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DISSERTATION

Titel der Dissertation

„Development of a protein microarray for diagnosis of sepsis“

verfasst von

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angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr.rer.nat)

Wien, 2014

Studienkennzahl lt. Studienblatt:
Dissertationsgebiet lt. Studienblatt:
Betreut von:

A 791 419
Chemie
Univ.-Prof. Mag. Dr. Peter A. Lieberzeit

Acknowledgment

The presented thesis was part of the MNT ERA-Net Project “Valipro – Validation and production of a TIRF based protein chip system for diagnosis of sepsis” and financially supported by the Austrian Research Promotion Agency (FFG). The main work of the study was carried out at the AIT (Austrian Institute of Technology), Health & Environment Department, Bioresources at the group conducted by Dr. Claudia Preininger. The fabrication of conventional molecular surface imprints using acrylic monomers and the related QCM measurements presented in this study were performed at the University of Vienna, Department of Analytical Chemistry at the group of Prof. Lieberzeit.

I am deeply indebted to Dr. Claudia Preininger for her excellent supervision and guidance through this thesis. I am grateful for the liberty and confidence I received and for the possibility to bring forward own ideas. Additionally, I appreciate the many opportunities I perceived to present my work at national and international conferences.

I am very obliged to Prof. Lieberzeit for his trustworthy support that went far beyond sole university supervision, for many suggestions and for pointing me to the right direction.

I thank Mag. Ursula Sauer for her support in technical and scientific concerns at any times.

I'd like to say a big thank you to my former and current colleagues Geri, Sara, Selma and Anna for the excellent working atmosphere and for the many chats and hilarious time off the lab.

I am deeply indebted to my family, most notably to my parents Albert und Renate, who encouraged and supported me throughout my studies. I know that without them I wouldn't be where I am today.

Last but not least, I am deeply grateful to my friend Hannes whom I always know behind me for his support and accompany through all my ups and downs.

Abstract

Sepsis is a systemic body infection triggered by invading pathogenic microorganisms and constitutes a deadly threat in intensive care units. To provide a convenient diagnostic tool, a protein microarray that allows the simultaneous detection of the biomarker C-reactive protein (CRP), Interleukin 6 (IL-6), Procalcitonin (PCT), S-100 and E-Selectin was developed.

The assay was miniaturized to work with only 4 μL patient serum sample which is especially beneficial for diagnosis in neonates as it minimizes stress caused by excessive blood withdrawal. Due to diffusion limitations, the reduction of the sample volume is accompanied by losses in sensitivity. To restore the assay sensitivity, streptavidin coated magnetic particles were added that function not only as stirring components but also as detection elements.

In order to provide a customer-friendly “one patient – one chip” assay, the calibration was directly integrated on the chip. For this purpose, the detection antibodies were immobilized in increasing concentrations besides the capture antibodies to form an internal calibration curve.

The serum concentrations of the biomarker pool are very different: IL-6 occurs in the pg/mL, PCT, S-100 and E-Selectin in the ng/mL and CRP in the $\mu\text{g/mL}$ range. The low but specific binding affinity between CRP and phosphocholine [$K_D = 5 \mu\text{M}$] was exploited to develop a low-sensitive sandwich assay for CRP to ensure the simultaneous quantification of the differently abundant biomarkers via the integrated on-chip calibration.

Four assay designs were evaluated: (I) the classical biotin-streptavidin assay, (II) the rapid assay using directly fluorophore labeled antibodies, (III) the enzymatically amplified assay and (IV) the antibody amplified assay. All assay designs were similar in precision (CV_{intra} : 8 – 14 %, CV_{inter} : 18 – 34 %), while the sensitivity (LODs) increased by a factor of 10 for the ultra-sensitive assays (III, IV). The accuracy (recoveries) proved best for the classical (I) and the antibody amplified (IV) assays.

Besides biomarker detection, the bacterial surface marker LPS (lipopolysaccharide) and LTA (lipoteichoic acid) were exploited for the fast Gram-differentiation of the causative bacteria. Therefore, a surface-imprinted sensor layer was fabricated using the thermally curable ready-to-use resist material Epon 1002F and compared in QCM measurements to conventional molecular surface

imprints comprising acrylic monomers. The thermo-surface imprints exhibit excellent sensitivity of up to 13 times increased compared to the non-imprinted reference films and a very high selectivity as cross-reactivities to the tested unspecific analyte were negligible.

Kurzfassung

Sepsis ist eine systemische Infektion des Körpers und stellt vor allem in Intensivstationen eine tödliche Gefahr dar. In dieser Arbeit wurde ein Protein Microarray zur Diagnose von Sepsis entwickelt, der die parallele Bestimmung der Biomarker C-reactives Protein (CRP), Procalcitonin (PCT), Interleukin 6 (IL-6), S-100 und E-Selektin erlaubt.

Um den Anforderungen in der neonatalen Diagnostik gerecht zu werden, wurde ein Protein Chip entwickelt, der mit nur 4 μ L Serum in einer 1:10 Verdünnung der Probe auskommt. Die Reduzierung des Probenvolumens führte anfänglich jedoch zur Abnahme der Assay Sensitivität. Um die Sensitivität wieder herzustellen, wurden dem Assay magnetische Partikel, welche mit Streptavidin ummantelt waren, zugegeben. Diese magnetischen Partikel dienen einerseits zur Durchmischung des geringen Probenvolumens und sind andererseits direkt im Detektionsprozess involviert.

Zur leichteren Handhabung des Assays, wurde die Kalibrierung direkt in den Chip integriert. Dazu wurden die Detektionsantikörper in aufsteigenden Konzentrationen direkt neben den Fängerantikörpern immobilisiert.

Die Biomarker unterscheiden sich sehr stark in ihren Serumkonzentrationen: IL-6 kommt im pg/mL, PCT, S-100 und E-Selektin im ng/mL und CRP im μ g/mL Bereich vor. Um die parallele Bestimmung der Biomarker trotz unterschiedlicher Konzentrationen zu gewährleisten, wurde die geringe Bindungsaffinität [$K_D = 5 \mu$ M] zwischen CRP und Phosphocholine genutzt, um einen Sandwich Immunoassay mit geringer Sensitivität für CRP zu entwickeln.

Es wurden vier Assay Designs entwickelt und evaluiert: (I) der klassische Biotin-Streptavidin Assay, (II) der beschleunigte Assay über direkt Fluorophor markierte Antikörper, (III) der enzymatisch amplifizierte und der (IV) Antikörper amplifizierte Assay. Die Reproduzierbarkeit war für alle Designs sehr ähnlich (CV_{intra} : 8 – 14 %, CV_{inter} : 18 – 34 %), die beiden amplifizierten Assays (III, IV) waren um eine Größenordnung sensitiver (LODs) und der klassische und der Antikörper amplifizierte Assay waren am genauesten (Recoveries).

Die bakteriellen Oberflächenmarker LPS (Lipopolysaccharid) und LTA (Lipoteichonsäure) wurden für die schnelle Unterscheidung von Gram-positiven und Gram-negativen Bakterien genutzt. Durch

molekulare Oberflächenprägung der LPS und LTA Mizellen in einem Epon 1002F Film, einem gebrauchsfertigen Thermoresistmaterial, wurde eine Sensorschicht mit ausgezeichneter Sensitivität (bis zu 15-mal höher Signale im Vergleich zum ungeprägten Referenzmaterial) und Selektivität (vernachlässigbare Kreuzreaktionen mit den getesteten unspezifischen Analyten) entwickelt. Die thermo-geprägten Schichten wurden zu konventionell hergestellten molekularen Oberflächenprägungen mit acrylischen Monomeren in QCM Messungen verglichen.

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1 Aim of the thesis

Sepsis is a very serious condition caused by the dysregulated response of the immune system to an infection triggered by pathogens such as bacteria, viruses or fungi. The gold standard in the diagnosis of sepsis involves the microbiological culturing of blood samples. This standard diagnosis lacks of sensitivity and more importantly it takes a long time (24 – 48 hours) till a first test results are available. Since the early diagnosis essentially contributes to the patient's survival, diagnosis asks for faster and more accurate devices.

The scope of the thesis was to develop a protein microarray for the reliable diagnosis of sepsis that meets the following requirements:

- Simultaneous multiplexed detection of the heterogeneous biomarker panel comprising CRP, IL-6, PCT, E-Selectin and S-100
- Reduction of the sample volume to minimize blood withdrawal essential for neonatal diagnosis
- Internal calibration for a customer-friendly and rapid quantification of the biomarkers
- Improved sensitivity accomplished by high-density labeling for the reliable quantification of low abundance analytes such as IL-6 or PCT
- Fabrication of a surface imprinted sensor layer using ready-to-use UV- or thermal NIL resist materials for the detection of LPS and LTA to allow a fast differentiation of the causative bacterial Gram-type

Publication 1 presents the results regarding the development and miniaturization of a protein microarray for the parallel detection of CRP, IL-6, S-100 and E-Selectin for the diagnosis of neonatal sepsis.

Authors: Patricia Buchegger, Ursula Sauer, Hedvig Toth-Szekely, Claudia Preininger

Title: Miniaturized Protein Microarray with Internal Calibration as Point-of-Care Device for
Diagnosis of Neonatal Sepsis

State: Published in: Sensors (Basel), 2012, Vol. 12, pp. 1494 – 508

In Publication 2, the results related to the development of an integrated on-chip calibration and of a low-sensitive sandwich immunoassay for CRP are presented. Furthermore, four different assay designs that differ in their assay steps, sensitivities and accuracy are evaluated.

Authors: Patricia Buchegger, Claudia Preininger

Title: Four assay designs and on-chip calibration: Gadgets for a sepsis protein array

State: Analytical Chemistry, submitted in January 2014

In Publication 3, the results regarding the fabrication and characterization of molecular surface imprints using UV- and thermo nanoimprinting techniques of the bacterial surface markers LPS and LTA are presented.

Authors: Patricia Buchegger, Peter A. Lieberzeit, Claudia Preininger

Title: Thermo-nanoimprinted biomimetic probe for LPS and LTA immuno-sensing

State: Analytical Chemistry, accepted in January 2014

2 General Introduction

2.1 Sepsis

Sepsis constitutes a deadly threat in intensive care units (ICUs) and arises from the patient's response to bacterial, viral or fungal infections. In Europe, 30 % of all patients staying in ICUs develop severe sepsis. Unacceptably high mortality rates of 27 %, 32 % and 57 % are reported for patients suffering from sepsis, severe sepsis and septic shock [1]. A review from 2009 [2] characterizes the epidemiology of sepsis referring to 11,000 patients from 37 countries. From the collected data, 89 % of the infections were due to bacterial invasions and 11 % were due to fungal infections.

2.1.1 Definitions

Till 1991, no precise definition of sepsis was available and many terms, like infection, septicaemia, bacteraemia and sepsis syndrome were synonymously used which often led to confusions among physicians and researchers. Therefore, a consensus conference from the American College of Chest Physicians and the Society of Critical Care Medicine was held with the aim to agree on a set of definitions for the clear distinction between systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis and septic shock [3]. Accordingly, SIRS is defined as an acute response to trauma, burn or infectious lesions and is diagnosed by the occurrence of at least two of the four conditions: body temperature above 38°C or below 36°C, heart rate greater than 90 beats per minute, respiratory rate above 20 per minute or arterial carbon dioxide tension ($P_a\text{CO}_2$) below 32 torr and white blood cell count above 12 000 cells/mm³ or below 4000 cells/mm³. If SIRS is triggered by pathogenic microorganisms, patients are categorized as sepsis patients. Severe sepsis is manifested when in addition organ dysfunctions, hypoperfusion or hypotension appears. Severe sepsis further develops to a septic shock when an acute circulatory failure arises.

2.1.2 Pathogenesis

Sepsis emerges from the complex interactions between the invading pathogens and the maladapted systemic immune-response. The immune-response of the human body to infecting organisms is triggered by pathogen-related compounds, like lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan or flagellin. Those so-called pathogen associated molecular patterns (PAMPs) are recognized by the host's pattern-recognition receptors (PRRs) like toll-like receptors or lectin receptors. Binding of the PAMPs to the PRRs triggers the immune-response of the innate immune system as a first line of defense. This initial response leads to the unspecific release of various pro-inflammatory mediators including Tumor necrosis factor alpha (TNF alpha), Interleukin 6 (IL-6) and Interleukin 1 beta (IL-1b) [4]. This phase is followed by the release of complementary anti-inflammatory compounds such as IL-4 and IL-10 in order to restore the immune homeostasis and to prevent collateral immune-mediated host tissue damage. In septic patients, the immune-responses

are very intensive, unregulated and elongated, thus prevent the recovery of the immune homeostasis which leads to tissue destruction and moreover to organ failure [5].

2.1.3 Diagnosis & the role of biomarker

Microbial blood culture constitutes the gold standard in the diagnosis of sepsis. This method severely suffers from the delayed availability of test results and moreover from the low sensitivity. Due to the long incubation times, blood cultures deliver first test results not until 24 to 48 hours have passed. Furthermore only 20 – 40 % of septic blood samples can be classified as such [6], [7]. The vast number of false-negative blood culture events proceeds from the only occasionally occurring pathogenic invasion of the blood stream. And yet, the early-stage diagnosis of sepsis has a tremendous impact on the patients' survival rates since sepsis can be cured by the early administration of antibiotics.

Substantial research has been done in the field of sepsis diagnosis to address the needs for faster and more accurate diagnostic tools. Several groups developed real-time multiplex PCR systems for the identification of the causative pathogens from blood samples [8]. Renowned providers of real-time PCR devices are for example Roche Diagnostics (Rotkreuz, Switzerland), who developed the LightCycler® SeptiFast, or Seegene (Seoul, South Korea), who offer the MagiPLEX™ Sepsis Screening Test in their product portfolio. The usage of real-time PCR yields 1.5 to 2-fold increased true-positive diagnoses compared to the conventional blood culture test [9]. Nevertheless, these devices suffer from several drawbacks since positive results again demand for the microbial invasion of the blood stream. Additionally, since this method is DNA based, it fails to distinguish between transient and replicating microorganisms which often leads to misleading results. Furthermore, the assay has a relative high detection limit of 30 – 100 CFU /mL, hence is not sensitive enough for clinical purposes where the detection of <10 CFU/mL is required [10].

Methods such as microbial blood culturing or real-time PCR are based on the verification of the causative pathogen. Alternatively, host biomarkers are exploited for the diagnosis of sepsis. Various laboratory markers have been evaluated for their potential use to accurately determine, rule out or monitor sepsis. In the 1980s, the pro-inflammatory cytokines TNF alpha, IL-1 β and IL-6 as well as

CRP (C-reactive protein) have been investigated as potential biomarkers for the diagnosis of sepsis. In the 1990s, PCT (Procalcitonin) emerged as another potential biomarker [11]. In 2003, CRP, IL-6, TNF alpha and PCT were selected as recommended diagnostic biomarkers at the consensus conference of sepsis [12].

In 2010, Vincent and Pierrakos published a review on sepsis biomarker [13]. Out of 178 potential sepsis biomarkers including growth factors, cytokines and inflammation- and coagulation factors, only two, namely CRP and PCT, are routinely used in clinical practice. Despite the high potential of many biomarkers, only their combinatorial use will be sufficient in terms of specificity and sensitivity for the accurate diagnosis. [14].

Figure 1 shows the kinetics of the sepsis markers IL-6, IL-10, TNF alpha, PCT and CRP after endotoxin administration in human volunteers [15]. As a first response, the pro-inflammatory cytokines IL-6, IL-10 and TNF alpha are excessively released and their serum levels peak two hours after the initial endotoxin challenge. The elevated IL-10 and TNF alpha levels re-reach physiological values after 24 hours. IL-6 shows a similar kinetic behavior but remains slightly elevated. The PCT values begin to increase only 2 hours after the initial endotoxin exposure and peak after 12 to 24 hours and again decrease after 48 hours whereas the CRP concentrations start to rise after 6 hours and peak after 24 to 48 hours and remain elevated for at least 72 hours. This study of the biomarker impressively demonstrates the vital necessity of a heterogeneous biomarker pool to cover the entire period of time to ensure accurate and reliable diagnosis of sepsis at any time.

