison of both formats resulted in working ranges of 0.044 - 2.9 mg/ L for the sandwich assay and 0.13 - 22.9 mg/ L for the binding inhibition assay.

Two different approaches were evaluated: direct immobilization of CRP (Figure 2b) or CRP immobilized via a specific antibody. The latter one was either realized by crosslinking the CRP to a chip-bound anti-CRP (Figure 2c) or by the use of a specific antibody as probe, which is saturated with CRP during the assay (Figure 2d). First, CRP antigen directly immobilised on ARChip Epoxy was tested using Dy633 labelled anti-CRP as tracer antibody at a final concentration of 1 mg/ L. The working range achieved was between 0.2 and 2 mg/L CRP which did not cover pathologically elevated protein levels. In addition, reproducibility of measurement was poor: the average CV was >50%.

Signals were extremely low and only about 10% of the maximum signal strength obtained in the CRP sandwich immunoassay. This is probably due to partial denaturation of CRP during covalent immobilization to the epoxy surface. For oriented CRP immobilization 0.5 g/ L anti CRP (clone 5) was spotted and after blocking the chip was preincubated with 3 mg/ L CRP in order to saturate the immobilized antibody. To achieve stable CRP binding, the crosslinker BS3 was added to the incubation mixture. A binding inhibition immunoassay performed with this probe assembly leads to signal strengths increased by factor 10 and a working range extending from 0.2 mg/L to at least 40 mg/ L CRP (figure 3b, red curve). However the average CV amounted to about 40%, which is not acceptable for accurate quantification. Yet it was found that a calibration curve with a working range between 3 and 100 mg/ L CRP and a mean CV of only 20% could be obtained when omitting the CRP/crosslinker preincubation step (figure 3b, black curve). Beyond a concentration of 3 mg/ L CRP in the assay solution, antibody clone C5 at the chip surface gets saturated with CRP as can be seen in figure 3. Below an analyte concentration of 3 mg/ L CRP the curve features a low concentration hook, above it shows the binding inhibition behavior. Thus when using this assay in further experiments we always simultaneously performed the binding inhibition assay using the immobilized antibody and the directly immobilized CRP probe (figure 3a, green curve) in order to identify and estimate CRP concentrations of samples with analyte concentrations in the range of the hook. From a diagnostic point of view the obtained working range of the two variations of binding inhibition assay is ideal for CRP testing: For instance cut-off levels of 30 mg/ L and 60 mg/ L CRP have proven useful in differentiating unlikely infection, likely and proven infection and proven sepsis in neonatals [12].

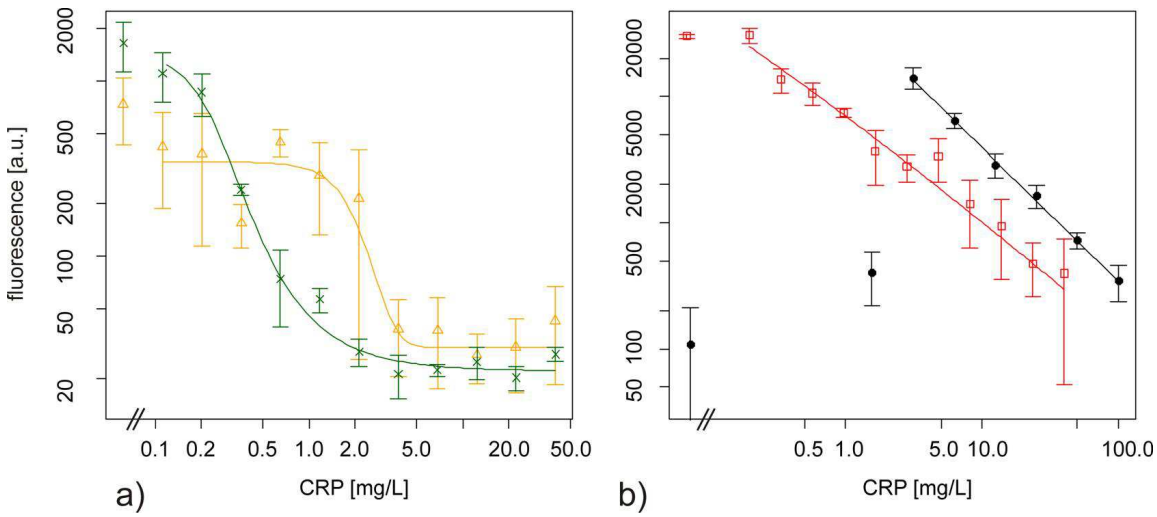

Fig. 3 Binding inhibition immunoassay for CRP. a) The CRP probe was covalently immobilized and the assay was conducted with CRP antibody clone C5 (Δ) or clone C7 (x) as tracer antibody. b) The probe either consisted of anti CRP antibody C5 alone (●) or the capture antibody was loaded with CRP in the presence of a crosslinker (□) prior to performing the assay. Anti CRP clone C7 was used as the tracer antibody.

3.2.3. Neopterin

Neopterin was conjugated either with bovine serum albumin (BSA) or thyroglobulin (TG) and immobilized on the chip. Those probes are competing with the target molecules in the sample for the labelled antibodies Dy633-mAb 3E2. Concentrations of neopterin-conjugates spotted onto ARChip Epoxy were 0.125 g/ L, 0.25 g/ L, 0.5 g/ L and 1 g/ L in 1x PBS (pH7.2) / 0.01% sodium-deoxycholate. With increasing probe concentration the mean coefficient of variation diminished to 10%, while the signal amplitude (i.e. the signal range between lower and upper asymptote of the 4 parameter model) was extended and hence was the working range, for example 0.46 to 81.92 µg/ L using 1 g/ L probe (BSA neopterin) and 0.1 mg/ L tracer antibody. Both conjugates were suitable for immobilization: thyroglobulin-neopterin conjugates show reduced signal strength by about 50 % but somewhat improved sensitivity, demonstrated by a left shift of the infliction point of the standard curve (0.87 µg/ L for the TG-conjugate compared to 1.32 µg/ L for the BSA conjugate).

In binding inhibition assays the range can be tuned by the concentration of the tracer antibody. To do so, 0.05, 0.1, 0.5 and 1 mg/ L Dy647-anti neopterin was used. As presented in Table 1 the amplitude of signals dramatically increases for higher tracer concentrations, but not so the working range. For the working range required to

distinguish normal (> 2 µg/ L) from elevated neopterin levels a concentration of 0.1 mg/ L was found optimal, higher concentrations resulted in lower sensitivity.

Table 1. Key parameters for a binding inhibition assay for neopterin using 1 g/ L immobilized neopterin-BSA conjugate. Dy647-anti neopterin concentrations are varied from 0.05 mg/ L to 1 mg/ L.

	Dy647-anti neopterin			
	0.05 mg/ L	**0.1 mg/ L**	**0.5 mg/ L**	**1 mg/ L**
IC50 (µg/ L)	1.35	1.15	2.88	3.98
k	8	8	7	5
LOD (µg/ L)	n.n.	0.46	3.14	0.88
ULOQ (µg/ L)	81.92	81.92	81.92	81.92
amplitude (a.u.)	6935	16481	28483	40419
mean CV (%)	14	12	10	11
R^2	0.91	0.94	0.84	0.92

3.2.4. IL-6, IL-8, IL-10, TNFα, and PCT

Assays for cytokines, TNF and PCT were set up according to the protocol developed for IL-6 and IL-10 [10] and integrated in the combined assay scheme following protocol I) described in 3.3., i.e. joint incubation of samples and competitive tracer antibodies, and subsequently incubation with sandwich tracer antibodies. Typical calibration curves are shown in Figure 4. Mean LODs and ULOQs obtained in ten experiments performed independently by two persons are listed in Table 2 and compared with normal levels in healthy adults and elevated levels reported for adults and infants in the literature. The chip is apt for detecting elevated levels of the biomarkers, except for TNFα, for which the assay's sensitivity needs improvement. For IL-8, PCT, neopterin and CRP quantification is also possible in the normal range of serum concentrations. However, we have to consider that both, serum concentrations regarded normal and so called 'cut off' values for predicting sepsis are extremely variable in literature.

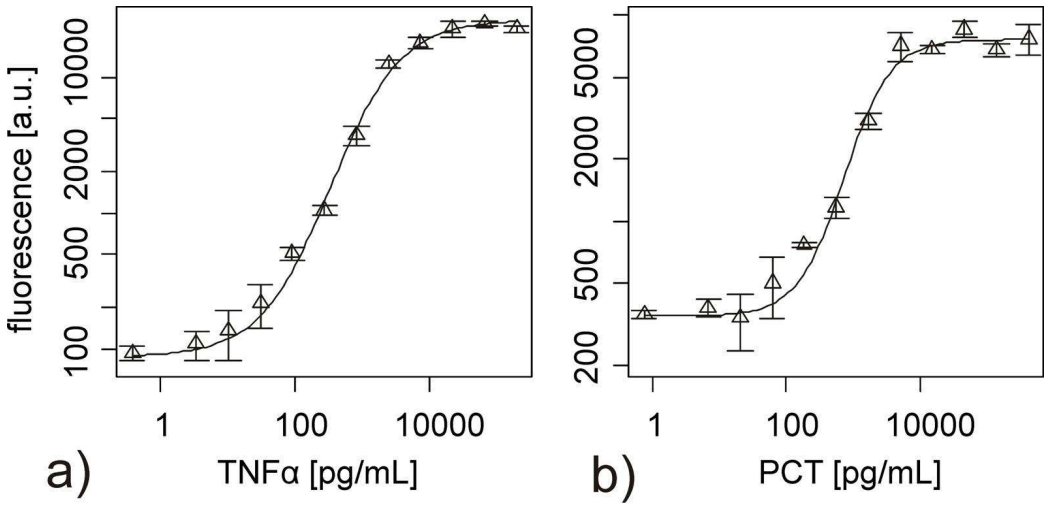

Fig. 4 Calibration curves of sandwich immunoassays for a) TNFα b) PCT.