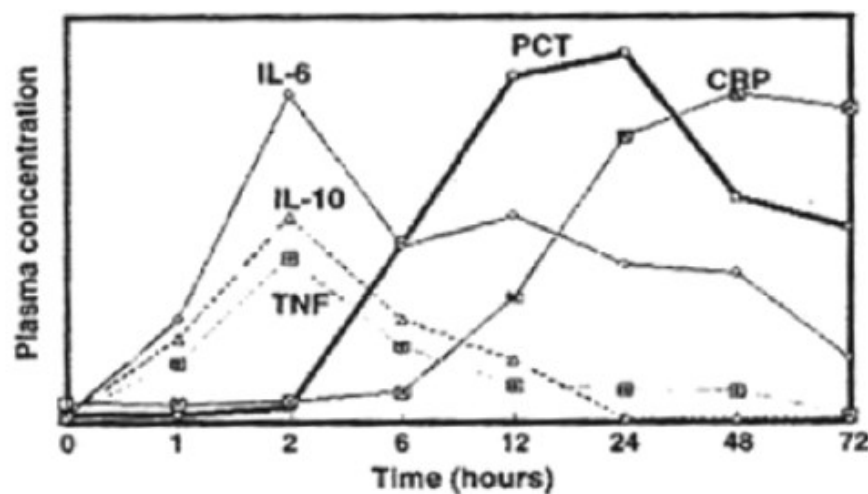


Figure 1: Kinetics of several sepsis biomarker after endotoxin exposure in human volunteers [15]

Several studies on the combinatorial usage of biomarkers have been published recently. For example, Kofoed and colleagues [16] examined the usage of a biomarker panel comprising of six sepsis biomarkers (suPAR, sTREM-1, MIF, CRP, PCT, neutrophils). The area under the receiver operating curve (AUC) was used for comparison of the respective single and combinatorial performances. The AUCs for the single analytes were between 0.5 for suPAR and 0.81 for CRP and improved to 0.88 when using the six markers in combination concluding that the summarized information derived from several markers improves the diagnostic accuracy.

Similarly, Irwin and coworkers demonstrated that the combinatorial use of NGAL, resistin and PCT improves the sensitivity in the diagnosis of serious bacterial infections. Particularly, areas under the curve (AUCs) improved from 0.79, 0.80 and 0.86 to 0.90 [17].

In the present thesis, a multiplex protein microarray was developed for the simultaneous detection of the proposed biomarkers IL-6, CRP, PCT, S-100 and E-Selectin that serve in combination as a specific and accurate biomarker panel for the early diagnosis of sepsis. The cut-off values of the respective biomarkers are outline in Table 1.

Table 1: Clinical cut-off values of the respective biomarkers

Biomaker	Clinical cut-off value	Biomaker	Clinical cut-off value
IL-6 (pg/mL)	31 [18]	S-100 (ng/mL)	10.4 [19]
PCT (ng/mL)	0.5 [20]	E-Selectin (ng/mL)	174 [18]
CRP (µg/mL)	10 [19]		

2.1.4 Bacterial surface marker

60 % of all sepsis cases are caused by bacteria. The incidences of Gram-positive and Gram-negative sepsis are balanced. Table 2 summarizes the most common Gram-negative and Gram-positive bacteria that cause sepsis.

Table 2: Most common bacterial pathogens that cause sepsis

Gram-positive Bacteria	Gram-negative Bacteria
<i>Staphylococcus aureus</i>	<i>Escherichia coli/Shigella sp</i>
<i>Staphylococcus epidermidis/capitis/caprae</i>	<i>Salmonella enteritidis/typhimurium</i>
<i>Streptococcus pneumonia</i>	<i>Klebsiella pneumonia</i>
<i>Streptococcus milleri group</i>	<i>Klebsiella oxytoca</i>
<i>Streptococcus pyogenes</i>	<i>Proteus mirabilis</i>
<i>Enterococcus faecalis</i>	<i>Serratia marcescens</i>
<i>Enterococcus faecium</i>	<i>Enterobacter cloacae/aerogenes</i>
Group B <i>Streptococcus</i>	<i>Pseudomonas aeruginosa</i>
	<i>Bacteroides fragilis</i>

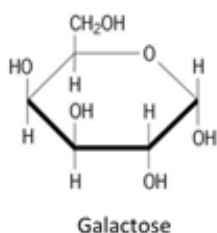
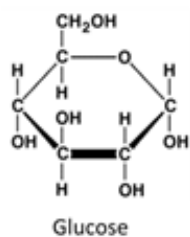
Gram-staining is a standard method in clinical and research practice and used to differentiate between Gram-positive and Gram-negative bacteria. This staining process involves the incorporation of the primary stain (crystal violet) that colors Gram-positive bacteria blue/violet and Gram-negative bacteria pink/red. This difference in color is due to the different chemical properties of the respective cell walls. Gram-positive cell walls are composed of a thick peptidoglycan layer (up to 95 % of the whole cell wall content) whereas the Gram-negative cell wall contains only a thin peptidoglycan layer (up to 10 %).

Gram-negative and Gram-positive bacteria do not only differ in their peptidoglycan content but can be also distinguished from each other by their surface markers: lipopolysaccharide (LPS) is specifically expressed on Gram-negative and lipoteichoic acid (LTA) specifically expressed on Gram-positive cell surfaces.

LPS and LTA are immunogenic molecules that trigger the response of the immune system during pathogenic invasion. Although LPS and LTA induce different patterns of cytokine release in the course of an infection, the clinical symptoms do not differ between Gram-negative and Gram-positive sepsis. Due to the different cytokine expression activation, the identification of the causative microorganism Gram-type may support clinicians to guide antibiotics medication and therapy.

LPS and LTA are both amphiphilic molecules with a hydrophobic lipid anchor and a hydrophilic polysaccharide moiety.

Hexoses:



Dideoxy sugars:

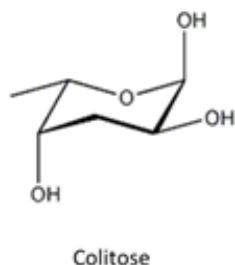
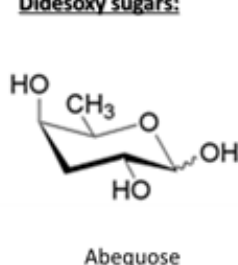


Figure 2: Most common sugars found in the O-Antigen of LPS

Particularly, LPS constitutes of 3 parts: the lipid part is called lipid A and is highly conserved among many species. It consists of several fatty acids that are linked to the so-called polysaccharide core which is partly conserved among few species. The external polysaccharide part that faces the environment is called O-Antigen. This part is highly variable and consists of hexoses, such as Glucose, Galactose and Mannose and dideoxy sugars, such as Abequose,

Colitose and Paratose (see Figure 2).

LTAs are polyanionic polymers and are responsible for the positive surface charge of Gram-positive bacteria [21]. The hydrophilic backbone consists of repetitive glycerophosphate units with D-alanine, Glucose, Galactose or N-Acetylglucosamine substituents. The hydrophobic lipid tail contains diacylglycerol (see Figure 3).

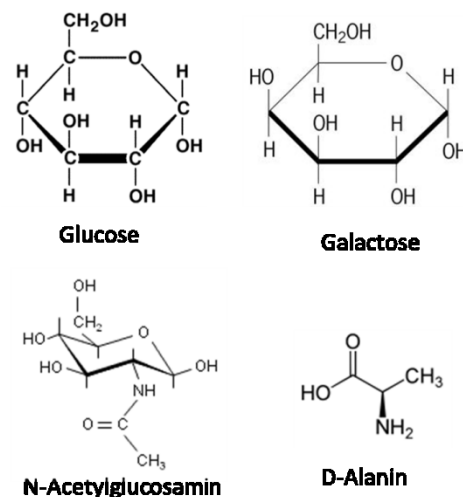


Figure 3: Most common compounds found in the outer moiety of LTA

LPS and LTA molecules are released from the bacterial cell mainly in the course of bacterial growth or cell death. Due to

their hydrophobic and hydrophilic moieties several LPS and LTA molecules form aggregates in solution. In a physiological environment (pH 7.2) micellar structures of approximately 20 – 30 nm are formed [22], [23].

2.2 Protein microarrays

Microarrays, also commonly referred to as biochips, comprise collections of biomolecules spatially immobilized on a solid platform that allow the simultaneous accomplishment of several analyses. Due to the outstanding properties regarding miniaturization, low consumption of materials,

parallelization, high-throughput and sensitivity, microarrays have been widely applied in various research fields. DNA microarrays emerged from DNA southern blot technology. This technique was further expanded and the first miniaturized microarray for gene expression profiling was introduced in 1995 [24]. Nowadays, genomic research cannot be imagined without DNA microarrays since they boosted large-scale genome wide screenings. DNA strands, for instance short oligonucleotides (15 – 25 nucleotides), long oligonucleotides (50 -120 nucleotides) or PCR products serve as capture molecules to target complementary DNA strands from a biological sample.

The sweeping advances of DNA chips and to a greater degree upcoming issues that went beyond genomic questions encouraged the transition of DNA microarrays to protein chips, thus realized the step from genomics to proteomics. Unlike DNA strands, proteins severely deviate from each other since they have different and rather complex tertiary or quaternary structures and a wide dynamic concentration range. Additionally, proteins often contain posttranslational modifications, such as introducing glycosyl- or phosphate groups. The fragile native structures as well as the modifications are prone to oxidation and other environmental influences which challenges the stable and operative immobilization of proteins [25].

Depending on the scientific question, protein microarrays can be accomplished as functional or analytical protein arrays.

Functional protein arrays, also referred to as target protein arrays, are mainly applied in basic research to support screening of large quantities of samples in parallel. Therefore, a large variety of proteins are immobilized to identify biomolecular interactions of the whole proteome such as protein-protein, protein-DNA, protein-RNA, protein-phospholipid or protein-small molecule interactions. Functional protein arrays found increasing popularity as high-throughput formats for drug discovery [26] and biomarker discovery [27].

Analytical protein arrays, also referred to as capture arrays, are applied to screen samples to specifically detect ligands of interest frequently via immunoassays and found applications in diagnosis and disease monitoring. Especially in the field of medical diagnostics, protein microarray

technologies are on the upgrade but yet failed to successfully establish in routine diagnostics since sensitivity, selectivity and reproducibility are not yet satisfactory.

2.3 Development of protein microarrays

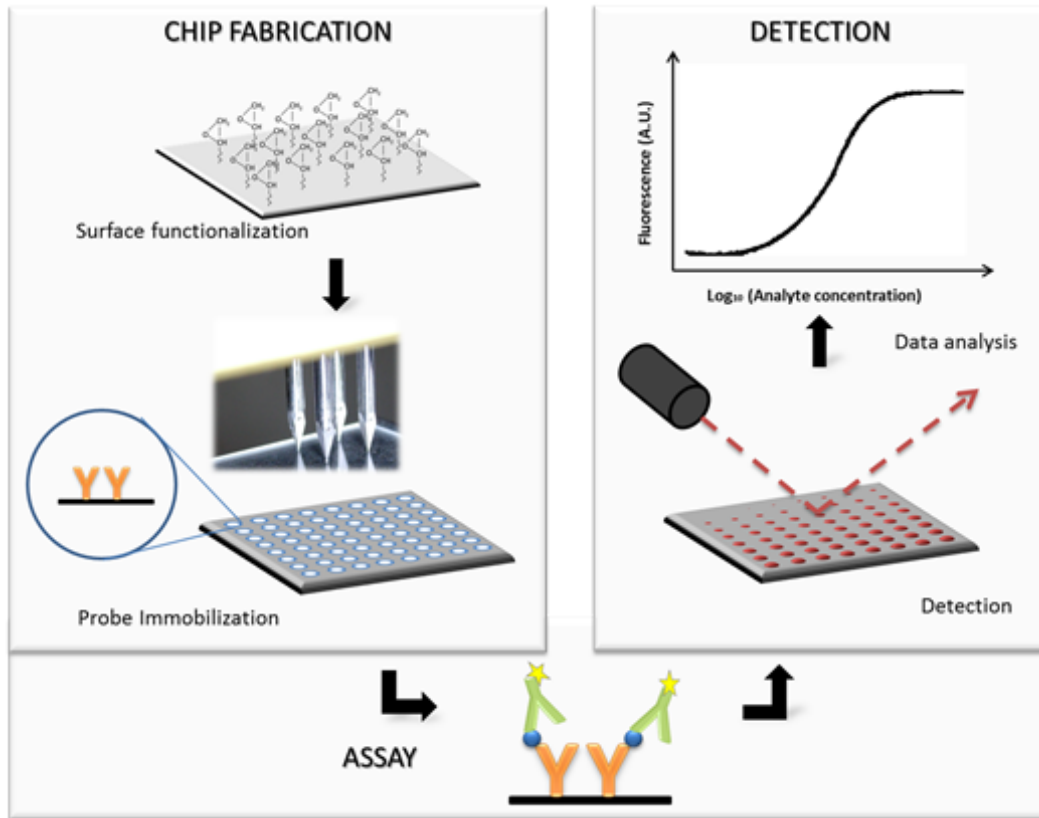


Figure 4: Process flow for the development of protein microarrays

The development of protein microarrays comprises several key steps including:

- Surface functionalization
- Probe immobilization
- Assay
- Detection
- Data analysis

Each step has to be optimized and adapted according to the respective assay requirements and the underlying analytical question. The following sections present the state-of-the-art of the techniques and methodologies applied for the development of protein microarrays.

2.3.1 Microarray printing

The process of transferring the probe to a solid substrate is referred to as microarray printing. Miniaturized features in micro- and even nanoscale are of outstanding importance for protein microarrays to ensure the multiplexed detection, high-throughput and enhanced sensitivity [28]. Sophisticated printing technologies emerged for the delivery of very low sample volumes. Generally, printing techniques can be categorized as “contact printing” and “non-contact printing” methods.

2.3.1.1 Non-contact printing

The delivery of the probe spots via non-contact printing occurs through dispersal of small sample volumes. Prominent examples for non-contact printing are ink-jet printing devices based on piezoelectricity that originated from the ink-jet printing industry. Therefore, the samples are transferred to the end of tiny capillaries and the respective ejection is triggered by a piezoelectric impulse. These devices are particularly advantageous when fragile molecules are transferred as the non-contact dispersal is a gentle delivery system.

2.3.1.2 Contact printing

Contact printing devices transfer the probes to the solid support via direct contact. The most prominent and automatized contact printing method is contact pin printing.

In contact pin printing, also referred to as spotting, high definition pins that deliver the sample through capillary forces are used. Frequently, solid pins or split pins are applied (Figure 5). Solid pins are very robust steel pins but require re-dipping after each deposited spot which makes their employment rather time-consuming. Split pins constitute a more efficient alternative. As shown in Figure 5b, split pins harbour a small channel that serves as probe reservoir allowing the repeated consecutive probe transfer without the need for re-dipping in between. One disadvantage of split pins is their susceptibility to clogging.

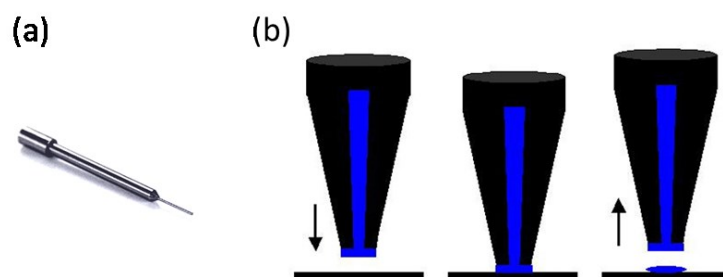


Figure 5: (a) solid pin; (b) principle of probe deposition using split pins (www.arrayit.com)

The diameter of the deposited features varies between 50 μm and 500 μm depending on the wettability properties of the solid substrate, the particular print buffer composition, humidity during printing and on the pin sizes. Typically, picoliter to low nanoliter volumes are deposited during contact pin printing. The deposition of such low volumes results in a large surface-to-volume ratio. Therefore, evaporation occurs immediately after deposition and needs to be controlled in order to minimize accompanying destabilizing drying effects such as changes in solute concentration, ionic strength, pH and surface energetic that alter the stability and activity of proteins [29]. Different print buffer additives have been evaluated in literature to maintain the water content of the proteins intended to preserve their native structure. Hydroxylated additives such as trehalose, sucrose, glucose, poly(ethylene glycol) and glycerol are reported to increase the stability of the immobilized probes. Hydrogen bonds between water and the hydroxyl groups keep the proteins hydrated. Hydrophobic interactions further stabilize the native form of the proteins. Lee and researchers demonstrated that the addition of more than 10 % PEG 200 significantly improves the protein stability [30]. A similar effect was shown by the addition of glycerol to the printing buffer [31]. Nevertheless, several researchers reported that the addition of glycerol leads to decreased signal intensities [32]. Additionally, detergents such as Tween 20 are often added to the print buffers in order to avoid protein aggregations [33]. In [34], the addition of zwitterionic detergents such as sodium deoxycholate or CHAPS yielded promising results regarding protein stability as they are less denaturing but also prevent undesired intermolecular interactions.