Table 2. Sepsis biomarkers, their serum levels considered normal in healthy individuals and elevated values indicating sepsis in adults and infants [11], and the working range of the combined sepsis chip following protocol I.

			sepsis chip	
Biomarker	**normal levels**	**elevated values**	**LOD**	**ULOQ**
IL-6 (ng/ L)	<10	68.5 [5]; 31 [11]	15	40 960
IL-8 (ng/ L)	< 125	31.5 [5];	26	40 960
IL-10 (ng/ L)	< 3	87.3 [12]	65	24 000
TNFα (ng/ L)	< 3.5	11.5 [5]; 17 [11]	40	6000
PCT (µg/ L)	0.1 [*] 0.5 [5]	2-10 [*]; 2.4 [5]	0.078	4.5
neopterin (µg/ L)	1.34 [13], 1.77 [14]	3.04 [15]	0.46	81.92
CRP (mg/ L)	1-10	14.5 [4]; 12 [11]	3	100

* www.procalcitonin.com

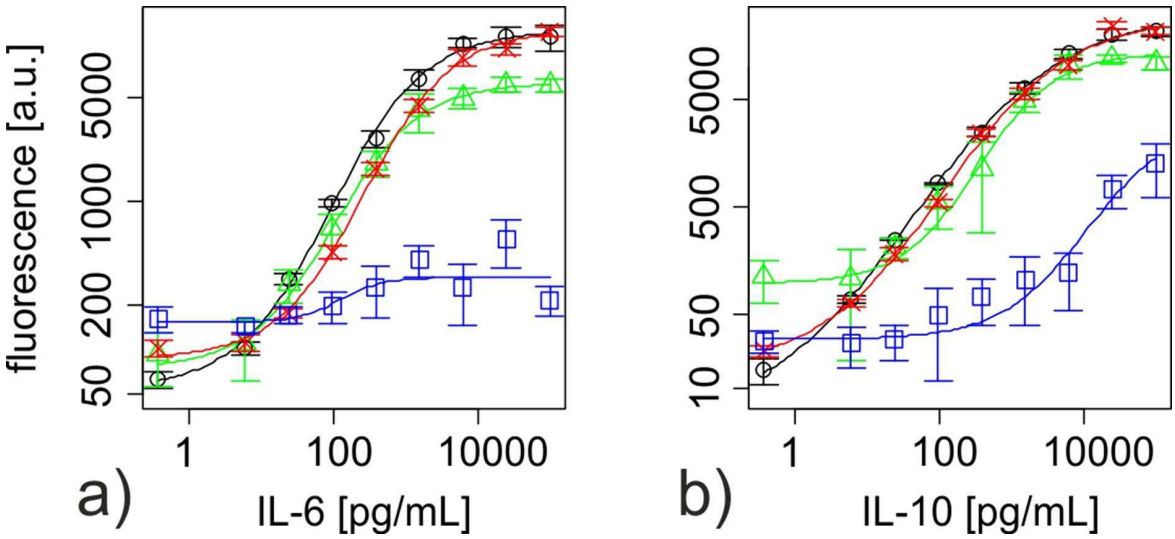

Fig. 5 a) IL-6 and b) IL-10 calibration curves for different combinations of sandwich and competitive immunoassay steps. The protocol can be realized as a I) three-step (■), a two-step II) (○), III) (▲) or as a one-step IV) process (▽). For details refer to the roman numerals in the text.

3.3. Speeding up: assay time versus sensitivity

In order to simultaneously measure multiple biomarkers in parallel, we combine biomarker assays described in 3.2.2, 3.2.3 and 3.2.4. on a single chip using various formats:

I) Firstly incubation of the chip with a mixture of sample and competitive tracer antibodies, secondly incubation with biotinylated sandwich detection antibodies and thirdly application of the streptavidin-dye conjugate (three-step protocol). The protocol reduces to two steps if fluorescently labelled detection antibodies are used.

II) The process can be reduced to a two-step protocol by combining step one and two, i.e. mixing the sample with the competitive tracer antibodies as well as with the sandwich detection antibodies for the first incubation step. As the second step the streptavidin-dye conjugate is applied.

III) Firstly incubation with a mixture of sample and tracer antibodies, secondly incubation with a mixture of biotinylated sandwich detection antibodies and dye-streptavidin.

IV) All-in-one: Incubation with a mixture of sample tracer antibodies, sandwich detection antibodies and dye-streptavidin conjugate.

In the following calibration curves obtained for IL-6, a monomer being the most valuable cytokine marker in sepsis, and IL-10 a homodimer are presented in detail using these formats (Figure 5).

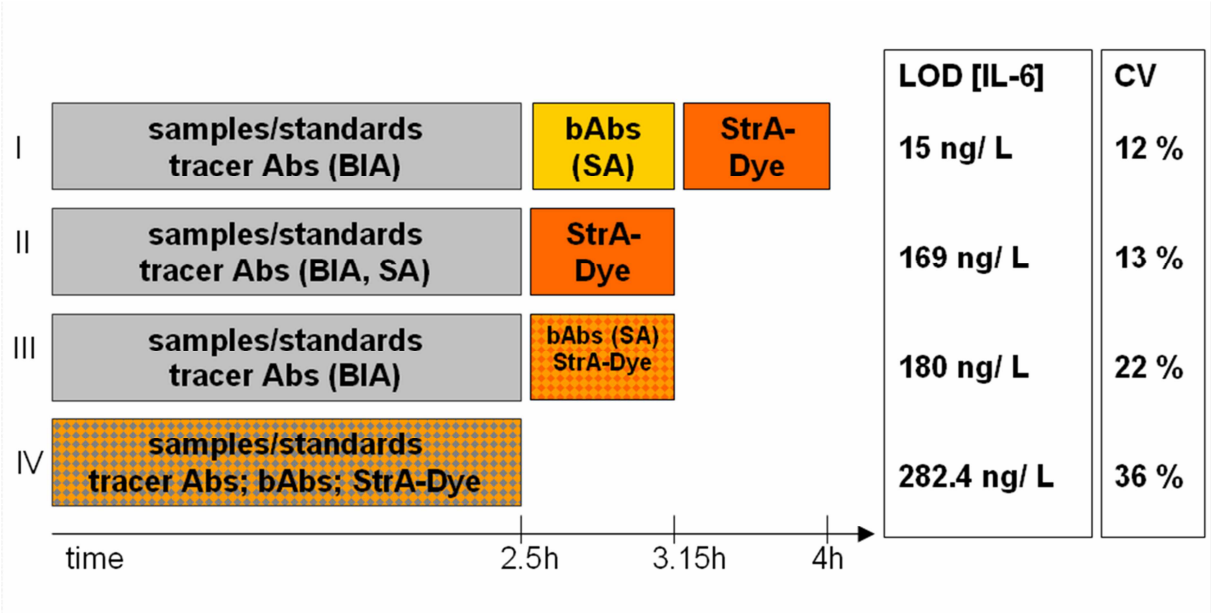

Fig. 6 Assay protocols for a combined format of sandwich (SA) and binding inhibition immunoassays (BIA). Assay times, LOD and CV given exemplarily for IL-6.

Protocol I) performs all three assay steps separately and standard curves are perfectly comparable to individually assayed sandwich tests.

When both sandwich and binding inhibition antibodies are added to the sample in the first incubation step (treatment II), the limit of detection (LOD) for the IL-6 assay increases by a factor of about 10 (mean LOD = 169 ng/ L, [27 - 317 ng/ L, n=6]) in comparison to treatment I (mean LOD = 15 ng/ L, between 2 and 32 ng/ L). This effect may be caused by the binding of the detection antibody to IL-6 which slightly changes the analyte's conformation and thus decreases its affinity for the immobilized capture antibody. For IL-10 no decrease in assay sensitivity was detected.

When adding the biotinylated sandwich detection antibodies and streptavidin-dye in a second incubation step (treatment III) the LOD for IL-6 and IL-10 increases by factor 10 and factor 40 respectively in comparison to treatment I. Also the mean coefficient of variation rises to values between 20% and 40%. Decreased sensitivity and higher variability was also observed for the other on-chip sandwich assays. This behaviour can be explained by the fact that streptavidin can bind up to 4 biotin molecules. Therefore the dye-streptavidin conjugate can capture up to 4 biotinylated antibodies (which are not necessarily of the same specificity) before the mixture is added to the chip. These bulky detection complexes lead to decreased assay performance. Sticking to the assumption that the mixture of dye-streptavidin with the biotinylated antibodies leads to the formation of multi-antibody conjugates the outcome of treatment IV, which uses a mixture of all

reagents in one step, can be explained: The respective analytes are crosslinked by the detection conjugates and a sensitive and reproducible detection is hampered.

Thus depending on the required sensitivity for IL-6 either protocol I or II can be chosen. If a less sensitive detection for IL-6 still meets the requirements of the experiment (e.g. in toxicity tests), protocol II offers the advantage of lower total processing time. In Figure 6 we compare directly the 4 protocols in terms of assay time and sensitivity for IL-6.

3.4. Conclusions and Outlook

The combination of two assay formats in one single protein biochip is demonstrated with a panel of seven biomarkers for sepsis (C-reactive protein, interleukin-6, interleukin-8, interleukin-10, TNFα, PCT and neopterin), for two reasons: First, it is of high diagnostic value for clinical applications and secondly it is most suitable for demonstrating the unique advantages of a combined assay format. The chip allows the quantification of high and low abundant proteins in parallel due to the different sensitivity levels exhibited by sandwich and binding inhibition format. In addition, the chip can detect proteins of different quaternary structure and size such as a monomer (IL-6) and a pentamer (CRP) but also neopterin, a pteridin of low molecular mass.

In contrast to microfluidic [18] and multichannel arrays [19] assay times reported here refer to a more or less static system. Integration of the sepsis chip into a point-of care system with a microfluidic set-up can reduce assay times significantly due to the reduced diffusion distance of target analytes to the immobilized probes, as has been shown in [18].

Longer assay times are also a result of the multiplicity of analytes determined at the same time under harmonized conditions. For example, in order to detect CRP (µg/L) and cytokines (pg/L) in parallel - two biomarkers of extremely different detection range - compromises in the measurement set-up and experimental conditions need to be made. Assay formats, printing solution and especially assay buffer were therefore optimized relating to the whole group of selected biomarkers, not just the individual markers.

In the present set-up, the chip uses 20 µL patient sample. The sample is diluted with 180 µL assay buffer, the resulting 200 µL sample solution is divided into four solutions of 50 µL each for 4 replicate incubations. By reducing the volume per incubation from 200 µl to 40 µl (i.e. four times 10 µL per well), the protein biomarker chip is especially attractive for diagnosis of sepsis in neonates and infants, where a reliable and fast test system with small sample volumes is an urgent need. First tests with smaller incubation chambers showed that a reduction of total volumes is possible, but is accompanied by loss of signal

which may be attributed to bad wetting conditions in the smaller chambers and possibly overcome by a more effective mixing during incubations.

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5 Critical role of the sample matrix in a point-of-care protein chip for sepsis

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Research paper

Critical role of the sample matrix in a point-of-care protein chip for sepsis

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ABSTRACT

Both highly specific antibodies and appropriate assay buffers are key elements in the development of sensitive multi-analyte diagnostic tests and essential assay components to minimize interferences from the sample matrix.

Herein, we investigate the influence of 0.1 M Tris (pH 7.4)/0.1 M NaCl/10 mM $CaCl_2$/0.1% Tween-20 used as assay buffer and diluent for serum, plasma and saliva samples in a protein biomarker chip for the diagnosis of sepsis. In detail, on-chip sandwich assays for detection of IL-6 and PCT are established using pure assay buffer and serum, plasma, and saliva, each diluted by a factor of 10 and 100 with assay buffer. The dilution linearity as well as the cross-reactivity to immobilized IL-8, IL-10 and TNF-α antibodies (< 1.8% in plasma and serum) is investigated; furthermore the influence of immunoglobulin G, fibrinogen and lysozyme, highly abundant proteins in serum, plasma and saliva. This effect is two times more pronounced in serum than in plasma and saliva and strongly decreases with increasing analyte concentration. Though the matrix proteins bind unspecifically to the immobilized receptors, they do not prevent the analyte binding; on the contrary, the analyte is reliably detected with high sensitivity, featuring limits of detection of 16 ng/L and 0.31 μg/L, and coefficients of variation of 18% and 29% for IL-6 and PCT in 10% serum.

1. Introduction

Molecular recognition of disease-specific biomarkers in complex biological fluids is a fundamental challenge in point-of-care (POC) testing and of great interest in medical practice. Biomarker diagnosis is based on analyte detection in serum, plasma, saliva and other biological fluids such as urine, tears and cerebral fluids. The attraction of human physiological fluids for POC testing is given by the fact that they comprehensively sample the state of the body at a particular point in time. There is a strong demand for POC testing in primary care facilities and outpatient clinics, in hospitals, workplaces and homes. Protein microarrays are a remarkable solution for POC applications as they fulfill important demands for a POC analyzer, such as reduced sample and reagent consumption and a high level of specificity and sensitivity.

The proteome of human plasma and serum holds the promise of disease diagnosis and therapeutic monitoring. Apart from the "classical proteins", plasma contains all tissue proteins (as leakage markers) and numerous distinct immunoglobulin sequences. Plasma is the most difficult protein containing sample to characterize on account of the large proportion of albumin (55%), the wide range in abundance of other proteins, and the tremendous heterogeneity of its predominant glycoproteins (Anderson and Anderson, 2001). Serum is very similar to plasma: pro-thrombin is cleaved to thrombin, fibrinogen is removed (to form the clot), and a limited series of other protein changes takes place. A third prominent class of biological fluids for disease diagnosis is human saliva, which is composed of the fluids secreted by the major and minor salivary glands, gingival fluid, and resident oral flora. Human saliva is finding increasing interest for proteomic and biomarker-discovery studies, due to the potential to be of

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Abstract

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Keywords: protein array, IL-6, PCT, matrix effect, serum, saliva, plasma

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The proteome of human plasma and serum holds the promise of disease diagnosis and therapeutic monitoring. Apart from the “classical proteins”, plasma contains all tissue proteins (as leakage markers) and numerous distinct immunoglobulin sequences. Plasma is the most difficult protein containing sample to characterize on account of the large proportion of albumin (55%), the wide range in abundance of other proteins, and the tremendous heterogeneity of its predominant glycoproteins (Anderson and Anderson, 2002). Serum is very similar to plasma: pro-thrombin is cleaved to thrombin, fibrinogen is removed (to form the clot), and a limited series of other protein changes take place. A third prominent class of biological fluids for disease diagnosis is human saliva, which is composed of the fluids secreted by the major and minor salivary glands, gingival fluid, and resident oral flora. Human saliva is finding increasing interest for proteomic and biomarker-discovery studies, due to the potential to be of significant diagnostic value in screening and monitoring disease state (Schramm and Smith, 1991; Hofman, 2001), and to have advantages over other biological fluids, such as sample collection in a non-invasive manner, and no risks associated
with blood sampling (Hofman, 2001). As saliva is reported to be a reservoir of immunoglobulins, clinical tests have been developed for HIV and Hepatitis B infection, as well as test for antibodies against specific viral proteins (Hodinka et al., 1998; Fisker et al., 2002; Moe et al., 2004). Moreover, assays for detection of non-infectious diseases (e.g. C reactive protein for inflammatory diseases) and specific cancers (e.g. IL-8 as an oral cancer marker) have been explored in saliva (Van Nieuw Amerongen et al., 2004; Christodoulides et
al., 2005; Yang et al., 2005). However, it is known that salivary protein composition may vary
with physiological and environmental factors. Moreover, currently the main challenge of using saliva as diagnostic fluid is its inherently low concentration of biomarkers.
Biomarkers reported for inflammation and sepsis are pro-inflammatory cytokines, such as IL-
6, IL-8, IL-1beta and TNF-α and acute phase proteins such as to C-reactive protein (CRP), and procalcitonin (PCT). Cytokine levels were assessed in numerous studies in order to evaluate their diagnostic importance. IL-6, with serum levels below 100 ng/L, is referred to as
a cytokine with important prognostic value in sepsis (Livaditi et al., 2006). Among a wide array of cytokines assessed so far, it was found that IL-6 stimulates a wide spectrum of acute phase proteins such as CRP and is crucial for the initiation of the innate immune response. Peak IL-6 serum levels are reached within 2 hours, and are often elevated before the onset of clinical symptoms and before routine laboratory tests, such as

measurement of highsensitivity CRP (hsCRP), turn positive (Volante et al., 2004). Procalcitonin is a peptide hormone of high sensitivity and specificity for bacterial infection and sepsis. Values up to 0.1 µg/L are considered as normal, whereas values between 2 and 10 µg/L indicate a severe sepsis (www.procalcitonin.com). Single biomarkers are limited in their usefulness due to the heterogeneity of disease occurrence and patient populations. A combination of early markers (IL-6) and acute phase proteins (PCT) can enhance the reliability of disease diagnosis and facilitate clinical decision-making concerning the appropriate therapy.

The presented protein microarray offers such a high quality testing for these multiple biomarkers in undiluted/ diluted body fluids (serum, saliva and plasma) using monoclonal capture antibodies in a sandwich assay format. The relevant analytical range of the biomarkers to be detected is quite different: while PCT concentrations are in the µg/L range, cytokines develop in the ng/L range. It is a great challenge detecting such analytes present among high abundant serum-, plasma-, saliva- proteins present in the g/L range. Consequently high abundant matrix components such as fibrinogen in plasma, human IgG in serum and enzymes in saliva are often removed from biological fluids to avoid cross-reactivity concerns and to specifically detect low abundant analytes. We tested the applicability of the developed protein microarray for fast and reliable POC analyte testing in human biological fluids without implementing a prior purification step. For cross-reactivity testing, high abundant proteins non-specifically bound to the chip surface were detected with Dy-547-labelled antifibrinogen, anti-IgG and anti-lysozyme.

2. Experimental

2.1. Materials and reagents

ARChips Epoxy are proprietary slides developed at AIT (EP 02799374; US 10/490543). Phosphate buffered saline (PBS) and Tris methylaminomethane (Tris) were purchased from Invitrogen (Austria). Sodium deoxycholate (minimum 97%) and polyoxyethylene-sorbitan monolaurate (Tween-20; Molecular Biology tested) were from Sigma (Austria). Dy-647- Streptavidin was from Dyomics (Germany). Recombinant human IL-6, biotin-conjugated anti human IL-6 (MQ2-39C3) as well as anti human IL-6 (MQ2-13A5) and anti IL-10 (JES3- 12G8) were obtained from eBioscience (USA). Anti IL-8 (Mab H8A5) was from Biolegend (USA). PCT was from ProSpec Tany Technogene Ltd. (Israel). Monoclonal capture anti-PCT (Mab 16B5) and monoclonal biotinylated anti-PCT antibodies (Mab 42) and anti TNF-α (B- 7F) were purchased from HyTest (Finland). Anti-lysozyme and anti-fibrinogen were from Biozol (Germany) and labelled with Dy-547 (Dyomics, Germany). Pooled human serum and pooled human plasma (anticoagulant Na

heparin) was purchased from Innovative Research (USA). Human saliva was collected from healthy volunteers and cleared by centrifugation at 4500 rpm for 30 min at 4 °C. The saliva supernatant, pooled human plasma and serum were used for spike-in experiments. The binding buffer for matrix dilution was 0.1 M Tris (pH 7.4)/
0.1 M NaCl/ 10 mM CaCl2/ 0.1% Tween-20.

2.2. Chip fabrication

0.4 g/L anti-IL-6, anti IL-8, anti IL-10 and anti TNF-α and 0.5 g/L anti-PCT in print buffer 1x PBS (pH 7.2)/ 0.01% sodium-deoxycholate were arrayed on ARChip Epoxy at a relative humidity of 50% using the contact spotter OmniGrid from GeneMachines (4 pins; pin SMP3, spotting volume: 0.6 nL/ spot). The resulting amount of antibody per spot was 0.24 ng and 0.3 ng respectively. For cross-reactivity testing undiluted saliva, serum and plasma were spotted. The spot to spot distance was 400 µm and the array to array distance was 8950 µm. All probes were arrayed in triplicate and 12 identical arrays were spotted per slide. After spotting, the slides were kept at 4°C for a couple of days to ensure complete receptor immobilization.

2.3. On-chip immunoassay

Surface blocking was performed in 1x PBS (pH 7.2)/ 0.1 % Tween-20 for 30 minutes to remove any non bound receptors and deactivate reactive surface groups. Afterwards the slides were washed twice in 1x PBS (pH 7.2) and dry-centrifuged for 4 minutes (900 rpm). After blocking, the slides were mounted into the Fast Frame (Whatman) composed of silicone chambers which create 12 separate reaction wells on each slide. Further details of the chip layout are given in Sauer et al., 2011. In this way a high throughput processing of the chips was enabled by pipetting and withdrawing the target and washing solutions with a multipipette (50 µl/ well).

For a nine-point calibration curve (including the zero standard) three chips composed of 12 arrays each were used, resulting in 12 replicates (4 arrays x 3 replicates) per analyte concentration and probe. The calibration standards of the antigens IL-6 [standards S1: 0 ng/L, S2-S9: 2.5 – 40950 ng/L] and PCT [standards S1: 0 µg/L, S2-S9: 0.025 – 409 µg/L] were spiked in human serum, plasma, and saliva. These biological fluids were analyzed undiluted, and diluted by a factor of 10 and 100. The spotted antibodies to IL-8, IL-10 and TNF-α alpha were used as negative controls for cross reactivity testing.

The antibody chip was incubated at room temperature (RT) for 2 hours with the serial dilutions of antigen standards using the dilution factor 4. After incubation, the slides were washed three times with 1x PBS (pH 7.2)/ 0.1% Tween-20. For analyte detection, a two-step sandwich assay was performed using a mix of biotinylated antibodies [1 mg/L per

antibody] and after washing, Dy647-Streptavidin [4 mg/L]. For cross-reactivity testing either Dy547 labelled anti-lysozyme [1 mg/L], or Dy547 labelled anti-fibrinogen [1 mg/L], or Dy547 anti IgG [1 mg/L] was added in the last incubation step. Both incubation steps were performed at RT for 45 minutes each. Finally, the slides were demounted from the Fast Frame and washed once with 1x PBS/ 0.1% Tween-20, twice with 1 x PBS (pH 7.2) and dry-centrifuged for 4 minutes (900 rpm).

Slides were scanned immediately after performing the immunoassay using the GenepixTM 4000B non-confocal scanner from Axon Instruments at appropriate sensitivity levels of the photomultiplier tube (PMT) voltage ($\lambda_{ex/em}$=635/670 nm; $\lambda_{ex/em}$=532/550 nm). The scan images were analyzed with the GenePix 6.0 software.

2.4. Data analysis

Fluorescence signals were background corrected and filtered for non valid spots, i.e. those given a flag not found by the image analysis software or excluded by an outlier test. Mean values of remaining spots were used for down-stream data analysis.

Calibration curves were set up using a four-parameter logistic model (Origin Pro 8G). To evaluate the immunochip sensitivity, the limit of detection (LOD) was determined. The LOD is defined as the lowest concentration of an analyte that the chip can reliably detect and yields a response higher than that of the zero standard + 3 standard deviations (SD). Moreover for evaluation purposes the coefficient of determination R2 of the fit, the inflection point of the curve (IC50) and the slope of the tangent at IC50 (k) of the logistic fit were used as key parameters. The coefficient of variation (%CV = SD / mean * 100) was a measure for data reproducibility.