2.3.2 Capture elements

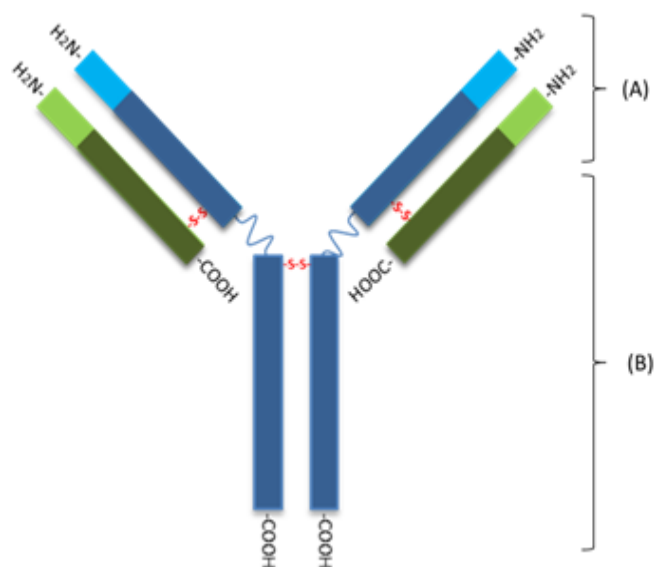
A large variety of receptors such as antibodies, aptamers, proteins, peptides and small organic molecules can be exploited for the specific recognition of target analytes.

The choice of the capture element depends on the particular analytical question. Besides the classical biological recognition elements, artificial biomimetic probes, like molecularly imprinted polymers (MIPs), have been recently exploited as capture elements in immunosorbent assays.

2.3.2.1 Antibodies

Antibodies constitute the most frequently applied recognition elements in analytical protein arrays due to their high affinities and to the well-established assay protocols. Antibody arrays may consist of monoclonal or polyclonal antibodies, antibody fragments or synthetic polypeptide ligands [35].

Antibodies, also referred to as Immunoglobulins (Ig), are large glycoproteins with a molecular mass of approximately 150 kDa and belong to the immune system where they identify and neutralize foreign molecules. Igs are produced and secreted by B-cells and can be divided into five main isotypes called IgM, IgG, IgA, IgD and IgE. Each isotype harbors different biological properties and plays a different role in the course of an immune response. 73 % of the circulating Immunoglobulins are of class G as they are the principal agents against invading pathogens. Due to their high



abundance after immunization, excellent target affinities (K_D in the lower nM range) and good stability during purification, IgGs are most frequently used in immunoassays. Figure 6 schematically depicts the typical Y-shaped structure of IgGs. It consists of 4 polypeptide chains that are covalently linked via disulfide bridges: 2 identical heavy chains (blue) and

Figure 6: General IgG structure

2 identical light chains (green). IgGs consist of a constant region at the lower two-thirds (B) and a variable region at the upper end (A). The variable region composes of 110 – 130 amino acids and is responsible for the distinct specificities of IgGs to distinct antigens. One IgG can bind two identical antigens; in fact one per arm. This antigen recognition region is also referred to as Fab (fragment antigen binding) Fragment. The constant region is called Fc region and can be cleaved with a protease to obtain Antibody Fab fragments [35].

Antibodies are produced by injecting the antigen, mostly proteins or polysaccharides, into a mammal. As the antigen is foreign, the immune system of the mammal gets activated and induces the B-cells to produce antigen-specific antibodies that are secreted into the circulation.

Polyclonal antibodies are purified from the mammal's serum containing a mixture of antibodies that show specificity to the same antigen but are derived from different B-cells, thus exhibit distinct epitope specificity.

In contrast, monoclonal antibodies are derived from clones of the same B-cell and show specificity to the same epitope. Therefore, B- cells are isolated and fused with fast-growing myeloma cells to form so-called hybridomas. After cultivation of the hybridoma cells, the monoclonal antibodies can be harvested from the respective media supernatant.

Generally, polyclonal as well as monoclonal antibodies are purified using prominent purification techniques such as size-exclusion chromatography, Protein A/Protein G chromatography or immunoaffinity chromatography [35].

To sum up, polyclonal antibodies are easier, faster and cheaper generated but suffer from limited specificity and yield. Therefore, monoclonal antibodies that exhibit higher sensitivities are preferably used in immunoassays.

2.3.2.2 Aptamers

Aptamers are the synthetic counterparts of the naturally occurring antibodies and compose of single-stranded DNA or RNA molecules that are selected *in vitro* for their binding properties to a target analyte. The *in vitro* selection process is called SELEX (systematic evolution of ligands by

exponential enrichment) and was independently developed by three laboratories in the 1990s [36], [37], [38]. Figure 7 shows the scheme of one SELEX cycle: First, (i) the target analyte is incubated with a pool consisting of 3D folded random nucleic acids, (ii) followed by the removal of unbound sequences and (iii) elution of the bound sequences that are (iv) finally re-amplified via PCR. In order to obtain highly selective and affine aptamers, this cycle is typically repeated 6 to 18 times [39].

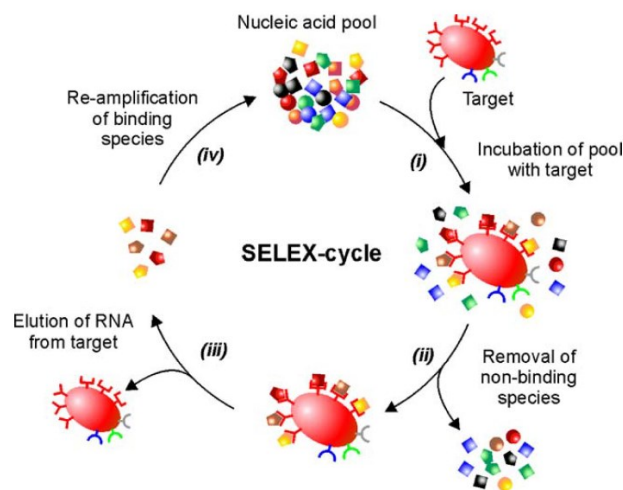


Figure 7: Schematic depiction of the SELEX process (taken from [39])

Similar to antibodies, the affinity constants of aptamers are in the low nanomolar range. The properties of aptamers can be adjusted and modified as desired, which is especially useful for applications in non-physiological conditions. Furthermore, aptamers can be developed against small molecules or ions that exhibit low or no immunogenicity whereas the development of specific antibodies is limited to immunogenic compounds.

Nevertheless, due to the large variety of analytes the selection process has to be adapted for each analyte which makes them rather expensive. Furthermore, stability problems may occur when using aptamers as capture elements in biosensors as they get easily degraded by nucleases.

2.3.2.3 Small organic molecules

In literature, only few reports on analytical protein chips that use low molecular weight molecules as capture elements for the specific detection of target analytes are available since low molecular weight analytes typically exhibit significantly lower binding affinities compared to antibodies, aptamers or MIPs.

For functional protein microarrays the situation is different: Here small organic molecules are frequently immobilized to study interactions with proteins to identify potential pharmaceutical compounds or drug targets. Especially in protein function profiling studies, small molecule microarrays are widely applied as they constitute an appreciated high-throughput tool [40].

2.3.2.3.1 Phosphocholine-CRP binding

CRP is a pentamer consisting of 5 identical subunits of 23 kDa each that are non-covalently linked. Each subunit has two Ca^{2+} binding sites and one binding site for phosphocholine (Figure 8). The binding of phosphocholine to CRP is Ca^{2+} dependent and occurs with rather low affinity ($K_D = 5 \mu\text{M}$) at a physiological Ca^{2+} concentration of 10 mM [41]. By comparison, antibodies usually have K_D s in the lower nM range. Although antibody affinities are significantly higher, the specific phosphocholine-CRP binding constitutes an alternative and interesting approach for the CRP detection. In this work, especially the rather low but specific binding affinity between CRP and phosphocholine is advantageous and exploited for the development of a low-sensitive sandwich assay. Since CRP occurs at rather high serum concentrations, the weak binding affinity allows the simultaneous detection of CRP with low abundance analytes.

As mediator of the complement system, CRP binds pneumococcal C-polysaccharide and other bacterial polysaccharides that contain phosphocholine. Besides phosphocholine, CRP binds to several biologically important ligands including O-phosphorylethanolamine, histones and polycations. Table 3 shows a selection of publications of recently developed analytical assays that exploit low molecular weight analytes instead of antibodies for the specific detection of CRP.

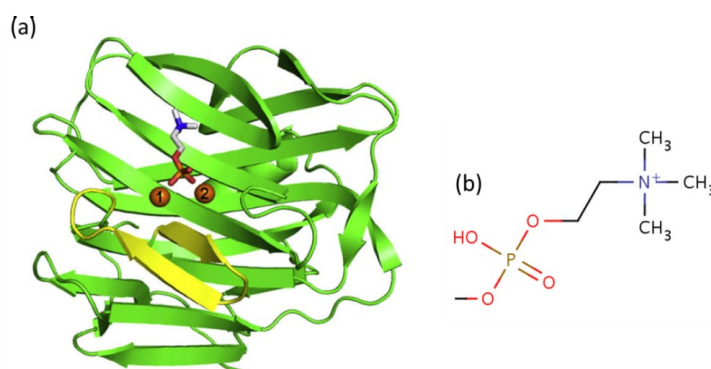


Figure 8: (a) Structure of CRP (PDB ID: 1B09) showing two bound Ca^{2+} ions (orange) as well as bound phosphocholine; (b) chemical structure of phosphocholine

Table 3: Assays using CRP ligands for the specific recognition

Reference	Analytical method	Capture element	Immobilization
[42]	Fluorescence-quenching assay	O-Phosphorylethanolamine	Coupled to Quantum dots
[43]	Sandwich ELISA	Pneumococcal C-polysaccharide	No coupling, Physically adsorbed
[43]	Sandwich ELISA	<i>P</i> -aminophenyl phosphorylcholine	Coupled to BSA
[44]	Competitive ELISA	Cytidine 5'-diphosphocholine	Coupled to BSA
[45]	Sandwich ELISA	Cytidine 5'-diphosphocholine	Coupled to Dendrimers

2.3.2.4 Molecularly imprinted polymers

Molecularly imprinted polymers (MIPs) using silica matrices were first introduced by Dickey et al. in 1949 [46]. From that point on, the field of molecular imprinting has evolved remarkably and aroused a great deal of interest in the scientific community and in the industry. Nowadays, several companies such as MIP technologies AB (Sweden) or POLYIntell (France) provide customized molecularly imprinted polymers.

MIPs are synthetic polymers that feature selectivity and specificity towards the imprinted target analyte. That is why they are often referred to as artificial or plastic antibodies. MIPs exhibit several advantages over their biological counterparts since they are simply and fast prepared, reveal high thermal and mechanical stress tolerance and have excellent storage stabilities.

Two major strategies based either on covalent or non-covalent interactions can be applied for the fabrication of MIPs. Additionally, there exist several hybrid techniques like semi-covalent imprinting that try to combine the advantages of both strategies for the development of an advanced imprinting technique. The key principle is the same for all approaches: MIPs are fabricated by the co-polymerization of monomers, crosslinkers, initiators and the template molecules. After elution of the template, binding sites that exhibit chemical and steric complementarity to the target analyte are obtained.

In covalent imprinting, the target molecule is covalently linked to the monomers frequently via ester bonds. This strategy generates highly selective recognition sites with excellent sensitivity accomplished through the high output of homogeneous binding sites [47]. Nevertheless, the use of covalent imprinting is restricted since their fabrication is very time-consuming and complex as it requires the formation of specific reversible covalent linkages.

The non-covalent imprinting approach was pioneered by Mosbach and Arshady in 1981 [48]. In that case, the target recognition is exclusively realized by the formation of non-covalent interactions between the monomers and the template molecule. This strategy is more versatile, easier to accomplish and has been applied for a large variety of analytes. One major drawback of this strategy is that it usually results in highly heterogeneous binding sites. The monomers contain functional groups and self-assemble around the template molecule through non-covalent forces. This interplay between various functional groups is fixed by the polymerization of the monomers induced by the initiator and carried out by the crosslinker. The rebinding process is similar to the naturally occurring host-guest interactions during antibody-antigen or enzyme-substrate binding following the key-lock principle. Hence, rebinding occurs by the reconstruction of the non-covalent interactions including hydrogen bonding, van der Waals, ionic, electrostatic, dipole-dipole and hydrophobic interactions. These weak interactions in their entirety mediate the specificity and selectivity of the imprints. The choice of the functional monomers and crosslinkers is therefore crucial for target selectivity.

Generally, acidic functional monomers are preferentially used for the imprinting of basic template molecules and vice versa. The most commonly used functional monomer is methacrylic acid due to the amphoteric structure which allows it to act as both as Lewis acid and base and as hydrogen donor and acceptor and furthermore the involvement in ion-ion or dipole-dipole interactions. Besides methacrylic acid, acrylic acid is another commonly used acidic functional monomer. 4-vinyl pyridine constitutes the most commonly applied basic monomer. The selectivity of the recognition sites can be further tuned by the incorporation of neutral monomers like for example acrylamide or methyl methacrylate or by the introduction of alkyl chains to support hydrophobic interactions.

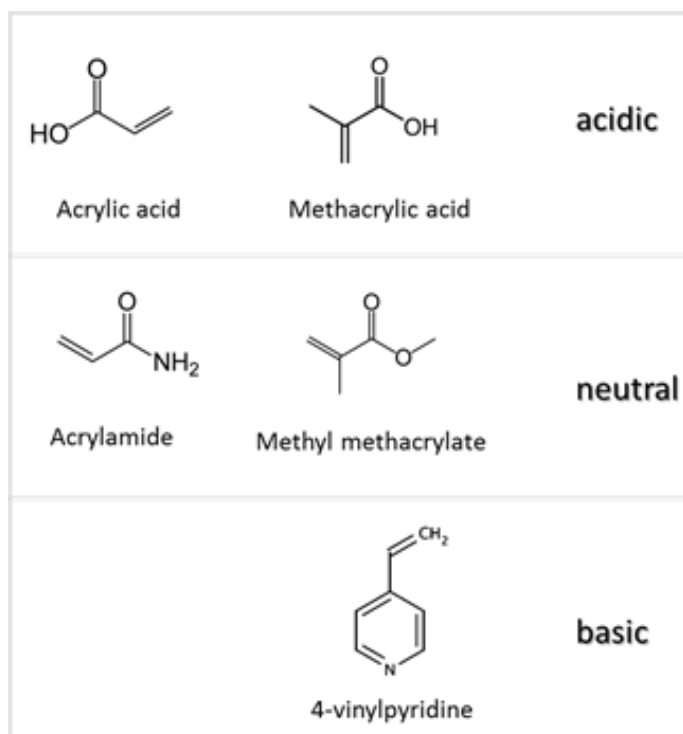


Figure 9: Frequently used functional monomers for the development of non-covalent imprints

Besides the selection of the appropriate functional monomers, the choice of the crosslinker plays a critical role in the development of molecularly imprinted polymers since it stabilizes the polymer network including the target recognition sites. For the imprinting of low molecular weight analytes, a high crosslinking degree of up to 95 % is aspired. This is frequently accomplished using ethylene glycol dimethacrylate (EGDMA) [49].

Additionally, the molar ratios of the ingredients as well as the choice of the polymerization reaction, the solvent and the reaction temperature have to be considered during MIP preparation.