3. Results & Discussion

3.1. Standard curves in biological fluids & linearity of dilution

Standard curves for IL-6 and PCT were set up in the body fluids serum, plasma and saliva, which were applied undiluted and diluted with 0.1 M Tris (pH 7.4)/0.1 M NaCl/10 mM CaCl2/ 0.1% Tween-20 by a factor of 10 and 100. Tris buffer was chosen in a comparative testing of 10 assay buffers used for sample dilution. Criteria for selection were the assay sensitivity and reproducibility of result (Domnanich et al., 2009). Similar to the work done by Pfleger et al. (2008), who tested the commercial sample diluents DY997 and RD6, the quality and effect of the diluent was investigated using multiplexed calibration curves of cytokines. Figure 1 depicts typical calibration curves for IL-6 in neat and diluted matrices and Table 1 summarizes the LOD, k, R^2 of the fit and the average CV for IL-6 in saliva, serum and plasma. In Figure 2 and Table 2 standard curves for

PCT in 1:10 diluted serum, plasma and saliva and the key parameters for these curves are shown. As obvious from Figure 1 and Table 1 the sensitivity of the IL-6 assay represented by the slope of the standard curve (k) does not change much with the dilution factor of the biological fluid. Only the slopes of assays in buffer and in neat saliva are slightly increased. In fact, good linearity is achieved between the 1:10 and 1:100 dilution curves for IL-6 detection. This indicates a high flexibility of the assay allowing to reliably measure and to compare samples of significantly different IL-6 concentrations: samples with high levels of biomarkers can be diluted several fold to make sure that the analyte falls within the dynamic range of the calibration curve, while a sample of low IL-6 concentration can be measured with a lower dilution factor using the same calibration curve. Another practical benefit of linearity between standard curves of various dilutions is the elimination of high dose hook effects. Even the calibration curves obtained for IL-6 in diluted plasma, serum and saliva are similar (Fig. 1). This means that there is also good linearity between the three different bodily fluids. By contrast the calibration curves for PCT in 1:10 diluted serum, plasma, and saliva are clearly different concerning all important assay parameters (signal intensity, slope, LOD, measurement range).

Table 1. LOD, k, R^2 and CV (%) for IL-6 calibration curves in buffer and diluted and undiluted serum, plasma, and saliva. Values are means of 3 to seven experiments.

Matrix	Dilution	LOD [ng/L]	k	CV [%]	R^2
buffer	no	9	1.2	18	0.97
serum	no	350	1.0	24	0.99
serum	1:10	16	1.1	18	0.94
serum	1:100	14	1.1	15	0.99
plasma	no	183	1.0	33	0.99
plasma	1:10	80	1.1	31	0.96
plasma	1:100	39	1.1	25	0.87
saliva	no	85	1.5	43	0.97
saliva	1:10	25	1.0	21	0.94
saliva	1:100	7	0.9	18	0.97

In contrast to the quite stable assay parameter k (slope), the LOD, another measure for sensitivity, significantly drops by diluting the pure matrix. This can be explained by a lower blank signal (standard S1) due to less unspecific binding of matrix components compared with neat matrices. Additionally a higher off-set may be attributed to IL-6 and PCT present in the commercially available matrices used in our spiking experiments. Low sensitivity was achieved for IL-6 in neat serum and plasma, while PCT performed best in serum, featuring a 10 times reduced LOD compared to saliva. Diluting the plasma samples by a factor of 100 resulted in a five times improved assay sensitivity for IL-6. A low LOD in a diluted matrix however does not necessarily mean that one can work in clinically relevant ranges unless the LOD drops with at least the dilution factor. R2 values calculated for the individual curve fit were close to 1 – irrespective of the dilution factor - illustrating a good fit of the model. Upon diluting the biological fluids data reproducibility increased up to 2.5-fold, indicating that diluting the matrix minimized the effect of interfering substances. Certainly, plasma is the most complex matrix tested in this study, reflected by higher CVs in spiked matrices (Table 1 and 2). Generally, the data reproducibility was improved with increasing matrix dilution factor (see Table 1). Assays in 1:100 diluted serum and saliva show as high data reproducibility as assays in buffer, the average CV for assays in buffer was 18%, in 1:100 diluted serum, plasma and saliva it was 15, 25, and 18 %. Total protein content of saliva was reported to be 92.59 mg/100 mL compared to 200 - 400 mg/100 mL for plasma (Shetty and Pattabiraman, 2004). Considering background signals, undiluted matrices caused up to two times greater background fluorescence than diluted biological fluids. A high dilution factor is very likely to contribute to reducing interferences originating from matrix components shielding the analyte and hence hinder specific binding of detection antibodies. Moreover, adding binding buffer to the samples positively influences the assay, in terms of stability, accessibility of the analyte and optimal wetting of the chip surface. Besides of ionic strength, pH and salt concentration also the viscosity of solution plays an important role in the antibody-antigen kinetics, especially when operated under non-equilibrium conditions (Morgan et al., 1998).

Considering all the above mentioned arguments especially in multiplexing approaches we recommend an up-to 1:10 sample dilution, as a trade off between reducing matrix effects, improving reproducibility and having still detectable biomarker concentrations available.

Table 2. Key parameters k, CV, R^2 and IC 50 of calibration curves for PCT in 1:10 diluted matrices. Values are means of 3 experiments.

Matrix	LOD [µg/L]	k	CV [%]	R^2	IC 50 [µg/L]
serum	0.31	1.0	29	0.92	10
plasma	0.77	1.2	39	0.86	11
saliva	3.85	1.5	27	0.73	80

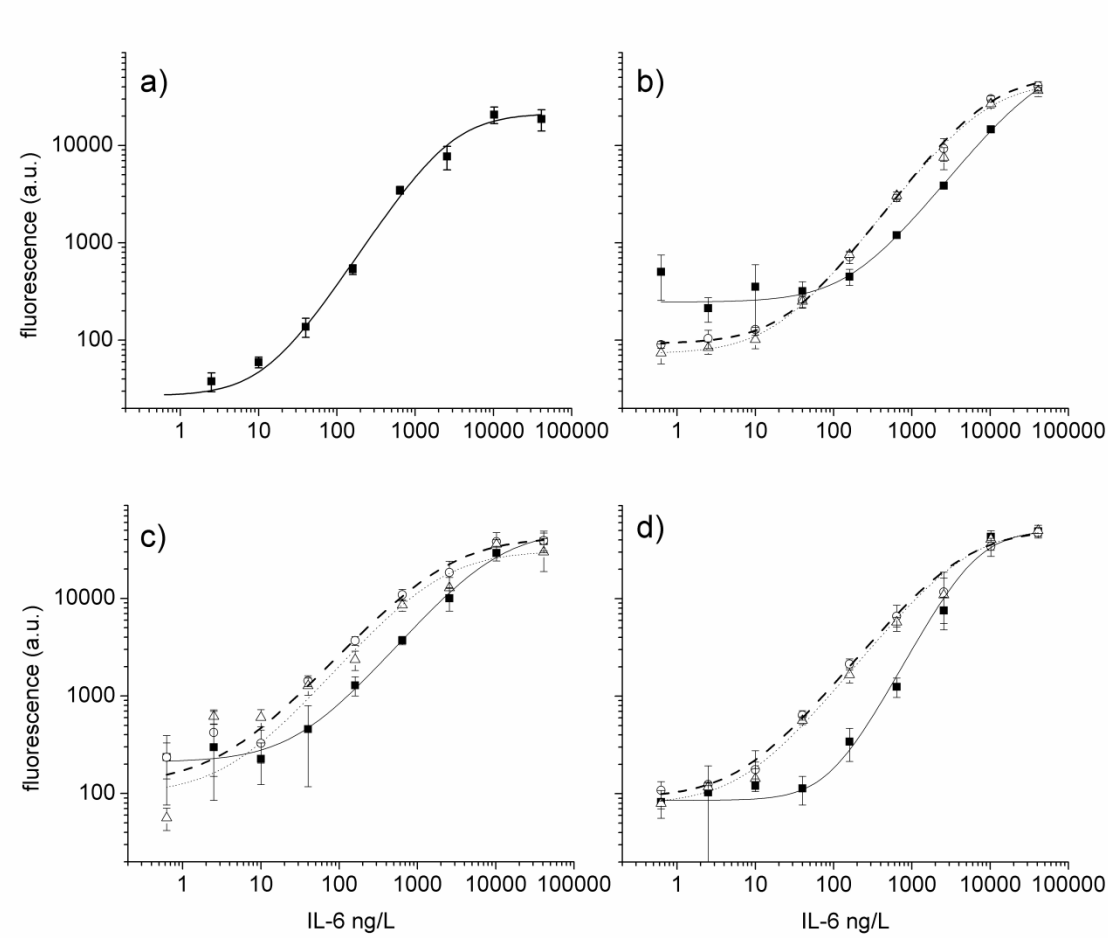


Figure 1. Calibration curves for IL-6 in **a)** buffer; and in undiluted (solid curve), 1:10 diluted (dashed curve) and 1:100 diluted (dotted curve) **b)** serum, **c)** plasma and **d)** saliva.

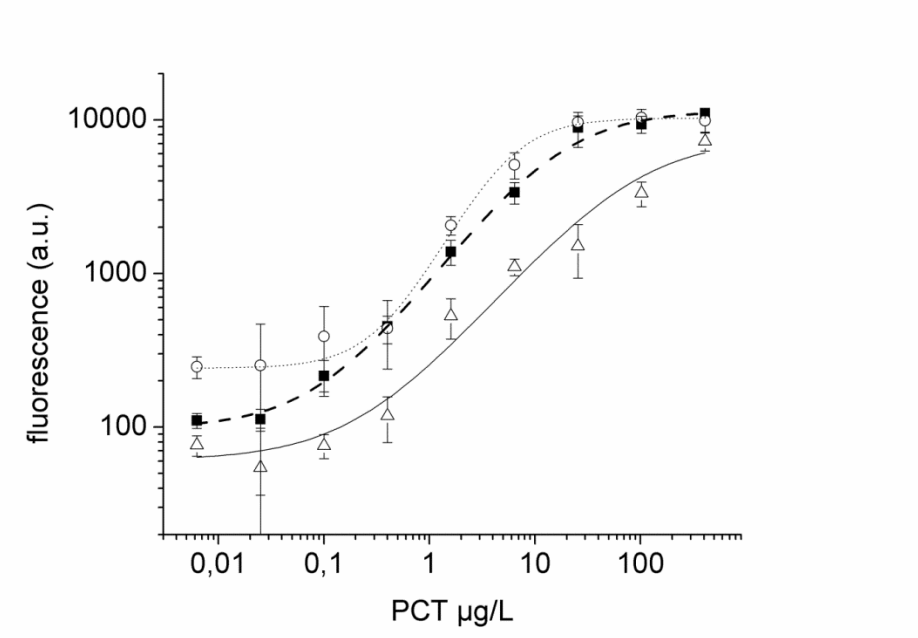


Figure 2. Calibration curves for PCT in 1:10 diluted serum (dashed line), plasma (dotted line) and saliva (solid line).