Besides the chemical makeup, the structural integrity of the template molecule during the imprinting process has to be considered since the empty cavities exhibit strong shape-memory. Therefore, it is important to maintain the native structure of the imprinted template during the whole imprinting process. The native structure is very prone to temperature, pH and the solvents used during the imprinting process.

The field of artificially created antibodies has enormously evolved during the last decades giving them the potential of replacing antibodies. MIPs found applications in various analytical applications such as solid phase extraction [50], chromatography [51], catalysis [52] and molecularly imprinted

sorbent assay [53]. A large number of low molecular weight analytes has been successfully imprinted so far advancing techniques such as separation, sensing and drug delivery. Nevertheless, the fabrication of macromolecularly imprinted polymers is complex due to the increased complexity of macromolecules.

2.3.2.4.1 Macromolecularly imprinted polymers

Imprinting of macromolecules is challenging and differs from imprinting of low molecular weight analytes due to their increased sizes, complex native structures and their susceptibility to non-physiological conditions. For the specific rebinding of small analytes, few strong interactions are required. Differently for large molecules: Here the rebinding capabilities of the MIPs are accomplished through a large variety of weak interactions.

Much effort has been undertaken to develop macromolecularly imprinted polymers. Four different approaches are introduced in literature for the preparation of macromolecular imprints (Figure 10):

- Bulk imprinting
- Particle polymerization
- Epitope imprinting
- Surface imprinting

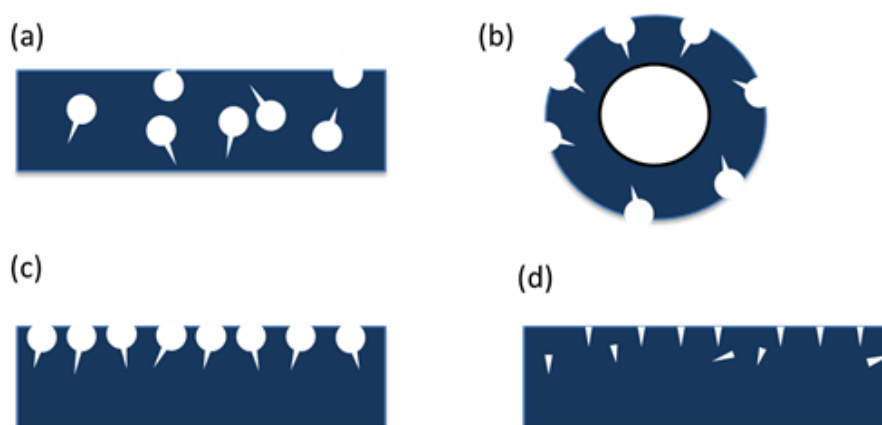


Figure 10: Schematic depiction of the most frequent approaches to create macromolecularly imprinted polymers (a) molecular bulk imprinting, (b) molecular particle imprinting, (c) molecular surface imprinting and (d) molecular epitope imprinting

2.3.2.4.1.1 Bulk imprinting

Bulk polymerization is most commonly used for the imprinting of low molecular weight analytes as it results in a relatively dense network. Bulk polymerization is realized by the simple co-polymerization of the monomers, the crosslinker, the initiator and the template molecules. After polymerization the template is washed out leaving template-specific but very heterogeneous cavities in a highly cross-linked 3D network. Nevertheless, bulk imprinting is also applied to create macromolecularly imprinted polymers. Therefore, the cross-linker density is especially critical since high amounts restrict the mobility of the molecule whereas low amounts lower the re-binding capabilities of the imprinted sites. [54], [55], [56].

2.3.2.4.1.2 Particle polymerization

The particle polymerization approach is derived from the surface imprinting approach. Here, the template is covalently linked to the surface of particles like silica, gold or magnetic particles. A polymer network is allowed to envelop the particles. After polymerization the template is washed away leaving complementary cavities in the shell of the particles [57], [58], [59].

2.3.2.4.1.3 Epitope imprinting

Epitope imprinting constitutes another approach that is widely used for the preparation of macromolecularly imprinted polymers. Therefore, a short structural fragment that represents a larger molecule is exploited for imprinting during polymerization. The small target-representative analyte is mostly easier removed from the polymer than the whole macromolecule. Additionally, by using one only representative epitope the resulting sensor layer exhibits a monoclonal antibody-like nature. To ensure high selectivity of the imprints to the target molecule the selection of the epitope to be imprinted is critical and depends on the uniqueness of the sequence [60], [61].

2.3.2.4.1.4 Surface imprinting

The so-called molecular surface imprinting approach constitutes the most widely used technique in the preparation of macromolecularly imprinted polymers. Approximately 60 % of all publications related to macromolecular imprinting use the surface imprinting approach [62]. The template is adsorbed on the surface of a planar stamp that is subsequently brought into contact with a thin prepolymerized film. After completion of the polymerization, the stamp is removed leaving template-specific 2D cavities on the surface of the polymer film (Figure 11). The surface imprints have high mechanical stability and are compatible with many sensor platforms. Molecular surface imprinting prevents analyte entrapment and is therefore suited for many molecules regardless of their size.

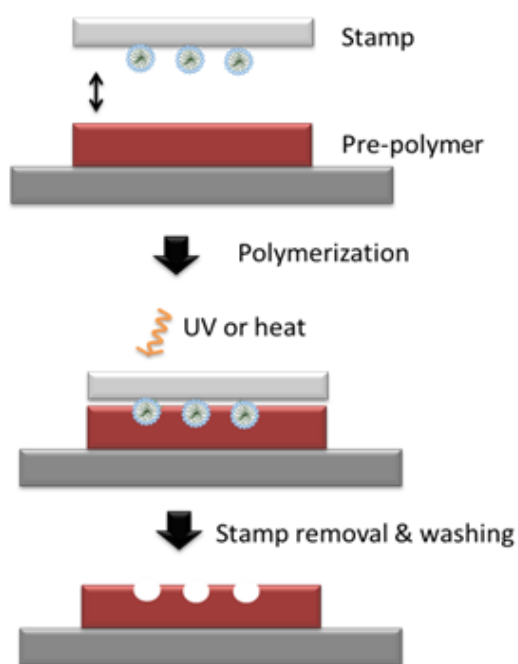


Figure 11: Fabrication of molecular surface imprints

The polymers used are frequently the same as in the bulk polymerization approach. 60 % of the monomers used for imprinting are acrylamide, methacrylic acid, aminophenylboronic acid, acrylic acid and N-isopropylacrylamide. The most common cross-linkers are methylenebisacrylamide (MBA) and ethylene glycol dimethacrylate (EGDMA).

Table 4 summarizes recent research that was published on molecular surface imprinting, also referred to as “protein stamping” or “microcontact” approach.

Table 4: Selected examples for macromolecularly imprinted polymers

Reference	Application	Analyte	Monomers	Crosslinker	Initiation
[63]	Spectroscopy	BSA	2-(dimethylamino)ethyl methacrylate,	N,N-methylenebisacrylamide, PEG4000DMA	UV
[64], [65]	Microcalorimetry	Lysozyme, RNAse A, Myoglobin	Methacrylic acid, hydroxyethylmethacrylate, dimethylaminoethyl methacrylate	EGDMA, TEGDMA, PEG400DMA or PEG600DMA	UV
[66]	ELISA	CRP	O-(4-nitrophenylphosphoryl)choline	EGDMA	UV
[67]	ELISA	RNAse A	Styrene	PEG400DMA	UV
[68]	MS	Myoglobin	methyl methacrylate	TEGDMA	UV
[69]	ELISA	Kreatin Kinase	Methacrylic acid	PEG400 DMA	UV
[70]	ELISA	Ovalbumin	methacrylic acid	PEG400 DMA	UV
[71]	QCM	ribonuclease A	2,2,3,4,4,4-hexafluorobutylmethacrylate, N-methacryloyl-histidine	trimethylolpropane trimethacrylate	UV
[72]	QCM	Lysozyme	methyl methacrylate	trimethylolpropane trimethacrylate	UV

2.3.3 Immobilization strategies

One key feature in the development and optimization of microarrays is the method of probe immobilization to ensure the activity and proper orientation of the same. Assay performance strongly depends on the accessibility of the binding sites and demands for the conserved native structure of the immobilized probes. In order to retain the biological functionality, accessibility and additionally the longevity of the immobilized proteins, several immobilization strategies were investigated in the past: physical, bioaffinity and covalent immobilization [73]. The choice of the immobilization strategy depends on the chemical and structural properties and of the desired orientation of the immobilized probes.



Figure 12: Schematic depiction of the various immobilization strategies: (a) physical, (b) bioaffinity and (c) covalent attachment

2.3.3.1 Physical adsorption

Physical immobilization is based on the adsorption of proteins on surfaces via intermolecular forces such as electrostatic forces and hydrophobic or polar interactions. Probe adsorption is frequently used in ELISA and western blot technologies. Due to its simplicity, adsorption is also frequently applied in protein microarrays especially for the immobilization of large proteins. Therefore, various surface materials are available to support the effective adsorption such as nitrocellulose [74], poly-L-lysine [75] or polyvinylidene fluoride (PVDF) [76]. Nevertheless, adsorption suffers from several drawbacks like the random orientation of proteins which may result in masked active sites or protein denaturation that often occurs as a result of hydrophobic interactions which further leads to the loss of the biological functionality. Another disadvantage of physically adsorbing the probes arises from the often weak attachment properties of proteins to the surface so that they may easily be removed throughout the washing steps. The low binding capacities lead to reduced signal intensities compared to covalent attachment techniques [77].

2.3.3.2 Bio-affinity immobilization

Bio-affinity immobilization is a very elegant attachment method that exploits the specific binding properties of affinity pairs for the oriented immobilization of the recognition elements. Therefore, one binding partner (e.g.: (strept-)avidin) is pre-coupled to the respective support whereas the second binding partner (e.g.: biotin) is linked to the probe. In this case, the immobilization of the probe is accomplished by the specific (strept-)avidin-biotin binding. Besides (strept-)avidin-biotin, a large variety of binding partners such as lectin-sugar [78], Protein A or Protein G-antibody [79] or other

common affinity tags like His-tags [80] were used so far. The (strept-)avidin-biotin system is the most frequently applied system. It takes advantage of the extremely strong binding affinity ($K_D = 10^{14} \text{ M}^{-1}$) and exhibits high resistance to harsh assay conditions [81]. Furthermore, biotinylated proteins are widely available. To all intents and purposes, the site-specific bio-affinity immobilization strategy impresses with oriented, homogeneous and reversible attachment of the immobilized probes.

2.3.3.3 Covalent immobilization

Most commonly proteins are covalently immobilized via functional groups of accessible amino acid side groups as it constitutes a robust linkage between the proteins and the respective surface. For covalent immobilization, a large variety of chemical surfaces and different functional groups can be used as summarized in Table 5. Covalent immobilization requires functionalized supports containing the respective reactive groups and is often achieved via glutaraldehyde-functionalized surfaces that exhibit high reactivity against amine groups [82]. Similarly, epoxy-groups are highly reactive towards amine groups and form very stable bonds over a wide temperature range and at a neutral to high pH. A large variety of solid supports like glass, polymer or gold surfaces can be used.

Table 5: Functional groups in proteins and required functionalities of the surface for covalent coupling [73]

Functional groups of proteins (Amino acids)	Required surface functionalities
-NH₂ (Lysine, Glutamine, Arginine)	Epoxy
	Carboxylic acid
	Aldehyde
	Active ester (NHS)
-SH (Cysteine)	Maleimide
-COOH (Aspartic acid, Glutamic acid)	Amine
-OH (Serine, Threonine)	Epoxy

Covalent immobilization results in a statistical orientation of the proteins due to the even distribution of the functional groups within a protein. But also when using covalent immobilization, the orientation can be partly controlled as proposed in [83]. Prior to covalent coupling, the proteins are physically

adsorbed on the solid surface via non-covalent interactions. Since only non-ionized amino acid residues will participate in the coupling reaction, the efficiency and the orientation of the probes are dependent on the surface pK_a , the isoelectric point of the proteins and on the pH of the immobilization buffer. By taking these considerations into account, the immobilization can be partly controlled. Proteins terminate with an α -amino group but harbor many other ϵ -amino groups as residues of the amino acids lysine, glutamine and arginine. At a neutral pH and assuming proper accessibility, the terminal amino group is likely to initially react since it exhibits a pK_a value between 7 and 8 whereas the ϵ -amino groups are protonated ($pK_a > 10.5$), hence have a significantly reduced reactivity.

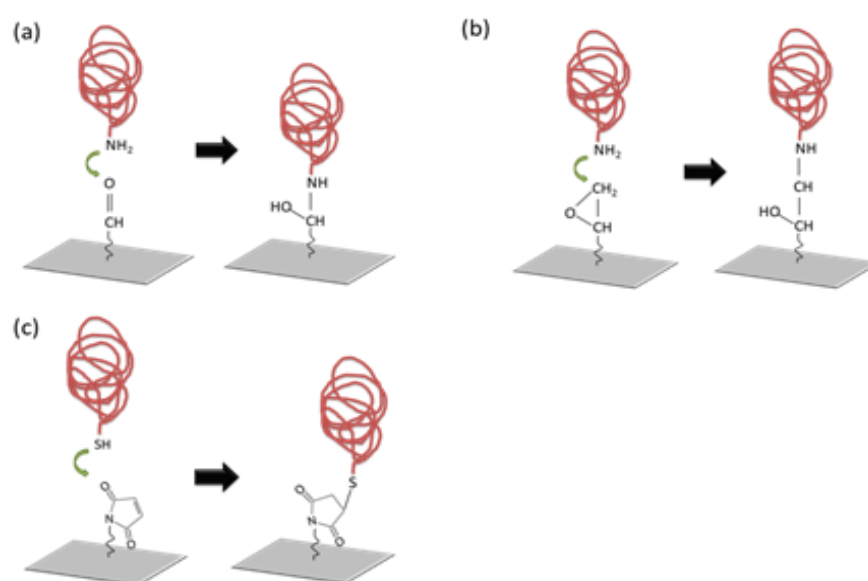


Figure 13: Covalent immobilization strategies via (a) Aldehyde, (b) Epoxy and (c) Maleimide groups

2.3.3.4 Immobilization strategies for small molecules

In order to support the immobilization of low molecular weight analytes, the small molecules are typically conjugated to carrier-proteins like BSA (bovine serum albumin) or KLH (Keyhole Limpet Hemocyanin). Otherwise, the carrier protein can be replaced by any other supportive macromolecule like oligonucleotides [84], polypeptides [85] or polymer dendrimers [45]. Other applied approaches for small molecule immobilization are in-situ synthesis [86] or direct covalent attachment to the surfaces [87].

Most immobilization strategies for small molecules are based on the preparation of small molecule-carrier conjugates. Therefore, the low molecular weight analyte has to carry at least one functional group for the covalent linkage to a carrier molecule. Typically, functional groups such as amines ($-NH_2$), carboxyls ($-COOH$), sulfhydryls ($-SH$) or carbonyls ($-CHO$) are exploited for coupling.

EDC is a carbodiimide and widely used as zero-length crosslinker. It first reacts with carboxyl groups to form reactive but unstable intermediates that further react with amino groups to form stable amide bonds. This coupling method is very efficient for proteins or polypeptides since both, carboxyl- and amino groups, are ubiquitary reactive groups. The reaction is often supported by the addition of N-hydroxysuccinimide (NHS) that stabilizes the labile intermediate by the formation of an amine-reactive sulfo-NHS ester. The chemical reaction scheme of EDC coupling is depicted in Figure 14.