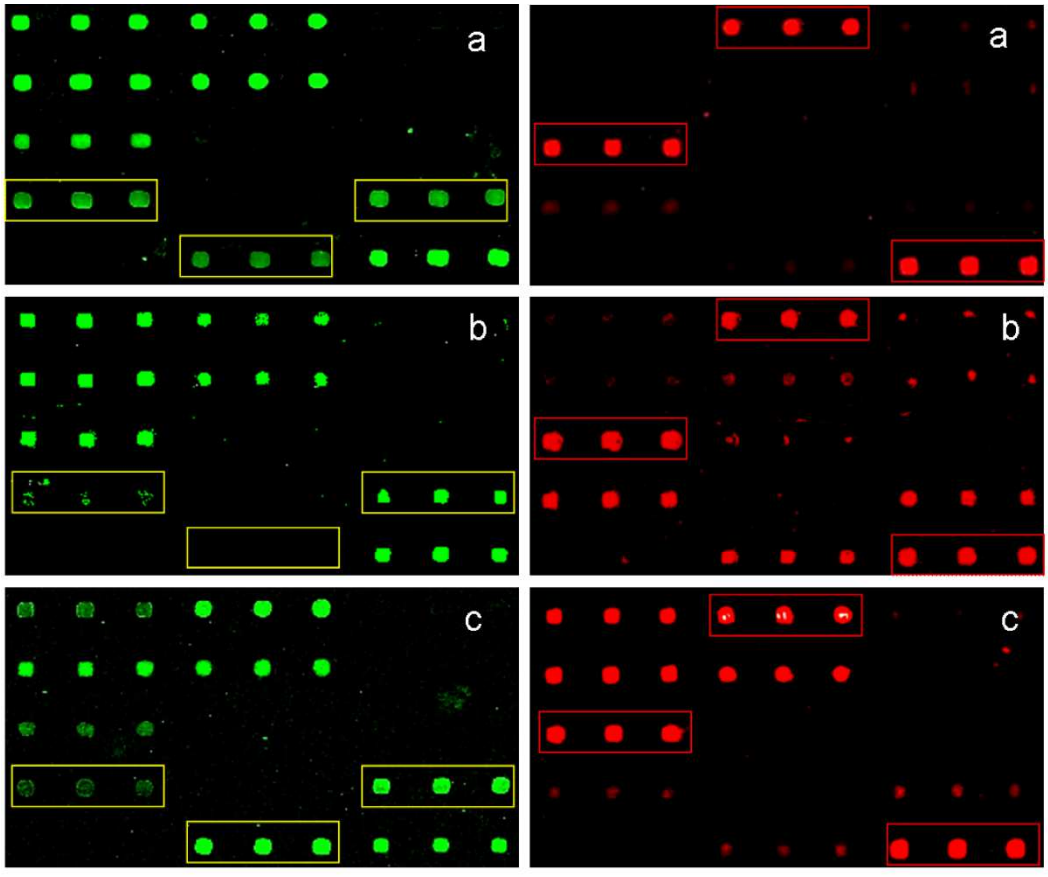
3.2. Interference effects

Several types of interferences negatively affect immunoassays. They are typically caused by the composition of the sample matrix, either by heterophilic antibodies, different kinds of interfering substances such as albumin, fibrin, drugs and lipids, as well as the viscosity of the solution, the pH value and salt concentration. Especially in bodily fluids the choice of sample diluent is critical to minimize these negative effects and improve assay sensitivity and reliability of results.

3.2.1. Matrix effects

In order to study the effect of non-specific binding of plasma components on immobilized receptors we added Dy-547-anti-fibrinogen together with Dy-647-streptavidin in the final incubation step. Accordingly, to assays in saliva Dy-547-anti-lysozyme and in serum Dy-547- anti-IgG was added. Matrix components binding non-specifically to the immobilized antibodies, analytes or detection antibodies, were detected by those labelled antibodies and a signal was recorded in the green channel at 570 nm. With this procedure we aimed at making a portion of unspecific binding visible, without interfering with specific signals in the red channel. Figure 3 shows images of arrays processed in 1:10 diluted a) serum b) plasma and c) saliva, performing immunoassays for IL-6 and PCT. The array consists of biomarker specific antibodies (highlighted in red in Figure 3 right) and spots of serum, plasma and saliva (highlighted in yellow in Figure 3 left). Fluorescence scans

(λex= 532 nm) on the left of Figure 3 show considerable signals for anti IgG (Fig. 3a) in serum, plasma and saliva spots, while fibrinogen is mainly detected in plasma spots (Fig. 3b) and anti-lysozyme binds to plasma and saliva spots (Fig. 3c).



anti TNFα	anti IL-6	Blank
anti IL-8	anti IL-10	Blank
anti PCT	Blank	Blank
Serum	Blank	Plasma
Blank	Saliva	Guide

anti TNFα	anti IL-6	Blank
anti IL-8	anti IL-10	Blank
anti PCT	Blank	Blank
Serum	Blank	Plasma
Blank	Saliva	Guide

Figure 3. Fluorescence scans of arrays processed in 1:10 diluted matrix in presence of Dy547 labelled antibodies added to a standard multi analyte assay for IL-6 and PCT. a) serum and anti IgG b) plasma with anti-fibrinogen and c) saliva with anti-lysozyme. Left: λ_{ex}=532 nm, right: λ_{ex}=635 nm, bottom: layout of the array. The position of specific antibodies for IL-6 and PCR as well as the red labelled guide dots are marked in red, reversed phase dots of serum, plasma and saliva are marked in yellow. Other probes are either buffer blanks or negative controls.

All three Dy-547 labelled matrix-specific antibodies can be detected in capture antibody spots specific for cytokines and PCT as well, as can be seen in Figure 3 left.

Figure 4 shows the unspecific signal in anti IL-6 spots for standard concentrations S1 - S9 working in serum (a), plasma (b) and saliva (c) as well as in buffer (black bars) as a

result of binding of Dy547-labelled antibodies to IgG, fibrinogen and lysozyme. Interestingly, also assays in buffer give rise to unspecific signals in the green channel, meaning that the antibodies for matrix components also unspecifically bind to capture antibodies and analytes. A smaller portion of this unspecific signal may be attributed to light scattering. Unspecific signals were in average 3, 1.6 and 1.5 times higher in 1:10 diluted serum, plasma and saliva than in assay buffer alone. The impact of interfering substances is two times higher in serum than in plasma and saliva. Nevertheless biomarker measurement in serum samples is reliable and sensitive, as the biomarker chip meets the clinical requirements. It's not necessary to either separate serum components (Altintaş, 2006) or use dramatically increased salt concentrations to avoid separation (Seiichi, 1983). The immobilization strategy for capture antibodies plays an important role for avoiding interferences too. Heterophilic antibodies may cross-react with reagent antibodies and compete with adsorbed secondary antibodies. DeForge et al. (2010) employed blocking agents in order to reduce interference from heterophilic antibodies when the capture elements were adsorbed to polystyrene ELISA plates. A direct covalently immobilized capture antibody as we used it herein or immobilization via streptavidin-biotin chemistry outperforms adsorption of biorecognition elements on a solid support (Wu et al., 2001).

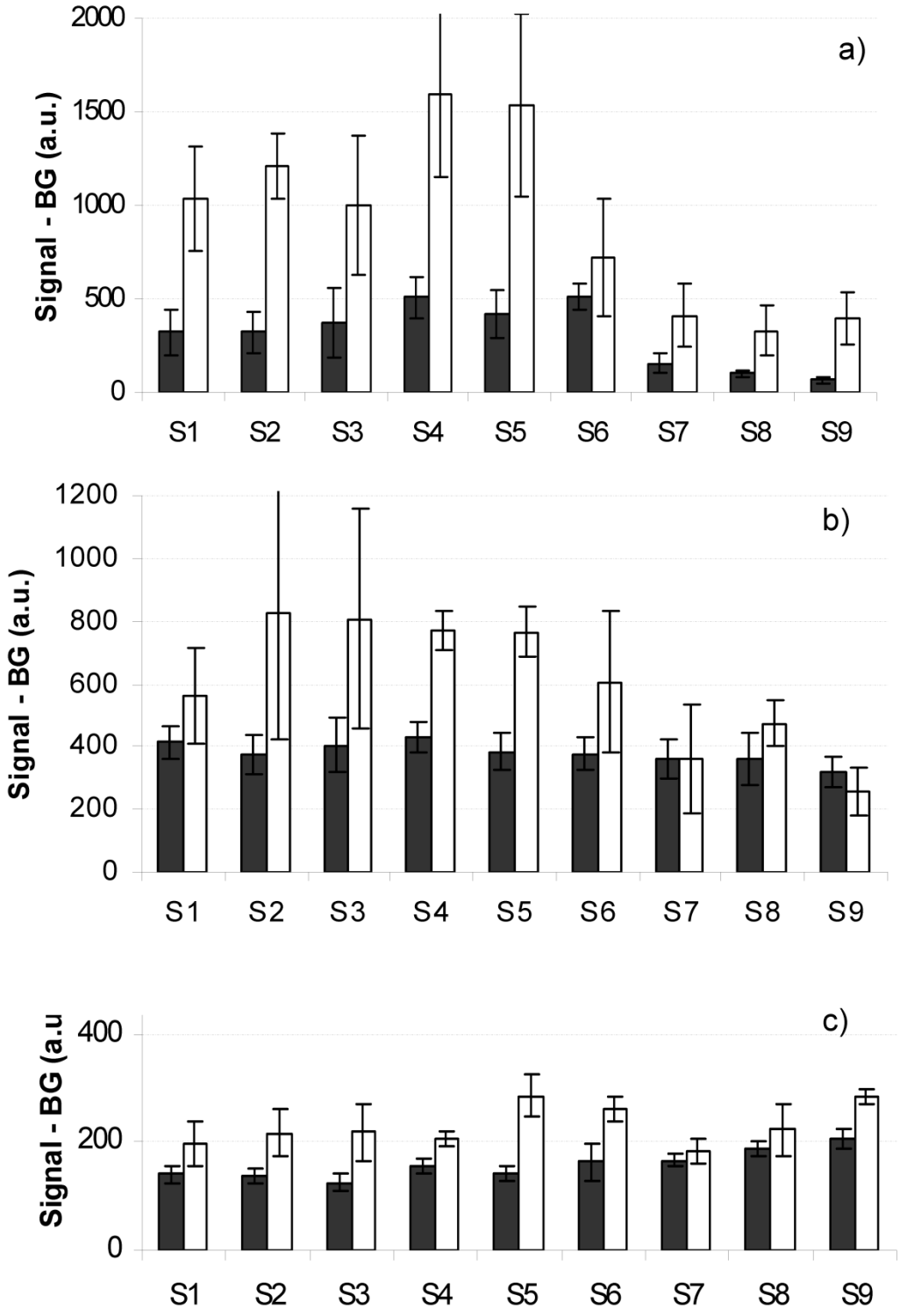


Figure 4. Unspecific signals of anti IgG, anti fibrinogen and anti lysozyme in IL-6 antibody spots when performing IL-6 assays for standards S1 - S9 in buffer (grey bars) and body fluids (white bars). a) Dy547 anti human IgG signal in buffer and serum b) Dy547 anti fibrinogen in buffer and plasma and c) Dy547 anti lysozyme in buffer and saliva. Matrices were diluted 1:10.