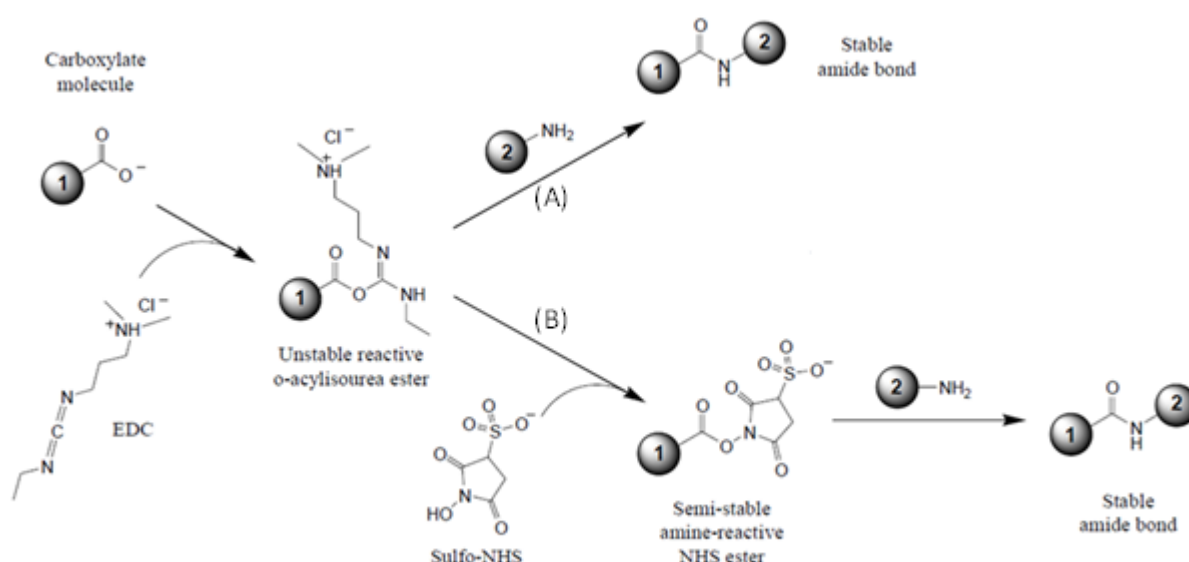


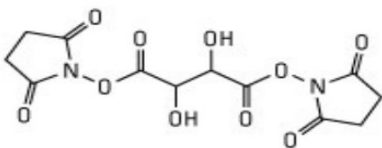
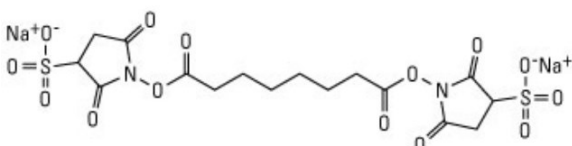
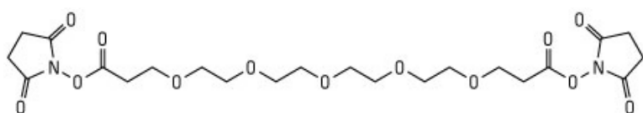
Figure 14: Reaction scheme of crosslinking using (A) EDC or (B) EDC + Sulfo-NHS (taken from [88])

Due to the high diversity of proteins it is often necessary to test different crosslinkers. This is especially true when the small molecules are intended to function as capture elements in analytical assays since the binding of the target analyte to the small ligands is accompanied by weak non-covalent interactions like electrostatic, hydrophobic or polar interactions. Therefore, diverse crosslinkers with varying spacer arms and different chemical properties may modulate and influence the binding properties differently. Hence, the evaluation of the appropriate crosslinker is a critical

step in the development of conjugates as the chemical make-up often decides about the final binding behavior.

Table 6 shows some selected amine-reactive crosslinkers with NHS ester ends that feature different spacer lengths and chemical properties, like hydrophobicity.

Table 6: Frequently applied Amine-reactive crosslinkers with different spacer lengths

Name/spacer arm length	Chemical structure	Linkage
DST Disuccinimidyl tartrate Spacer Arm: 6.4 Å		Amine-to-amine
BS3 Bis(sulfosuccinimidyl)suberate Spacer Arm: 11.4 Å		Amine-to-amine
BS(PEG)₅ Bis(succinimidyl)penta(ethylene glycol) Spacer Arm: 21.7 Å		Amine-to-amine

2.3.4 Immunoassay formats

Different on-chip immunoassays can be applied including direct, competitive and sandwich assays (Figure 15). The choice of the immunoassay format strongly depends on the availability of reagents and on the particular assay requirements.

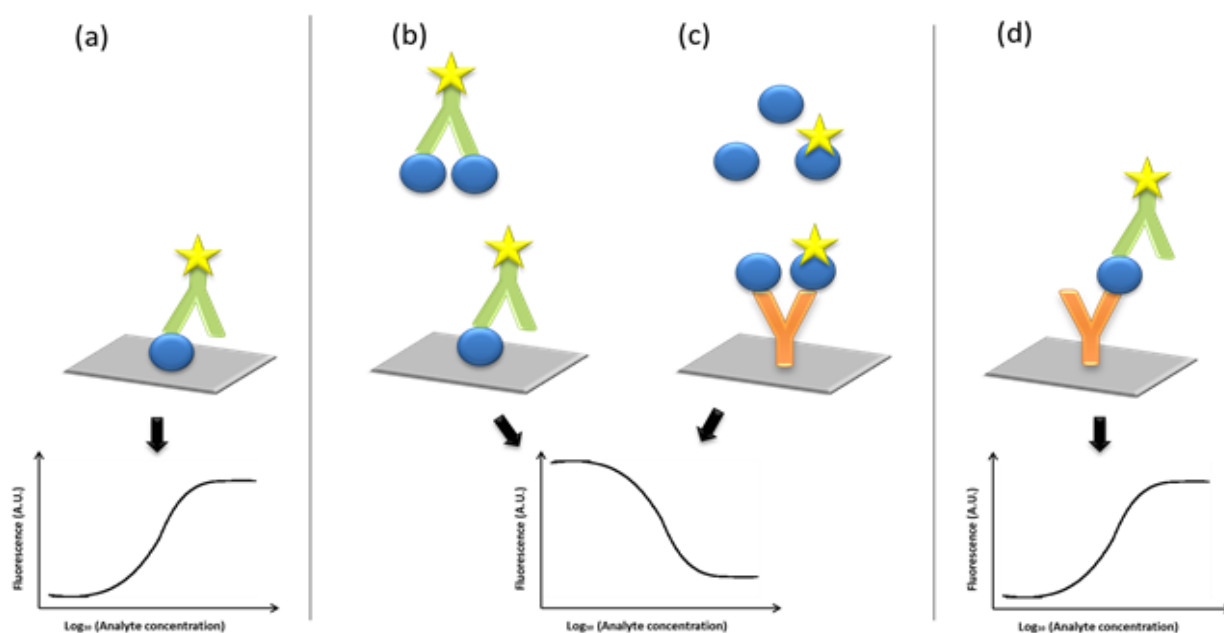


Figure 15: Schematic view of the different on-chip assay formats and the respective typical sigmoidal calibration curve obtained: (a) direct, (b) & (c) competitive and (d) sandwich immunoassays

2.3.4.1 Direct immunoassay

Direct immunoassays are performed to detect target molecules such as antibodies. Therefore, the antigen is immobilized and interacts directly with the target molecule of the sample. Due to the positive correlation between the binding event and the target concentration, the signals are directly proportional to the target concentration.

2.3.4.2 Competitive Immunoassay

Competitive immunoassays are frequently applied to detect analytes where only one antibody is available. This is the case especially for small molecular weight analytes (< 10 kDa) due to the lack of different epitopes that are necessary to develop distinct antibodies. Also, the accomplishment of a sandwich assay would fail due to steric hindrance that occurs at the attempt of binding two antibodies. In other cases, sandwich antibody pairs are not available forcing the accomplishment of a competitive assay. A competitive immunoassay is based on the competition of labeled and unlabeled ligands for a limited number of binding sites and can be performed in two variants: either the analyte or the specific antibody is immobilized. The target analytes are simultaneously incubated with defined amounts of the specific antibody or of labeled analytes respectively. In the first variant,

less analyte in the sample leaves more antibodies available to bind to the immobilized analyte and in the second variant, less analyte in the sample leads to a relative excess of labeled analytes that can bind to the immobilized antibody. Due to the negative correlation between the target analyte binding events and the amount of the target analyte, the signals are inverse proportional to the target concentration.

Generally, competitive assays are less sensitive than sandwich immunoassays and are therefore often applied for the detection of high abundance proteins. In [89], it is demonstrated that the combinatorial use of sandwich and competitive on-chip immunoassays allows the parallel detection of low and high abundance proteins. In addition, this assay format allows the adjustment of the dynamic range by modifying the detection antibody concentration.

2.3.4.3 Sandwich Immunoassay

In order to perform sandwich immunoassays, two target-specific antibodies that detect distinct epitopes of the same analyte are required. One antibody, the so-called capture antibody, is immobilized on the surface and specifically captures the target analyte from the sample. In a subsequent step, the second antibody, the so-called detection antibody, is added to specifically bind the captured analyte and thereby sandwiches the target analyte. The binding events of the detection antibody are directly proportional to the amount of bound target analyte. Consequently, there is a positive correlation between the obtained signal and the target concentration. The assay performance in terms of sensitivity and specificity depends on the affinities of the applied antibodies but is generally enhanced compared to the competitive immunoassay.

2.3.5 Calibration

For the precise quantification of the target analytes in immunoassays, calibration curves, also referred to as standard curves, have to be generated. The calibration curve depicts the relationship between the concentration in the sample and the corresponding signal and allows the interpolation of unknown samples. The most traditional method to generate calibration curves requires the preparation of a standard dilution series but some efforts to develop internal calibration curves have

recently been done. As the development of an internal calibration is also part of this study, the characteristics and differences are outlined below.

2.3.5.1 External calibration

An external calibration is generated from a standard dilution series from the respective analytes of interest. Therefore known concentrations of the analytes are spiked into a sample matrix. This sample is consecutively diluted by a known dilution factor to obtain a serial dilution. The externally prepared calibration curve is rather time consuming and often inaccurate especially for complex biologic samples like blood, plasma, serum, saliva or urine due to occurring matrix effects [90].

2.3.5.2 Internal calibration

The standard addition method is an elegant way to address matrix effects in complex biological samples. Therefore, known amounts of the analyte of interest are additionally spiked to unknown sample aliquots. A linear calibration graph is obtained by plotting the added analyte concentrations against the obtained signals. The unknown analyte concentration is derived as the negative value from the x-intercept [91]. This quantification method is frequently applied in chromatography or electrophoresis but found applications in other assay formats as for example in bioluminescence assays [92]. Nonetheless, this method again requires the preparation of a standard dilution series and as each calibration point consumes the same sample aliquot, this method is very sample-wasting.

More efficient approaches for internal calibrations have been introduced by Konovalova et al. [93] and Mezzasoma et. al [94]. In both studies, an internal calibration curve is generated by the immobilization of the analytes of interest in increasing amounts. The signals from the immobilized analytes are used to set up a dose-response calibration curve allowing the interpolation of unknown samples. This approach originates from colorimetric lateral flow assays where indicator ranges are frequently used for the semi-quantitative analyte detection. There, different cut-off concentrations of the respective analytes are immobilized and the obtained color intensity is compared to the respective indicator ranges after processing.

In this study, two different approaches for the development of internal calibration curves are developed (Figure 16): On the one hand (a) the analytes are coupled to the carrier protein BSA in order to support the immobilization of the rather small analytes and subsequently immobilized in increasing concentrations. On the other hand (b), this approach is extended in a cost-cutting and time-saving manner and improves long-term stability of the internal calibration by immobilizing the respective analyte-specific detection antibodies in increasing amounts.

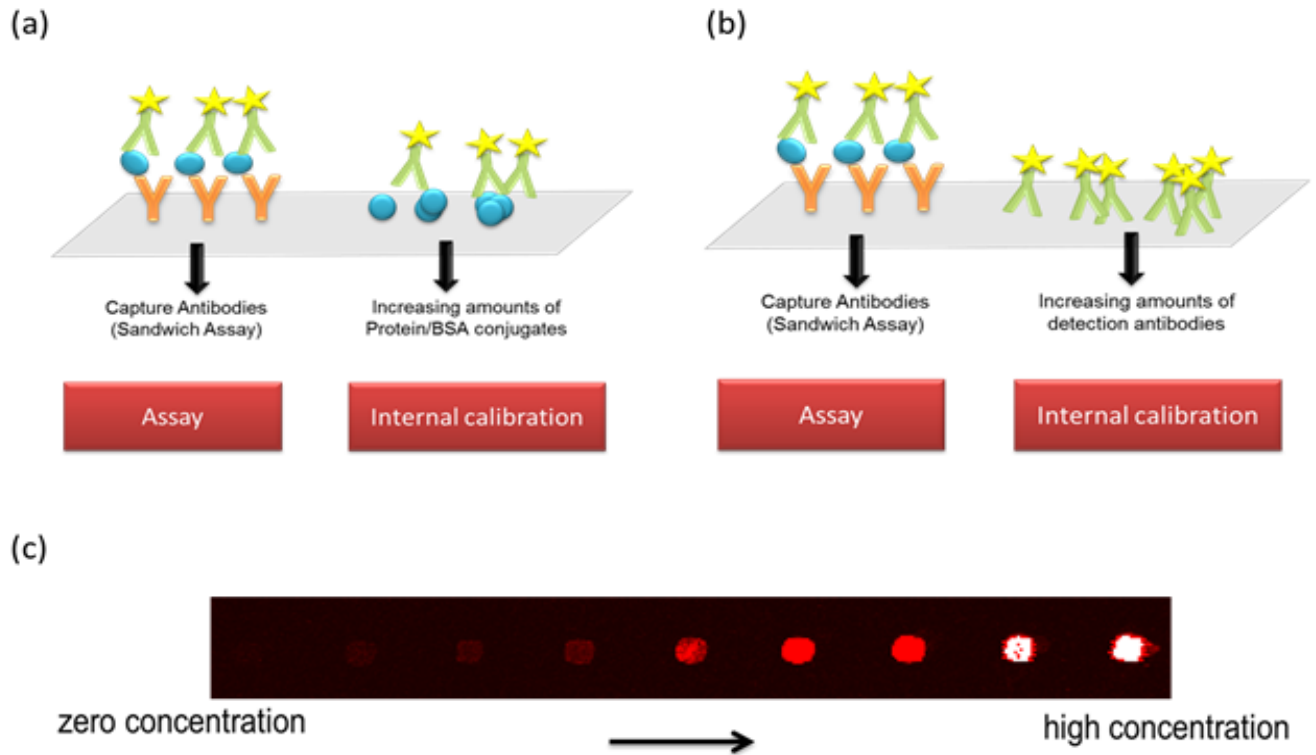


Figure 16: Schematic view of the applied on-chip calibration principles (a) using protein/BSA conjugates or (b) detection antibodies in increasing concentrations; (c) processed spots of an internal calibration

For both, the internal and the external calibration, the signals obtained from the standard dilution series and from the increasing amounts of immobilized analytes or antibodies respectively are used to set up the respective calibration curves. Ligand binding assays are typically characterized by a nonlinear relationship using the 4-Parameter-Logistic-Fit or the 5-Parameter-Logistic-Fit as reference models that result in a sigmoidally shaped calibration curve [95]. Equation (1) describes the 4-Parameter-Logistic-Fit:

$$Y = D + \frac{A-D}{1+(\frac{x}{C})^B} \quad (1)$$

Where Y is the signal, D is the signal from the bottom plateau, A is the signal from the top plateau, B is the slope parameter, C is the inflection point of the curve and x is the concentration.

The 5-Parameter-Logistic-Fit contains an additional parameter (G) to account for the asymmetry of the curve. Equation (2) describes the 5-Parameter-Logistic-Fit:

$$Y = D + \frac{A-D}{[1+(\frac{x}{C})^B]^G} \quad (2)$$

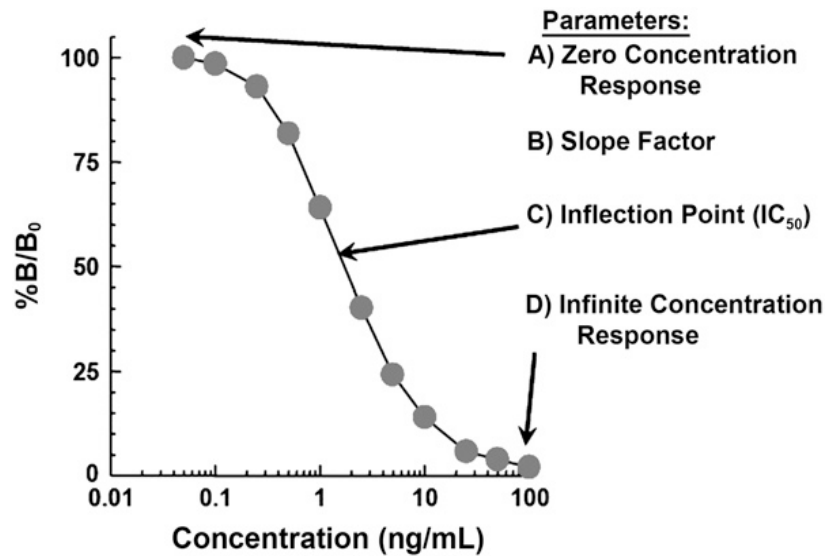


Figure 17: Typical calibration curve for a competitive immunoassay using the 4-parameter logistic fit [95]

2.3.5.3 Assay parameter

To assess the assay performance and compare different assay treatments, several parameters can be calculated from the calibration curves and from the raw data. The most prominent parameters for the evaluation of assays for clinical purposes include the calculation of the CV (coefficient of variation), recoveries, LODs (limit of detection) and LOQs (limit of quantification). Table 7 summarizes the calculation of the parameter as well as the respective significance for assay evaluation.

Table 7: Parameter used for assay evaluation

Parameter	Significance	Calculation
CV	Precision/Reproducibility	$CV (\%) = \frac{\text{mean SD}}{\text{mean signal}} \times 100$
Recoveries	Accuracy	$Rec (\%) = \frac{\text{calculated concentration}}{\text{expected concentration}} \times 100$
LOD	Sensitivity	$LLOD = \text{mean blank signal} + 3 \times \text{mean SD}$
LOQ	Quantification limit	$LLOQ = \text{mean blank signal} + 10 \times \text{mean SD}$

The CVs are calculated to assess the assay precision that allows the interpretation of the repeatability of consecutive measurements. For clinical purposes, the CVs should be below 20 %. The recoveries are used to evaluate the assay accuracy and should be within 70 and 130 %. The differences between precision and accuracy are outlined in

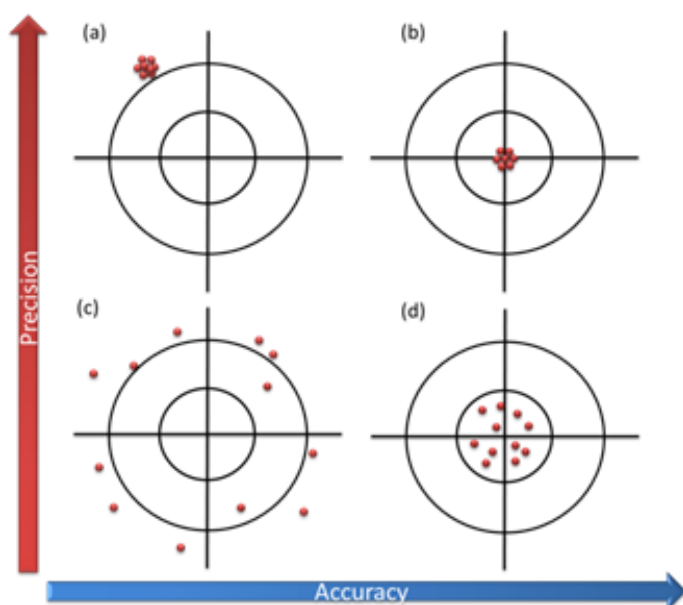


Figure 18. The LOD is the lowest concentration that can be distinguished from the blank sample. The LOQ is the lowest concentration that can be reliably measured with a defined extent of accuracy and precision [96].

Figure 18: Exemplary depiction of precision and accuracy: (a) low accuracy, high precision; (b) high accuracy, high precision; (c) low accuracy, low precision; (d) high accuracy, low precision

2.3.6 Detection

The binding events in microarrays can be verified either via label-free or label-based methods. Both approaches have their assets and drawbacks regarding sensitivity, applicability and instrument availability. Label-based detection methods require the labeling of the detection molecules such as antibodies or involve direct labeling of the target analytes. Various labels such as fluorescent dyes,

radioisotopes or enzymes have been used. By using label-free methods the tagging of the targets or of the specific detection elements can be avoided by measuring inherent properties of the target analyte.

2.3.6.1 Label-free detection

Label-free detection methods impress with the possibility to study molecular kinetics and binding interactions in real-time without the need to label analytes or detection molecules. Thereby, interferences that may arise from labeling like modifying or masking the binding recognition sites can be completely avoided. Nevertheless, label-free detection methods often suffer from sensitivity and specificity problems [97]. One promising label-free method frequently applied in protein chips (besides surface plasmon resonance (SPR)) is the mass sensitive quartz crystal microbalance (QCM).

2.3.6.1.1 QCM immunosensors

QCM sensors have been widely applied to detect a large variety of analytes ranging from small low-molecular weight analytes [98] to whole cells [99].

The piezoelectric effect was first described in 1880 by the brothers Pierre and Jacques Currie. The word “piezoelectricity” comes from the Greek and means “electricity by pressure”.

Mechanical deformation of a piezoelectric material leads to the formation of microscopic dipole moments within primitive cells. The summation of the microscopic dipole moments, thus the summation of the associated electrical fields results in a macroscopic, measurable voltage. This phenomenon is referred to as the direct piezoelectric effect which is reversible meaning that the application of a voltage conversely leads to the deformation of a piezoelectric material (inverse piezoelectric effect). The piezoelectric effect occurs exclusively in crystals that lack symmetry along at least one of their crystallographic axes. One prominent material that exhibits piezoelectric properties is quartz. QCM sensors exploit the piezoelectric properties of quartz for the mass sensitive detection. The crystal structure of quartz is hexagonal and has no symmetry along the y-axis (Figure 19a). For sensing, the so-called AT-cut quartz discs are used. AT-cut refers to the bias

angle used during production of the quartz discs. The cutting angle is a critical parameter in the preparation of the discs as it determines the thermal stability of the resonance frequency as well as the ductility of the quartz discs. In the case of AT-cut quartzes, a bias angle of $35^{\circ}15'$ related to the y-axis is used. Figure 19b depicts the frequency changes due to temperature changes for three different cut angles showing that with the AT-cut quartz the obtained resonance frequency is insensitive to temperature changes between $0 - 50^{\circ}\text{C}$.

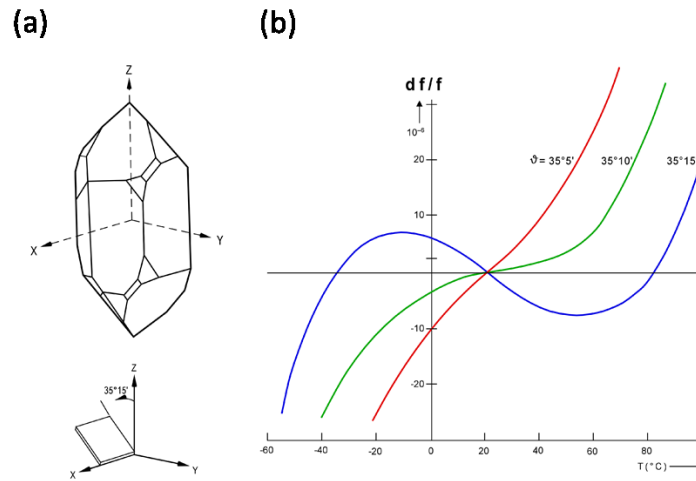


Figure 19: (a) Depiction of an AT-cut quartz crystal, (b) Relationship between frequency and temperature for three different cut angles (taken from [100])

The quartz crystal microbalance is an analytical device that records frequency changes in response to mass loading on quartz crystal resonators. By applying an alternating voltage, the quartz crystal starts oscillating in a shear mode generating transversal acoustic waves.

The resonant frequency depends on several factors including the thicknesses of:

- the quartz disc
- the deposited metal electrodes
- the deposited sensor layer

The resonant frequency decreases in response to mass deposition on the sensor surface and can be described by the Sauerbrey equation (3). Where Δf is the resulting frequency shift (Hz), f_0 is the resonant frequency (Hz), A is the piezoelectrically active crystal area (cm^2), ρ_q is the density of quartz ($\rho_q = 2.648 \text{ g/cm}^3$), μ_q is the shear modulus of quartz ($\mu_q = 2.947 \times 10^{11} \text{ g/cm.s}^2$) and Δm is the mass change in grams.

$$\Delta f = - \frac{2f_0^2}{A\sqrt{\rho_q\mu_q}} \Delta m \quad (3)$$

A typical working station for QCM measurements requires the following electronic interfaces (

Figure 20a):

- Measuring cell that contains the quartz crystal with electrodes deposited on both sides
- Oscillator circuit as electronic driver
- Frequency counter
- Power supply that passes on a constant alternating current
- PC for data collection and processing

The quartz crystal contains two facing electrodes deposited on its top and bottom side

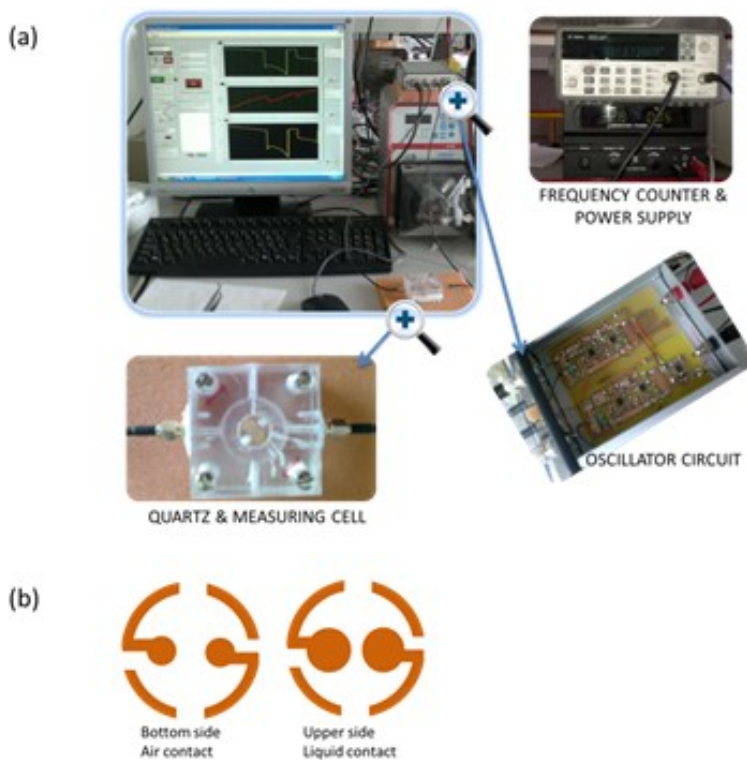


Figure 20b). To allow QCM measurements in liquids, the opposite electrodes are typically of different sizes: the smaller one is exposed to the air and the larger one faces the analyte solution. This setup minimizes the dissipation of electrical field lines out of the liquid.

The power supply generates a constant alternating voltage that triggers the oscillation of the quartz crystal. This oscillation is amplified via the oscillator circuit and detected by the frequency counter that is connected to the PC for further data processing [101].

Figure 20: (a) Electronic interfaces for QCM measurements containing of a power supply, the measuring cell and the quartz crystal, the oscillator circuit, the frequency counter and a computer (b) Quartz crystal with different opposite electrode sizes

2.3.6.2 Label-based detection

Despite the efforts to develop label-free devices, label-based detection systems are dominant in the field of microarray technologies. Two general strategies for label-based detection are used: direct or indirect labeling. In the first variant, the analytes of interest are directly tagged. The labeling process often lacks efficiency due to the heterogeneous chemical makeup of analytes which makes this method complex to accomplish in a reproducible manner. Furthermore, direct labeling may alter the binding behavior of the analyte to the recognition element. In contrast, the indirect labeling strategy requires the tagging of a detection element like detection antibodies. Since all antibodies have a similar chemical structure, the labeling process can be performed under standardized conditions. A large variety of labels are available allowing colorimetric, (electro)chemiluminescence or fluorescence detection.

2.3.6.2.1 Fluorescence detection

Since fluorescence spectrometry exhibits high sensitivity and high resolution, fluorescent dyes are the most prominent tags in microarray analysis. For fluorescence labeling, the antibodies can be directly tagged with a fluorescent dye or otherwise with a biotin residue that subsequently binds fluorophore labeled streptavidin.

Fluorescence is defined as the emission of light in response to the excitation of a fluorescent molecule. More precisely, a photon of the excitation light ($h\nu_{\text{ex}}$) is absorbed by the fluorophore which increases the energy level of the fluorophore and stimulates an external electron from the ground state (S_0) to the excited state (S'_1). The increased energy is partly evolved non-radiatively and the electron enters the relaxed excited state (S_1). The remaining energy is released as the electron relaxes back to the ground state. During the return of the electron, a photon of light ($h\nu_{\text{em}}$) is emitted. The particular electron transitions are shown in Figure 21a. Due to the initial non-radiative relaxation the excited photon is of higher energy than the emitted photon. Therefore, the emitted wavelength is of lower energy than the incident wavelength. Figure 21b shows a typical absorption and emission spectrum of a fluorophore. The difference between the excitation maximum and the emission

maximum is called Stokes shift. Generally, a large Stokes shift is preferable for detection since it allows the sharp distinction between incident and emitted light.

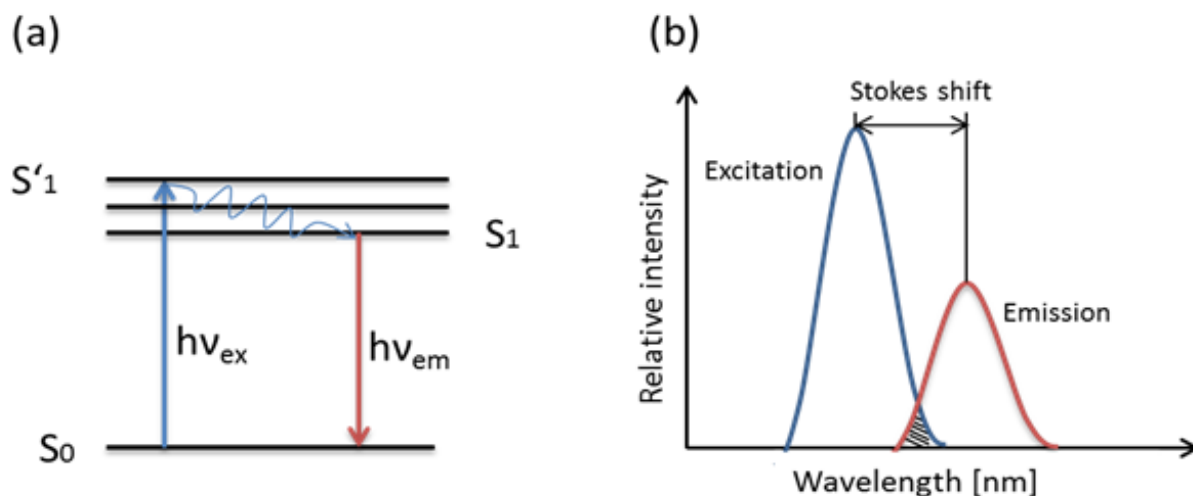


Figure 21: (a) Energy diagram of fluorescence and (b) typical absorption and emission spectrum of fluorophores

Despite the outstanding advantages and widespread usage of fluorescence detection, the method suffers from several drawbacks. Photobleaching is often reported as a problem associated with fluorescence detection. It is caused by irreversible destruction of fluorophores by exposing the fluorophores too long or too extensively to the excitation light. Another problem that arises with fluorescence is the occurrence of quenching effects. Through quenching the fluorescence intensity of fluorophores is decreased. This phenomenon is often exploited for methods like FRET (Förster resonance energy transfer) or contact quenching. In protein microarrays, the unwanted self-quenching effect, triggered by the short-range interaction between two fluorophores, may occur which often leads to false negative results. Therefore, the density of fluorophores during the labeling and the detection process has to be controlled [102].

A large variety of fluorophores with distinct emitting wavelengths ranging from the blue-violet to the infrared is available from many manufacturers, for example Molecular Probes® from LifeTechnologies (USA), Dyomics (Germany) or Pierce/Thermo Scientific (USA).