The anti IgG signal in capture antibody spots decreased with increasing analyte concentration (Figure 4 a), in the negative control spots (IL-10 e.g.) however the signal was stable (not shown). The same was true for anti-fibrinogen signals (Figure 4 b): increasing analyte concentration resulted in an up to 69% decreasing unspecific signal. This goes together well with the results of the standard curves: in lower concentration ranges we observed a higher background expressed as an up-shift of the lower asymptote. Higher concentrations of analytes featuring high affinity to the capture antibodies displace unspecifically bound molecules. However, non-specifically chip-bound fibrinogen and lysozyme still allowed antigen binding to the corresponding receptor over the tested working range, indicating that analyte binding was not prevented by high abundant plasma proteins such as fibrinogen or saliva proteins such as lysozyme. Anti-lysozyme signal was detected in all capture antibody spots of the array whether involved in immunoassays or not. Those signals were constant on a very low level (on average 230 a.u.) over the whole standard curve, indicating that lysozyme plays a lesser role in matrix effects of saliva (Figure 4 c).

Plasma is the most complex human-derived proteome, containing proteins specific to blood, and proteins released either through leakage or injury (Anderson and Anderson, 2002). Albumin is present in plasma samples in the milligrams per millilitre range while cytokines, like IL-6, is present in the picograms per millilitre range (Bishop et al., 2000). These two proteins differ in their plasma abundance by a factor of 10 orders of magnitude. Still, in this study, 158 ng/L IL-6 concentrations were detected in undiluted plasma, without removing high abundant proteins.

Serum, which is derived by centrifugation of clotted plasma, contains 60-80 g/L protein, in addition to various small molecules such as salts, lipids, and sugars (Anderson and Anderson, 2002). Roughly 22 high abundant proteins, such as albumin, immunoglobulin, haptoglobin, and transferrin, to mention a few, comprise approximately 97% of the protein content of serum. The remaining 3% of proteins are present in low concentrations (like cytokines) and are referred to as low abundant proteins (Bishop et al., 2000). Still, human IL-6 was detected as low as 5 ng/L and PCT as low as 0.2 µg/L in 1:100 diluted serum samples. By contrast, the proteome of human saliva is less complex, containing glycoproteins (mucin) enzymes (peroxidase, amylases, etc.) and anti-microbial proteins such as lysozyme, present at mg/L.

3.2.2. Crossreactivity of antibodies

Since PCT and IL-6 are measured in complex matrices, assay specificity for each analyte is imperative and most prevalently a result of excellent antibody quality. Cross reactivity of cytokines was checked with spotted antibodies for IL-8, IL-10 and TNF-α.

The unspecific signal of those spots was examined when the analytes were not present in the calibration standards. The highest standard S9 in buffer containing 40950 ng/L IL-6 and 409 µg/L PCT, typically yielded unspecific signals of less than 0.2 % of the respective IL-6 signal. In neat and diluted plasma and serum unspecific signals did not rise significantly with increasing standard concentrations, the unspecific signals did not exceed 1.8 % of the specific IL-6 signal in the highest calibration standard. A stable unspecific signal, not rising above the level of the zero standard, results in elevated LODs for the respective analyte but not in false positive quantification of that. However, in 1:10 diluted saliva cross reactivity rose up to 17%, 23% and 11% for IL-8, IL-10 and TNF-α alpha and a signal increase with higher calibration standards was observed. Low unspecific signals in serum and plasma, and high crossreactivity in saliva can also be seen in Figure 3 right.

4. Conclusions

Like all clinical laboratory tests immunoassays are subject to interferences that may result in false-positive or false-negative readings. Undetected interferences lead to unnecessary or inappropriate treatment of patients (Kricka, 2000). Hence, every endeavour has to be made to recognize and eliminate erroneous measurements in order to avoid harm for the patient. Herein we demonstrate that using 0.1 M Tris (pH 7.4)/ 0.1 M NaCl/ 10 mM CaCl2/ 0.1% Tween-20 as diluent for real sample measurements in serum, plasma, and saliva minimizes interferences derived from matrix proteins, like IgG, fibrinogen and lysozyme in a biomarker chip for sepsis. Linearity of dilution however is dependent on the type of antigen in the sample solution: while the calibration curve for IL-6 in assay buffer is similar to those obtained in serum, plasma and saliva, each diluted by factor 10 and 100, this similarity in different dilutions and matrices was not observed for PCT. We recommend an up-to 1:10 sample dilution in order to reduce matrix effects, improve reproducibility and having still detectable biomarker concentrations available. Our most recent experiments have shown good results with addition of only 10% assay buffer (10:1 dilution)). We furthermore showed that matrix proteins bind non-specifically to the immobilized receptor without impeding the detection of the analyte, but are replaced by the analyte with increasing analyte concentration. Crossreactivity of antibodies is another potential source of interference and was tested in IL-6 and PCT-assays with spotted anti-IL-8, anti-TNF-α and anti-IL-10. Interestingly, crossreactivity was very low in serum and plasma (<1.8%), but enhanced in saliva (up to 23%).

In summary, the selected antibody pairs and the tested matrix diluent proved to balance well the various effects of matrix, protein content and protein conformation allowing

detecting and quantifying the analytes in various sample dilutions with high specificity and good reproducibility using a protein biomarker chip.

Acknowledgements

The authors thank the European Commission for financial support within CARE-MAN (NMP4-CT-2006-017333).

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6 Conference presentations

6.1. 4th Advances in Microarray Technologies

7-8 May 2008, Barcelona, Spain. Poster Presentation

A biochip for the detection of CRP, PCT and IL-6, the major biomarkers for inflammation

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INTRODUCTION

Cytokines, such as IL-6, acute phase proteins, like C-reactive protein and procalcitonin are biomarkers reported for inflammation and sepsis. The analytical range of the biomarkers to be detected can be quite different. while CRP may raise up to 1000 µg/mL in an acute phase, cytokines develop in the pg/mL range. In order to address the various relevant concentration ranges respective measurements are most often done using different assay formats and detection techniques, depending on what method covers best the required range.
Herein, a biochip for the parallel detection of biomarkers for inflammation and sepsis is presented. Three biomarkers varying in size, quaternary structure, and clinical range were chosen for demonstrating strategies to overcome obstacles in assay development. Improved immobilization of capture probes, combination of complementing biorecognition elements (antibodies, aptamers and peptide scaffolds), and usage of different immunoassay formats in parallel have been evaluated, showing the enhanced detection capabilities of our novel assay design.

On-Chip Immunoassays

0.25 mg/l to 1 mg/ml monoclonal antibodies and 50µM RNA aptamer were immobilized onto ARChip Epoxy and ARChip Gel. Antigens were spiked into human serum (Hytest) 1:10 diluted with assay buffer (0.1M Tris pH 7.4, 10mM $CaCl_2$, 100mM NaCl) and incubated on slides at RT in a humid chamber for 2 hours. Biotin-conjugated antibodies were applied for 30 min after a washing step. Subsequently, the slides were washed in 1x PBS (pH 7.2) and incubated with fluorescent Dy647-labelled streptavidin for 30 min. Assays were performed in FAST Frames (Whatman).

Scanning & data analysis
Fluorescence signals were recorded using the Tecan LS confocal scanner and analyzed using the Genepix Software 6.0 package. Mean values, SD, CV and LOD were calculated based on background corrected signals (FG –BG signals). Calibration curves were set up using the four parameter logistic model.

Parameter	Borderline value	LOD
CRP	8 µg/ml	0.18 ng/mL
Procalcitonin	2 ng/ml	0.1 ng/mL
IL-6	10 pg/ml	3.1 pg/mL

Clinical borderline values and LODs of the Sepsis Chip

Experimental Design

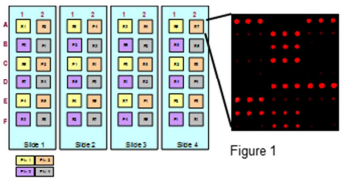

Figure 1

The biomarker chips were manufactured with 4 pins, each spotting identical arrays, an assembly of capture elements, normalization probes, blanks and guide dots. Each of these arrays was assayed with a sample, either a standard or a patient sample. Quality control by means of ANOVA revealed a significant effect of pins, print runs and slides. Consequently experiemtnal design and normalization strategies were developed. Figure 1 depicts an example of the sample distribution representing an 8 point standard curve and 4 patient samples in a set of 4 slides.

Procalcitonin

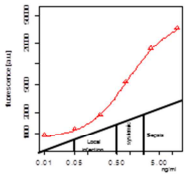

Figure 2. Standard curve for PCT in human serum 1:10 diluted with assay buffer. Antibody pair specific for PCT was developed by Petra Krämer, Helmholtz Zentrum München.

Interleukin-6

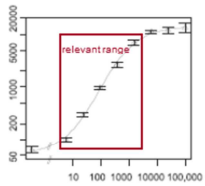

Figure 3. Standard curve for IL-6 in human serum 1:10 diluted with assay buffer (LOQ 10.3 pg/ml, R^2= 0.994, mean CV <20%)

CRP-aptamer and antibody

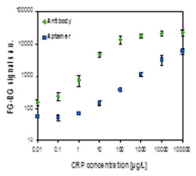

Figure 4. Standard curve for CRP using RNA aptamer specific for CRP and monoclonal capture antibody in a sandwich format.

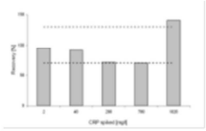

Figure 5. Aptamer as BRE: % Recovery for serum samples spiked with 2 to 1020 mg/l CRP (dilution factor 1:100). Dashed lines indicate the border lines for recovery acceptance.

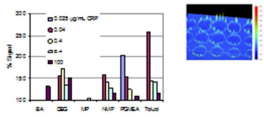

Figure 6. Nanowell arrays
Antibodies were spotted into nanowells etched with: butylacetate, diethyleneglycol, methoxypropanol, 1-methyl-2-propylacetate, 1-methoxy-2-propylacetate, toluol.

Strategies for improved antibody immobilization

Enhanced immobilization capacity can significantly increase analytical performance of the biochip.
•Surface chemistry with higher immobilization capacity: ARChip Gel, a 3D hydrogel was used for improved immobilization capacity.
•Suitable immobilization buffers: 1xPBS/0.01% Na-deoxycholate; 1x PBS/ 0.005% CHAPS/0.01% BSA.
•Increasing surface area: immoblization on particles; immobilization into microwells
•Crosslinking immoblized antibodies for higher probe density: Transglutaminase

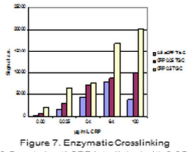

Figure 7. Enzymatic Crosslinking
0.5 mg/ml anti-CRP interlinked with 0.05 and 0.5 u transglutaminase spotted in 0.1 M Tris (pH 8), 0.005% Chaps, 2 mM CaCl2 onto ARChip Epoxy

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AUSTRIAN RESEARCH CENTERS

6.2. 5th International Conference on Advanced Materials and Nanotechnology

(AMN-5), February 7-11 2011, Wellington, New Zealand. Poster Presentation

AUSTRIAN INSTITUTE OF TECHNOLOGY

PROFACTOR

Fabrication of nanostructures for protein chips: effect of wettability on immobilization and assay performance

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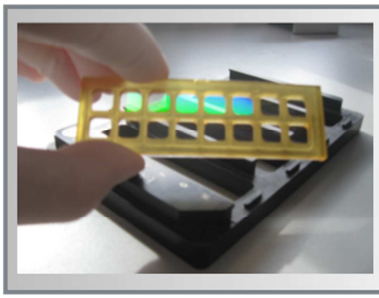
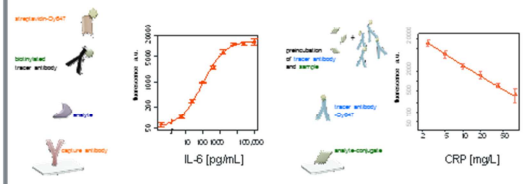


Abstract

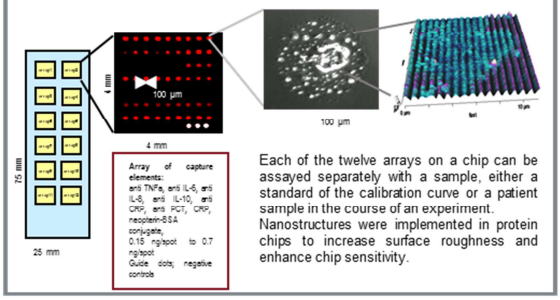
The major advantage of biochips over conventional biological techniques is the highly parallel, addressable, miniaturized array format which allows simultaneous detection of different targets, virtual automation and functional integration for high throughput screening. One of the major challenges to further developing biochip technology is to strengthen the signals produced by probe-target interaction, especially in cases of low probe and target concentrations.
Several approaches have been reported for achieving stronger signals and lower detection limits, including such based on more sensitive detection methods (e.g. evanescent wave technology), alternative labels (e.g. quantum dots), and novel surface chemistries. Among the available chip surfaces especially three-dimensional surfaces such as hydrogels and dendrimers promise high loading capacity and improved accessibility of targets.
Herein we follow a completely different and new approach: we use thermal nanoimprint lithography (thermal NIL) to nanostructure the chip surface to increase surface roughness and as a result significantly enhance protein immobilization capacity which has direct impact on the assay performance and subsequently the fluorescence signal.

Protein biochip for the quantification of biomarkers

Biochips are arrays of biomolecular probes immobilized onto solid supports for highly parallel miniaturized analysis systems. The protein chip for diagnosis of sepsis combines both sandwich (left) and binding inhibition (right) immunoassays in order to quantify high (CRP) and low abundant proteins (cytokines, PCT, neopterin) in parallel. Signal readout is performed using fluorescence detection.

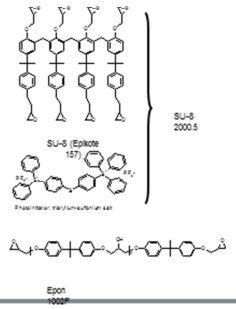


Dimensions of a nanostructured protein biochip



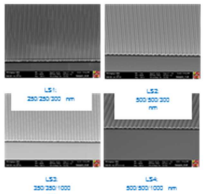
Each of the twelve arrays on a chip can be assayed separately with a sample, either a standard of the calibration curve or a patient sample in the course of an experiment.
Nanostructures were implemented in protein chips to increase surface roughness and enhance chip sensitivity.

Surface structuring using nanoimprint lithography (NIL)



For fabrication of the nanostructured chips we chose the negative photoresist SU-8 as it provides excellent chemical resistance and high optical transparency. Microscopic glass slides were spincoated and nanostructured by thermal NIL, which is basically a pressure induced transfer of topographic patterns from a rigid or flexible template into a thermoplastic polymer film heated above its glass transition temperature. Here, SU-8 2000.5 and EPIKOTE are monomers and the temperature used was higher than their melting points (82 ° C).

Structured surfaces and wettability

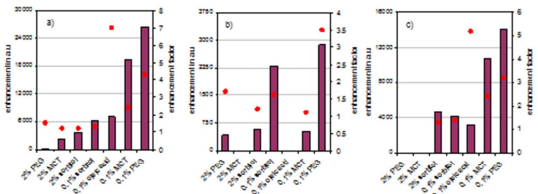


Print buffer	LS1	LS2	LS3	LS4	No structure
H2O	103	109	102	90	96
CHAPS/BSA	99	98	95	82	85
0.01%Na-deoxy.	100	100	97	86	92
2% SDS	62	62	60	55	15
0,1% SDS	53	59	57	53	26
2% DTAB	87	86	84	68	17
0,1%DTAB	80	79	80	73	43
2%Oleic Acid	77	78	74	71	58
0,1%Oleic Acid	88	79	87	75	40
2%PEG	96	94	95	82	68
0,1%PEG	94	95	94	82	65

Color code contact angle:
> 100
80 - 100
60 - 80
< 60

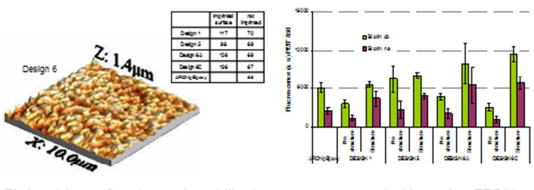
F... ...pace structures were compared regarding their hydrophobicity. As expected, contact angles increased upon nanostructuring, which is also reflected in the decreased spot size. Wettability and hence good contact between surface and probe is important to ensure optimal binding of biomolecules.

CRP binding inhibiton assay on structured epoxy surfaces



Signal enhancement (a.u) (columns) on nanostructured a) Epon1002F, b) SU-8 2000.5, and c) SU-8 (Epikote 157) surfaces compared to respective planar epoxy chips. The enhancement factor (•) indicates the signal increase by x times compared with the planar, non structured slides.

Biotin-strepatividin assay on nanostrucutred chips



Biotin (size ~ 2 nm) was immobilized onto nanostructured chips using PBS/Na-deoxycholate (na) and PBS/CHAPS/BSA (cb) as spotting buffers and detected with Dy647-strepatividin. Brightest signals were achieved on the most rough surface of Design 6.

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6.3. 12th International Conference on Nanoimprint and Nanoprint Technology

October 21-23, 2013, Barcelona, Spain. Poster Presentation

Gold discs produced by residue-free UV-NIL and subsequent lift-off for integration in biosensors.

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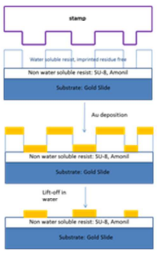
AUSTRIAN INSTITUTE OF TECHNOLOGY

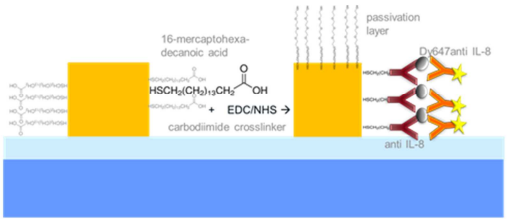
NILPlasmonics

austrian nano initiative



Fabrication of a nano-structured biochip surface including UV-NIL, layer deposition, and lift-off.

We aim to integrate Nanoimprint Lithography (NIL) in a high throughput production of plasmonic biochips to exploit the metal enhanced fluorescence effect for detection of protein markers. We propose the following assembly:
Au slides are coated with a non water soluble resist ("spacer layer") of 120 nm thickness onto which nanostructures of water soluble resist are created using NIL. Onto these nanostructures we evaporate Au and lift-off the resist to yield Au clusters of 110 nm diameter, 50 nm height and a period of 460 nm. Size, shape and pitch were identified through finite difference time domain (FDTD) simulations [Bauch et al. 2013].



Biosensor surface: Functionalization of metal via carboxy-functional thiols self-assembling on Au discs and activation with carbodiimide cross linker.

Approach 1: Spontaneous dewetting-induced residue-free imprinting

The process is controlled by the spreading parameter S. If S<0, the system gains energy by squeezing the intercalated liquid away to create an intimate contact between the solid and the rubber: the film is metastable and dewets by nucleation and growth of dry patches [Choi et al. 2009, Martin et al. 1997].

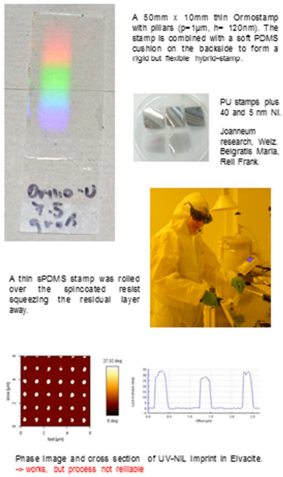
Approach 2: Water soluble resist ACMO for residue-free UV-NIL

Auner et al [2009] introduced a UV NIL process enabled by residue-free imprinting with ACMO and a water-based lift-off. During imprinting the resist is displaced because of the difference in polarity of resist and stamp. Hence we characterized surface energies of substrates, spacer layers, stamp materials and imprint resists.

Site specific immobilization of biomolecules

The spacer layer (SU-8) is blocked with ethanolamine inhibiting antibody binding between gold discs. If the upper part of the discs is passivized with e.g. 11-mercaptoundecanol, the walls can be selectively modified with carboxy-functional thiols and activated with carbodiimide/succinimid (EDC/NHS).

Working stamps and imprinting



A 50mm x 10mm thin Ormostamp with pillars (p=1µm, h= 120nm). The stamp is combined with a soft PDMS cushion on the backside to form a rigid but flexible hybrid-stamp.

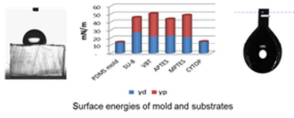
PU stamps plus 40 and 5 nm Ni.

Joanneum research, Weiz, Belgratia, Maria, Reil Frank.

A thin sPDMS stamp was rolled over the spincoated resist squeezing the residual layer away.

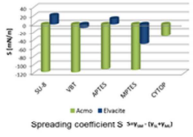
Phase image and cross section of UV-NIL imprint in Elvacite.
-> works, but process not reliable

Dewetting at the interface mold/ resist/substrate

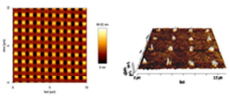


Surface energies of mold and substrates

PDMS and CYTOP show low surface energy with a very small polar component. SU-8, APTES, MPTES, and VBT feature a more balanced ratio of polar and dispersive component. weAP exhibits a positive spreading coefficient on SU-8 and APTES -> no dewetting.

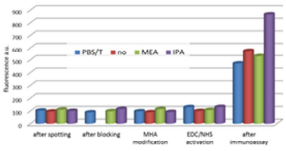


Spreading coefficient S

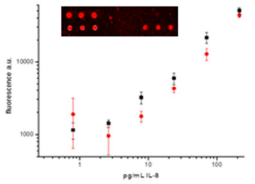


UV-NIL with Acmo 3 and sPDMS stamp, Cr/Au deposition (5/50 nm) (left) and lift-off in water (right). -> low gold discs, optimization needed.

Immunoassay on gold surfaces



Low background signal was recorded after various processing and activation steps. on plain gold surfaces. The immunoassay with a fluorescently labelled antibody yield s high signals



Fluorescence signal on gold surface (MHA modified, EDC/NHS activated): standard curve for IL-8. Activation buffer PBS(black) or imidazol (red). Insert: Scan image of sensor surface with antibody dots.

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6.4. 11th BBMEC, International Biosensor Conference

September 26 -30, 2015, Regensburg, Germany. Oral Presentation

Making protein biochips more attractive for real-life applications

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Abstract

The great impact of biochip technologies forecasted only partly came true so far. While biochips are frequently used in research, the routine application in clinical labs, point of care, or industrial applications is still unusual. The same is true for *in situ* detection of environmental pollutants and pathogens. Although biochips score with faster results, determination of multiple parameters in parallel, and reduced sample volumes, the technology could benefit from increased sensitivity, improved reproducibility and at the same time low production cost. Further, integration of biochip technology into portable instruments would promote market entry.