The resolution and sensitivity of fluorescence detection strongly depends on the properties of the applied fluorophores regarding quantum yield and susceptibility to photobleaching. The fluorescence quantum yield describes the amount of emitted radiation after excitation deducting non-radiative

dissipation. Organic fluorescent dyes are the preferred fluorophores due to their high stability and biocompatibility. Cy3 and Cy5 are sulfoindocyanines and constitute the standard dyes in microarray experiments. In contrast to the initially used dyes Fluorescein and Texas Red [103], the cyanine dyes are not prone to physiological pH changes. Nevertheless, the cyanine dyes suffer from photobleaching and quenching. Another class of fluorophores, the Alexa dyes, provides remedy of these troubles by exhibiting higher quantum yields and stability [104]. Since the excitation and emission spectra of the cyanine and Alexa dyes are almost identical they can be used interchangeable.

Table 8: Frequently applied fluorescent dyes

Dye	λ_{ex} (nm)	λ_{em} (nm)	Quantum yield
Cy3	552	570	0.15
Cy5	650	670	0.28
Alexa Fluor546	561	572	0.79
Alexa Fluor647	652	668	0.33

For the readout of fluorescence many different instruments are available like fluorescence scanners that are able scan the whole slides and allow the detection of localized fluorophores. These so-called microarray readers comprise of a light source for excitation, most commonly lasers, photodiodes or lamps, of emission filters to screen the designated emission wavelength of the particular fluorophore and of a detector that converts the emitted photons to an electrical signal for read-out. Frequently, a PMT (photomultiplier tube) serves as detector which amplifies the obtained signals for the enhanced detection.

2.3.6.2.2 Signal amplification

The sensitivity of the applied detection method is critical for the outcome and often enhancement strategies are required to enable the analysis of low abundance analytes. The state of the art of promising technologies for the ultrasensitive protein detection is outlined in the following.

2.3.6.2.2.1 Polymerase based amplification of nucleic acid based tags

Polymerases are enzymes that catalyze the synthesis of new complementary DNA or RNA strands from template strands. PCR (polymerase chain reaction) is a well-established in-vitro technique for the amplification of DNA. Since PCR is a very efficient amplification strategy, various polymerase-based protein detection methods have been developed including immuno-PCR or immuno-rolling circle amplification (Figure 22).

Immuno-PCR was invented by Sano et al. in 1992 and combines ELISA and PCR techniques [105]. A DNA-tagged antibody specifically recognizes the analyte of interest and the bound DNA sequence is amplified via PCR leading to the indirect detection of the analyte. Unique DNA sequences that encode for the respective Antigen-Antibody binding event are used. Initially, the PCR products were subsequently detected using gel-electrophoresis but this method is rather time-consuming and non-quantitative. Therefore, this approach was further enhanced using real-time PCR which proved to be more practical and allowed the quantification of the captured analytes in an extremely sensitive

manner [106]. Immuno-PCR yields sensitivities that are up to three orders of magnitude enhanced compared to conventional ELISAs.

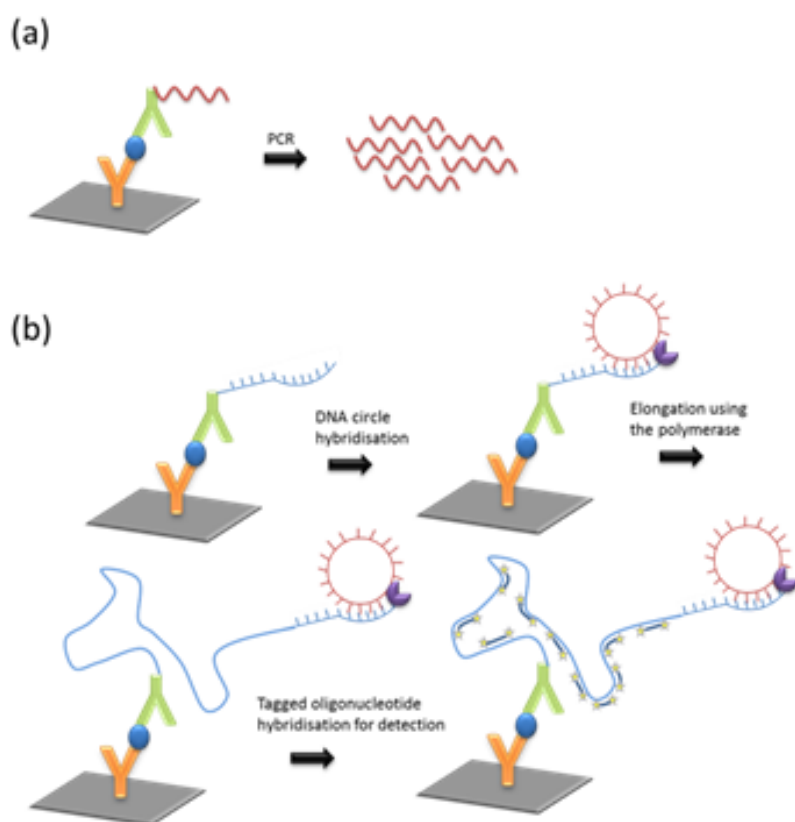


Figure 22: Polymerase based amplification methods

Immuno-rolling circle amplification generates high-density labeling by accumulating multiple dye molecules along a DNA strand and is based on the rolling circle reaction. Therefore an antibody is tagged with an oligonucleotide primer. A DNA circle binds the primer in a

complementary manner and in the presence of nucleotides and the DNA polymerase, the tagged primer gets extended resulting in a long single stranded DNA molecule. The so-created DNA strand consists of sequential complementary DNA circle sequences that can be detected via the hybridization of complementary tagged DNA strands or directly by the implementation of tagged nucleotides. The rolling circle amplification method is advantageous over the Immuno-PCR technique as it is an isothermal reaction, hence does not require any extra equipment such as PCR cyclers, and the reaction can be performed at constant temperature.

2.3.6.2.2.2 Dendrimers and nanoparticles

Nanoparticles [107] and dendrimers [108] have been used as carrier for labels. In both cases, the signal intensities are improved by the generation of high-density labeling accomplished by increasing the number of tags by exploiting the high surface to volume ratio of the particles and the dendrimers respectively.

2.3.6.2.2.3 Antibody amplification

The antibody mediated amplification method is based on the consecutive incubation of two fluorophore labeled antibodies. The first antibody is target specific and the second antibody targets the Fc portion of the primary antibody, hence is species specific. The application of two antibodies increases the local density of fluorophores and thereby enhances the signal intensity [109]. Figure

23 schematically depicts the working principle of the antibody mediated amplification.

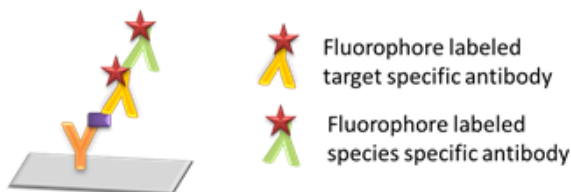


Figure 23: Working principle of the antibody mediated amplification

2.3.6.2.2.4 Tyramide signal amplification

Tyramide signal amplification (TSA) is an amplification method that increases the local density of fluorophores by exploiting the catalytic activity of the enzyme horse radish peroxidase (HRP). In the presence of hydrogen peroxide, HRP converts multiple tyramide molecules to instable tyramide radicals that are highly reactive to the phenol moieties of neighbored tyrosine residues. Tyramine is a phenolic low molecular weight compound that is coupled to fluorophores or biotins to accomplish TSA detection. TSA has found wide applications in immunohistology stainings like FISH [110], DNA [111] and protein chips [112] but can be adapted to other analytical techniques. Figure 24 schematically depicts the functional principle of TSA in immunoassays.

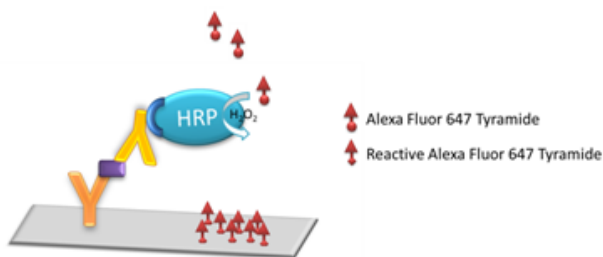


Figure 24: Functional principle of the tyramide signal amplification method

2.4 Nanoimprint lithography

Nanoimprint lithography (NIL) was first introduced by Chou et al. as a low-cost alternative to the expensive electron beam lithography or focused ion beam lithography. NIL is based on the mechanical deformation of a resist thin film and constitutes a high-throughput and flexible technique for the fabrication of nanostructured surfaces as for example used in electronic, optical or biosensing devices [113]. The implementation of nanostructured surfaces in protein chips is a very promising approach in order to increase the assay sensitivity. The usage of 3D structured surfaces increases the surface area available for protein immobilization which further increases the signal intensities during detection [114].

The traditional nanoimprint lithography approach established by Chou et al. in 1995 is nowadays known as thermoplastic nanoimprint lithography (T-NIL) and is accomplished as followed: First the thermoplastic resist is applied on a solid support like wafers or a glass slides frequently via spin-

coating. Afterwards the stamp that contains a predefined surface pattern is pressed onto the resist film that has been heated above its glass transition temperature (T_g). After cooling down, the stamp is removed leaving the negative stamp pattern on the surface of the cured resist thin film.

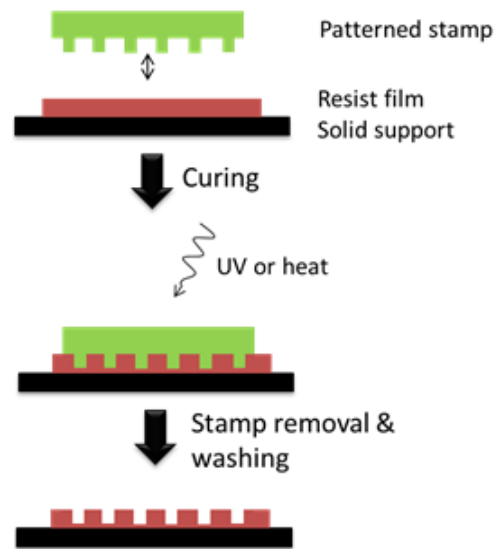


Figure 25: Scheme of the NIL process

For the UV-based nanoimprint lithography (UV-NIL) UV curable resist materials are applied. The principle of UV-NIL is also based on the mechanical deformation of the resist material: The resist material is applied onto a solid support, the nanopatterned stamp is mechanically pressed on the resist thin film and the polymerization is subsequently triggered via UV radiation that freezes the predefined structures of the stamp on the surface of the resist film and the stamp can be removed. UV-NIL has the advantage that it can be carried out at room temperature and at low pressure but UV-NIL requires UV permeable stamps like quartz glass or PDMS molds [115].

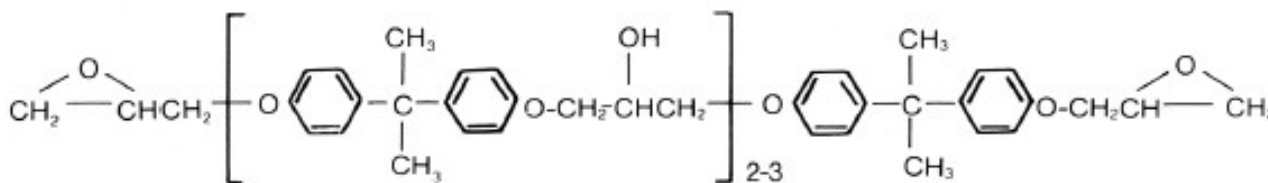
2.4.1 Resist materials

Several companies including micro resist technology GmbH (Germany), Amo GmbH (Germany) or Miller Stephenson Chemical Company (USA) commercially provide ready-to-use resist materials. For NIL applications, the resist materials should exhibit improved adherence to the solid substrate and reduced adherence to the stamp, low shrinking during curing and fast crosslinking speed. Depending on the NIL type, resist materials are classified as thermoplastic polymers used for T-NIL

or photocurable polymers applied in UV-NIL. Epoxy- and acrylate-based resins are most commonly used.

Acrylate-based resins are often used since acrylated monomers are highly reactive to a large variety of chemicals allowing the easy and fast fabrication of a NIL applicable resist system [116].

Epoxy resins are polyether resins that contain at least one epoxy group and are manufactured by the reaction of Bisphenol A with epichlorohydrin. A large variety of epoxy resins with varying numbers of epoxy functionalities, molecular weight and chemical backbone makeup were designed. The various epoxy resin formulations vary in their chemical and physical properties; hence differ in their glass transition temperature (T_g), viscosity, crosslinking density, stiffness and intermolecular interactions [117]. Due to the highly versatile nature of epoxy resins, they have found diverse applications in industry as protective coatings in the paint industry, as adhesives in the aircraft, automobile or boat industry and furthermore as casts, molds or laminates [118]. Various commercially available epoxy resins, like Epon 1002F, SU8 Epikote 157 or SU8 2000.5 were applied as coatings in bioanalytical applications [119] and used for nanostructuring to enhance protein immobilization in biochips [114].



2.5.1 Contact angle measurements

The measurement of the contact angle is one of the earliest methods for surface characterization but it is still widely used due to its simplicity, straightforwardness and high information content [121]. A small volume sample of a few μL is dropped on the surface of interest and the contact angle is determined from the tangent of the liquid/surface interface by assuming a thermodynamic equilibrium between the three phases (solid, liquid, vapor) via the equation from Young:

$$0 = \gamma_{SG} - \gamma_{SL} - \gamma_{LG} \cos\theta \quad (4)$$

where θ is the Young contact angle and γ_{SG} , γ_{SL} and γ_{LG} are the energies between the solid-vapor (SG), solid-liquid (SL) and liquid-vapor (LG) interfaces respectively (Figure 27 b).

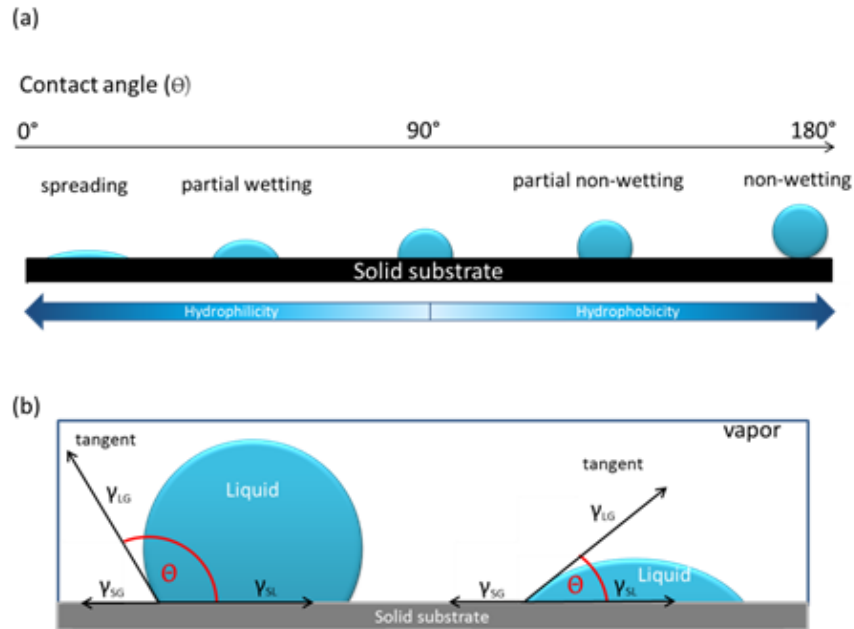


Figure 27: (a) Typical behavior of water on hydrophilic to hydrophobic surfaces (b) Determination of the contact angle according to Young's equation

From the obtained contact angle, the surface wettability can be categorized (Figure 27 a). When using water, a contact angle below 90° indicates a hydrophilic surface and a contact angle above 90° refers to a hydrophobic surface. Surfaces that exhibit contact angles above 150° are considered as superhydrophobic. Contact angle measurements are sensitive to contaminations on the surface and require smooth and homogeneous surfaces. Furthermore, for reliable measurements the dropped liquid should not dissolve or swell the surface [122].