In order to deal with extremely low detection limits as well as with the diverse chemistry of analytes, such as protein biomarkers in biological fluids or toxins in environmental samples, chip platforms of enhanced sensitivity and flexibility are urgently needed. To develop such platforms we propose biochips

- combining natural and artificial biorecognition elements in multiplexed assays
- coping with a number of real-life matrices
- exploiting metal-enhanced fluorescence in biochips fabricated by Nanoimprint Lithography (NIL)

Relevance

Integrating antibodies, aptamers, and molecularly imprinted polymers and combining different assay formats in one chip, makes the technology applicable for a wide range of analytes and samples with high and low abundant targets. Plasmonic amplification of fluorescence signals yielded a 5-fold increased sensitivity, a number that still can be increased by tuning the nanostructures for plasmonic chips to the optics of the commercial laser scanner.

Conclusions

Developed in the 1990s, protein microarrays are on the verge of becoming a routinely used tool for a wide range of applications be it in basic or applied research. Our aim is to bring them from big central research facilities to the clinical routine lab or point-of-care.

During our extensive preliminary research on protein microarrays for diagnostics a number of challenges emerged. Herein we identified them and focused our research on the most important ones, namely low reproducibility, insufficient sensitivity, analysis time, and integration of assays into portable systems. Other crucial topics include production cost, availability of biological or artificial biorecognition elements, crossreactivities, lack of standards, and alternative detection schemes allowing broader application of the technique.

The centrepiece of our microarray is the immobilization platform ARChip Epoxy, a SU-8 coating (dip-coated 1% Epikote 157 in 2-butanone). SU-8 is a material widely used in microsystems technology as a negative photoresist. In contrast to other commercially available microarray coatings utilizing functional epoxy groups we work with an organic coating solution of the resin rather than using a silane with epoxy groups (e.g. 3-Glycidyloxypropyl)trimethoxysilane). No photo initiator is added and the material is not cross-linked in order not to use up the epoxy groups. As a cost-effective, simple and stable surface chemistry, suitable for automated coating, ARChip Epoxy qualifies perfectly for both oligonucleotide and protein based microarray applications. For fluorescence based measurements the low autofluorescence, low unspecific binding after surface blocking and excellent immobilization capacity are valuable features. We were able to produce thin coatings of approximately 10 nm thickness which are not only suitable for fluorescence but also for TIRF, planar waveguide, and plasmonic chips where layer thickness plays a crucial role. A thin layer of epoxy resin has been shown to be a simple and efficient alternative to labor intensive and less efficient alkanethiol SAMs and EDC/NHS chemistry for probe immobilization on gold nanostructures. For site specific immobilization spotting of the epoxy resin provides an interesting new technique.

Our approach of combining sandwich and binding inhibition assay formats on one chip accounts for the heterogeneity of target molecules one may encounter in multiplexed assays. Small molecules as for example neopterin and highly abundant molecules (e.g. CRP) are detected by one antibody in a binding inhibition format. Larger proteins such as interleukins are detected in a sandwich format providing high sensitivity. Also different BREs were used to adapt to abundance of the target molecules, high sensitivity was

achieved using antibodies, while tuneable detection ranges are made possible with aptamers, MIPs or specific ligands.

Using 0.1 M Tris (pH 7.4)/ 0.1 M NaCl/ 10 mM CaCl2/ 0.1% Tween-20 as diluent for real sample measurements in serum, plasma, and saliva minimized interferences derived from matrix proteins, like IgG, fibrinogen and lysozyme in a biomarker chip for sepsis. A 1:10 sample dilution proved to provide a good balance between reducing matrix effects, improving reproducibility and having still detectable biomarker concentrations available.

Preliminary experiments for exploiting metal-enhanced fluorescence in biochips fabricated by Nanoimprint Lithography (NIL) showed the potential of plasmonic amplification of fluorescence signals. We yielded a 5-fold increased sensitivity, but this number is thought to be increased tremendously by further optimizing the production of nanostructures for plasmonic chips and tuning their dimensions to the optics of the commercial laser scanner.

Curriculum Vitae

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Date of Birth:	25 October 1968
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Biology degree M.Sc., University of Vienna, Institute of Ecology and Conservation Biology under the supervision of Prof. Maier.

Training abroad

June 2016	**Metrohm Inula User Training, Vienna**: ESEM and EDX
May 2012	**JPK User Training, Berlin**: AFM imaging and force spectroscopy with simultaneous optical observation under ambient and liquid conditions
Feb 2011	**University of Canterbury/MacDiarmid Institute**, Christchurch, New Zealand Bioimprint Technology for AFM investigations of cancer cells
Nov 2010	**University of the Negev**, Beer Sheva, Israel AFM and NSOM training
April 2004	**EURISKED/CREDO Cluster**, Mallorca Workshop *Multi-organic Risk Assessment of Endocrine Disrupters*
Feb 2003	**University of Aarhus**, Denmark Workshop on *Statistical Analysis of Microarray Data*
June 2002	**National University of Ireland**, Maynooth Bioinformatics Summer School
June 1999	**Instituto de Investigaciones Agrobiologicas de Galicia**, Santiago de Compostella, Spain Project *Induction of Androgenesis in Chestnut Hybrids*

Professional Experience

Since 2011	Scientist at the **Austrian Institute of Technologies**
Since Jan 2004	Researcher at the **Austrian Institute of Technologies**, Biosensor Technologies
Since Aug 2001	Freelancer **Austrian Research Centers**

	Biosensor Technologies – *Development and evaluation of new reactive coatings for biochips; assay development for protein biochips; data analysis*
July 2000-Oct 2000	Freelancer **International Atomic Energy Agency** (IAEA)
	Project *Publish reproducible operating procedures on tissue culture and mutation techniques*
June 2000	Freelancer **ARC Seibersdorf research** GmbH, Biotechnology
	Project *Cryopreservation of Oak*
June 1999-Dec 1999	Freelancer **ARC Seibersdorf research** GmbH, Biotechnology
	Project *Biocontrol of Chestnut Blight*

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Book Chapters

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Lecture

164.220 Biochip Technologies in (Bio)Analytical Chemistry

2014S, VO, 2.0h, 3.0 ECTS, togethter with G. Haberhauer, A. Sessitsch and P. Ertl

Technical University of Vienna

Conferences

Oral Presentations

Invited Lecture: Assessment of the Quality of Microarray Data; MolPAGE workshop: Statistical Analysis of Genetic and Gene Expression Data, Pavia, Italy, March 20-24, 2006.

U.Sauer, C. Preininger, J. Dostalek, K. Gier, S. Gogalic, S. Hageneder, M. Bauch, A. Solar. Making protein biochips more attractive for real-life applications. 11th BBMEC, International Biosensor Conference, Regensburg, Germany, September 26 -30, 2015.

U. Sauer, C. Preininger, H. Toth-Szekely, L. Riegger, Functional Functionalization of cyclo olefin polymer substrates for on-chip PCR applications, 10th workshop on biosensors and bioanalytical microtechniques in environmental and clinical analysis, Weimar, Germany, June 19 -22, 2011.

U. Sauer, C. Preininger, B. Koger, A. Brandenburg, L. Riegger and A. Telle, Functional Chip Surfaces in a Diagnostic Test System for Cancer Screening, Nanosens 2010, Vienna, Austria, December 2-3.

U. Sauer, Johanna Pultar, Ivo Cernoch, Milan Franek and Claudia Preininger: Biochips for rapid biomarker based diagnosis, Advances in Microarray Technology, Stockholm, Sweden, 19-20 May 2009.

U. Sauer, C. Preininger: Protein Chips zur Bestimmung Endocriner Disruptoren, Deutsches Biosensor Symposium, Regensburg, Germany, March 13 -16, 2005.

U. Sauer, Preininger C,Trombitas M, Obersriebnig S, Krumpel G, Kern W: Signal Enhancement of Protein Chips. 7th European Conference on Optical Chemical Sensors and Biosensors, Madrid, Spain, 4-7 April 2004.

Poster presentations

12th International Conference on Nanoimprint and Nanoprint Technology 2013, 21-23 October 2013, Barcelona, Spain. U. Sauer, A. Solar, C. Preininger, Gold discs produced by residue-free UV-NIL and subsequent lift-off for integration in biosensors.

39th International Conference on Micro and Nano Engineering 2013, 16-19 September 2013, London, GB. U. Sauer, A. Solar, M. Bauch, J. Dostalek, C. Preininger, Fabrication of gold-nanostructures for biosensor surfaces via residue-free NIL and lift-off.

38th International Conference on Micro and Nano Engineering 2012, 16-20 September 2012, Toulouse, France. U. Sauer, A. Salibasic, J. Bawitsch, C. Preininger, Nanoimprinted Polymer Surfaces for Signal Enhancement of Protein Biochips.

2nd International Conference on Bio-Sensing Technology 2011, 10-12 October 2011, Amsterdam, The Netherlands. U. Sauer, C. Preininger, M. Seibt, P. Buchegger, H. Toth-Szekely, P. Domnanich, M. Chouiki, R. Schöftner, Signal enhancement for a protein biomarker chip by integration of nanostructures and metal-enhanced fluorescence detection.

Fifth International Conference on Advanced Materials and Nanotechnology (AMN-5), February 7-11 2011, Wellington, New Zealand. U. Sauer, C. Preininger, M.Chouiki, R. Schöftner, Fabrication of nanostructures for protein chips: effect of wettability on immobilization and assay performance.

Slonano, October 20-22 2010, Ljubljana; Slovenia. U. Sauer, C. Preininger, B. Koger, A. Brandenburg, L. Riegger and A. Telle, Functionalization of Chip Surfaces for a Diagnostic Test System for Cancer Screening.

Advances in Microarray Technologies, 7-8 May 2008, Barcelona, Spain. U. Sauer, J. Pultar, P. Domnanich, C. Preininger, A biochip for the detection of C-reactive protein, procalcitonin and interleukin-6, the major biomarkers for inflammation.

The 11th Annual European Conference on Micro and Nanoscale Technologies for Biosciences 2007, Montreux, Switzerland. U. Sauer, L. Schorn & C. Preininger: Polymer nanostructures as immobilization matrix in protein chips for detection of inflammation and sepsis.

The Ninth World Congress on Biosensors 2006, Toronto, Canada. U. Sauer, C. Preininger, K. Derwinska, J. Pultar, C. Martin: Protein Biomarker Chip for TNF alpha, IL-6, IL-8, IL-10, IL-13, Fibrinogen, and Albumin.

Statusemiar Chiptechnologien 2005, Frankfurt am Main, Germany. U. Sauer, C Preininger. Strategies for signal enhancement of antibody arrays.

ÖGBM Joint Meeting 2002, Salzburg, Austria. U. Sauer, C. Preininger, Experimental Design and Quality Control of Oligonucleotide Microarrays.

Symposium on Advanced Techniques in Microscopy and Cellular Bioinformatics, 2002, Vienna, Austria. U. Sauer, C. Preininger, Data analysis and quality control of microarrays.

International Conference on Plant Embryology, 2001, Nitra, Slovakia. U. Sauer, E. Wilhelm, Improving Maturation and Germination Conditions for Somatic Embryos of European Chestnut.