In practice, a spectrum of contact angles is obtained ranging from the maximal (advancing) contact angle to the minimal (receding) contact angle. This phenomenon is called contact angle hysteresis and arises from the surface roughness and contaminations. The equilibrium contact angle is between the advancing and receding contact angle.

2.5.2 Atomic force microscopy

Atomic force microscopy (AFM) belongs to the family of scanning probe microscopy (SPM) that was initially introduced in the early 1980s by Rohrer and Binnig by the invention of the scanning tunneling microscope (STM) [123]. Unlike optical microscopy, where the images are generated by the illumination and optical magnification of the samples, in SPM, the images are created by the interaction of a scanning probe with the sample. AFM yields high resolution in the nanoscale and records a 3D profile of the sample surface. AFM works well in air and liquid environments allowing the analysis of biomolecules and even living cells or microorganisms in their natural environment.

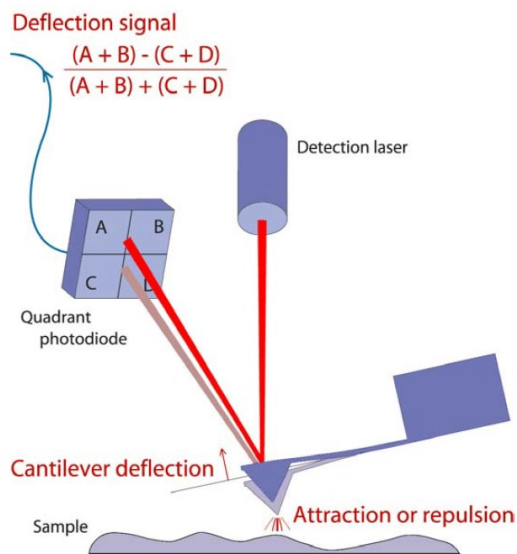


Figure 28: Detection principle of the AFM [taken from [124]]

The surfaces are scanned with a sharp tip of approximately 100 Å in diameter that is located at the free end of a cantilever. Typically, microfabricated silicon or silicon nitride cantilevers with integrated sharp tips are used. A piezoelectric element is often used for the accurate alignment of the scanning probe in all directions as it allows the precise scanning of the samples. During scanning, attractive or repulsive forces between the tip and the sample occur that reflect the scanned topography. Most

AFM devices use the optical lever principle for detection (Figure 28): A laser beam gets reflected from the back of the cantilever to a photodetector. Small angular displacements of the reflected laser beam due to the bending of the cantilever are recognized by the photodetector. Software algorithms convert these changes to a surface topography map of the scanned sample.

AFM can be operated either in the contact, non-contact or intermittent contact mode depending on the particular probe properties and the analytical issue.

In contact mode, the tip mechanically contacts the sample surface and thereby experiences strong electrostatic repulsive forces. The contact mode is especially suitable for flat and rigid probes as it often damages soft probe materials due to the occurring lateral forces.

In the non-contact mode, the scanning tip has no contact with the probe. In this case, the cantilever is excited to its resonant frequency with an amplitude generally below 10 nm. Attractive forces such as hydrophobic interactions that typically occur at a distance of 1 nm to 10 nm above the surface, decrease the oscillating frequency. The frequency decrease is recorded and balanced by the integrated feedback loop. One major drawback of the non-contact mode is that under ambient conditions it is very difficult to balance the sample-to-tip distance due to the attractive forces that are reinforced by a hydrated layer formed by the ambient moisture content. Therefore, the tip is likely to stick to the surface and switch from non-contact to contact operation mode. For this reason, non-contact mode measurement is frequently accomplished in vacuum or ultra-high vacuum conditions where atomic resolution images are obtained [125].

The intermittent contact mode, also referred to as tapping mode by one AFM manufacturer (Digital Instruments), is the most common operation mode since it combines the benefits of the contact and the non-contact modes. As for the non-contact mode, the cantilever is oscillated near its resonance frequency but with an amplitude approximately one order of magnitude higher than that for the non-contact mode. Within each oscillation circle, the tip contacts and diverges from the sample. Attractive and repulsive forces change the oscillation amplitude that is recorded and adjusted by an integrated feedback loop. Besides analysis of the surface topography, AFM allows the differentiation of two distinct materials within a sample when operating in the intermittent contact mode. The phase

shift caused by differences in the interactions between the tip and the sample are measured and depicted in phase images [124].

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3 Publications constituting the thesis

Publication 1

P. Buchegger, U. Sauer, H. Toth-Szekely, and C. Preininger, “Miniaturized protein microarray with internal calibration as point-of-care device for diagnosis of neonatal sepsis”, Sensors (Basel), 2012, vol. 12, pp. 1494-508

Publication 2

P. Buchegger, and C. Preininger, “Four assay designs and on-chip calibration: Gadgets for a sepsis protein array”, Analytical Chemistry, 2014, submitted

Publication 3

P. Buchegger, P.A. Lieberzeit, and C. Preininger, “Thermo-nanoimprinted biomimetic probe for LPS and LTA immunosensing”, Analytical Chemistry, 2014, accepted

Miniaturized Protein Microarray with Internal Calibration as Point- of-Care Device for Diagnosis of Neonatal Sepsis

P. BUCHEGGER, U. SAUER, H. TOTH-SZEKELY, AND C. PREININGER,
SENSORS (BASEL), 2012, VOL. 12, PP. 1494-508.

P. Buchegger carried out all microarray experiments and was responsible for data analysis and interpretation, U. Sauer and H. Toth-Szekely supported in technical concerns. C. Preininger contributed to data evaluation, planned the concept and supervised the work.

Article

Miniaturized Protein Microarray with Internal Calibration as Point-of-Care Device for Diagnosis of Neonatal Sepsis

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Received: 13 December 2011; in revised form: 13 January 2012 / Accepted: 29 January 2012 / Published: 3 February 2012

Abstract: Neonatal sepsis is still a leading cause of death among newborns. Therefore a protein-microarray for point-of-care testing that simultaneously quantifies the sepsis associated serum proteins IL-6, IL-8, IL-10, TNF alpha, S-100, PCT, E-Selectin, CRP and Neopterin has been developed. The chip works with only a 4 μ L patient serum sample and hence minimizes excessive blood withdrawal from newborns. The 4 μ L patient samples are diluted with 36 μ L assay buffer and distributed to four slides for repetitive measurements. Streptavidin coated magnetic particles that act as distinct stirring detection components are added, not only to stir the sample, but also to detect antibody antigen binding events. We demonstrate that the test is complete within 2.5 h using a single step assay. S-100 conjugated to BSA is spotted in increasing concentrations to create an internal calibration. The presented low volume protein-chip fulfills the requirements of point-of-care testing for accurate and repeatable ($CV < 14\%$) quantification of serum proteins for the diagnosis of neonatal sepsis.

Keywords: protein microarray; streptavidin coated magnetic particles; miniaturization; point-of-care testing; neonatal sepsis; internal calibration

1. Introduction

Sepsis is a life-threatening systemic body infection manifested by symptoms ranging from elevated heartbeat, breathing difficulties and fever to severe necrosis of the internal organs. It is caused by microbial pathogens including bacteria, virus and fungi. The survival rates increase enormously with an early stage diagnosis which allows timely administration of antibiotics. Neonatal sepsis is still a leading cause of death among newborns since symptoms are often wrongly interpreted due to their unspecific and late occurrence [1]. However, early-stage diagnosis in neonates is even more challenging and demands for a reliable and rapid point-of-care device that works with a low sample volume to keep invasive procedures on neonates to a minimum. Key requirements for point-of-care devices relate to short assay time, quality assurance through simple and stable calibration, convenient operation and cost effectiveness [2]. The standard diagnosis procedure for sepsis is based on the isolation of microorganisms from patient blood or urine samples and subsequent microbiological cultivation and identification. Disadvantages of the standard procedures are the low sensitivity, the processing time which may exceed 24 hours and the need for large sample volumes (>1 mL) [3–5].

Sepsis is a condition caused by pathogenic microorganisms but induced by the immune response through excessive release of growth factors, cytokines and inflammation- and coagulation factors into the systemic circulation. Those factors can be exploited as specific biomarkers enabling early and reliable diagnosis. The cytokines interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10) and tumor necrosis factor alpha (TNF alpha) and the serum proteins C-reactive protein (CRP), procalcitonin (PCT), S-100 and E-Selectin have been identified as a specific and accurate biomarker panel for the early diagnosis of sepsis. The clinically relevant ranges as well as the cut-off values of the respective serum proteins that allow discrimination between healthy and septic patients are outlined in Table 1. IL-6, IL-8, IL-10 and TNF alpha are proinflammatory cytokines secreted by cells of the immune system that mediate host defense [6,7]. E-Selectin is expressed by endothelial cells and is a main mediator in the migration of immune cells to the centre of infection [8]. CRP and PCT are acute-phase reactants expressed predominately as part of the immediate immune response to infections [9]. S-100 serum levels are significantly elevated in neuronal inflammations [10]. Neopterin is expressed by macrophages in response to infections [11].

Table 1. Cut-off values for the respective biomarkers.

Biomarker	Cut-off values	Biomarker	Cut-off values
IL-6 (pg/mL)	18 [7], 31 [6]	PCT (pg/mL)	500 [9], 36 [13]
IL-8 (pg/mL)	20 [12]	Neopterin (pg/mL)	16 [11]
IL-10 (pg/mL)	17 [13]	S-100 (ng/mL)	10.4 [10]
TNF alpha (pg/mL)	17 [6]	CRP (μg/mL)	10 [7,9] 12 [6]
E-Selectin (ng/mL)	174 [6]		

Herein we aim at the further development of a sepsis chip for point-of-care testing of neonatal sepsis. This is achieved by: (a) the reduction of required patient sample volume, (b) the reduction of assay steps and incubation times and (c) the integration of an internal calibration. The chip for biomarker detection of sepsis comprises multi-analyte on-chip immunoassays which include both

sandwich (cytokines, PCT, S-100, E-Selectin) and binding inhibition formats (CRP, neopterin). The combination of both assay formats allows analysis of the selected biomarkers on one chip despite the large heterogeneity of the analytes regarding cut-off concentration [14]. In our previous work we showed that LODs of 15 pg/mL (IL-6), 36 pg/mL (IL-8), 65 pg/mL (IL-10), 40 pg/mL (TNF alpha), 0.078 ng/mL (PCT), 0.46 ng/mL (neopterin) and 3 µg/mL (CRP) are achieved in this set-up [14].

In order to prevent excessive blood withdrawal from newborns, the biochip was further miniaturized to operate with a five-fold reduced sample volume. The assay performance of the miniaturized chip is then compared with the classical format and evaluated using assay parameters like analyte concentration halfway between the zero point and the upper asymptote (IC₅₀), limit of detection (LOD), limit of quantification (LOQ), and inter- and intra-array variations.

In general, assay miniaturization provokes loss in assay sensitivity due to e.g., diffusion limitations. While a sample volume of 50 µL, as used for the classical chip, is mixed properly during incubation on the rotational shaker, a sample volume of 10 µL, as used for the miniaturized chip, is too small to be moderately mixed under the same incubation conditions. Different theoretical considerations have been made concerning the impact of diffusion on the antibody antigen kinetics for immunoassays on solid surfaces [15–17]. For example, Kusnezow *et al.* [15] developed a mathematical model to simulate the impact of diffusion on the binding kinetics. This model was examined empirically comparing the development of signal intensities over time for anti-IFN-γ spots of 45 µm to 272 µm radius under stirring and non-stirring conditions. Clearly, stirring significantly accelerates the immunoreaction (up to hundreds of times), while the velocity increases with decreasing size of the spot. Hartmann *et al.* reported three-fold enhanced signals using surface acoustic waves (SAW) coupled through the glass slide into the reaction solution. To account for the antibody spot kinetics, thus the migration of the analytes in solution towards the spot and across the surface we included bi-functional streptavidin coated magnetic particles (Strep.MPs), which act as both micro-mixer and detection reagent for the biotinylated antibodies. Strep.MPs in the sizes of 130 nm, 500 nm and 1 µm were tested. In addition, the assay procedure was further optimized by reducing assay steps and incubation times. Furthermore the calibration was integrated on the chip (internal calibration) presenting a point-of-care device suitable for use in neonates.

2. Experimental Section

2.1. Materials and Reagents

Chip platform used was the proprietary ARChip Epoxy [14], Anti IL-6 (MQ2-13A5), recombinant IL-6 protein, biotinylated anti IL-6 (MQ2-39C3) as well as anti IL-10 (JES3-9D7), recombinant IL-10, biotinylated anti IL-10 (JES3-12G8), anti TNF alpha (MAb1), recombinant TNF alpha and biotinylated TNF alpha (MAbF6C5) were purchased from eBioscience (San Diego, CA, USA). Anti S-100 (MAb8B10), S-100 protein, biotinylated anti S-100 (MAb6G1), anti PCT (MAb16B5), biotinylated anti PCT (MAb42) and CRP-free serum were obtained from Hytest (Turku, Finland). Anti E-Selectin, human E-Selectin, biotinylated anti E-Selectin were purchased from R&D Systems (Minneapolis, MN, USA). Anti CRP (C5) and biotinylated anti CRP (C7) were from Exbio (Vestec, Czech Republic). Human procalcitonin was obtained from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel). Neopterin conjugated with bovine serum albumin (BSA) and antibodies mAb 3E2 were kindly

provided by Milan Franek, Veterinary Research Institute, Brno, Czech Republic and labelled with Dy647 by Exbio. Dy647 Streptavidin was from Dyomics (Jena, Germany) and Cy3-Streptavidin was from GE Healthcare (Chalfont St Giles, UK). Streptavidin coated magnetic particles with a 1 μ m diameter were purchased from Roche (Basel, Switzerland), Streptavidin coated magnetic particles with a 500 nm and a 130 nm diameter were from Spherotech Inc. (Lake Forest, IL, USA) and magnetic particles with a 500 nm diameter were from micromod (Rostock-Warnemuende, Germany). Biotinylated anti Cy3/Cy5 (Cy-96), Tween 20, CHAPS, glycidyloxypyltrimethoxysilane (GOPS), polyethyleneglycol, MW 6000 (PEG 6000), ethanolamine and sodium deoxycholate were purchased from Sigma (St. Louis, MO, USA). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was from Fluka Biochemicals (St. Louis, MO, USA). Phosphate buffered saline (PBS, pH 7.2) was purchased from Gibco (Invitrogen, Grand Island, NY, USA) and MES buffered saline packs were from Thermo Fisher Scientific (Portsmouth, NH, USA).

2.2. Dy647 Streptavidin Conjugation to Magnetic Nanoparticles

All washing steps were performed via centrifugation at 16,200 g-force number for 6 min (Eppendorf Centrifuge 5417C). Five hundred μ L of a 1 mg/mL 500 nm magnetic particles suspension are coated with functionalized silica by adding 200 μ L GOPS and sonicating for 90 min, then an additional 10 μ L of GOPS was added and the particles were sonicated (Branson Ultrasonics B.V. 2510, Soest, The Netherlands) for another 90 min. The particles were washed twice with 1 $\times$ PBS and resuspended in 1 $\times$ PBS. Particles were washed 3 $\times$ with 0.5 mM MES buffer (pH 5.0) by centrifugation and resuspended in 250 μ L MES buffer. 50 μ L Dy647-streptavidin (1 mg/mL Stock) and 50 μ L PEG 6000 were added and incubated for 2 h on a rotational shaker at maximum speed (Stovall, USA). The reaction was vortexed every 30 min. The particles were washed twice with PBS and resuspended in 30 mM ethanolamine containing 1% BSA for 30 min to quench the reaction. The particles were washed 3 $\times$ and resuspended in 500 μ L 1 $\times$ PBS containing 0.05% Tween 20 and 0.1% BSA (pH 7.4) and stored at 4 $^{\circ}$ C until use. The particle concentration was estimated to be 200 μ g/mL. The protocol was adapted from [18].

2.3. Chip Fabrication

Capture antibodies were diluted in printing buffer “na” (1 $\times$ PBS (pH 7.2)/0.01% Na-deoxycholate) to concentrations of 0.4 μ g/mL for IL-6, IL-8, IL-10 and 0.5 μ g/mL for TNF alpha, S-100, procalcitonin and E-Selectin. The proteins CRP and neopterin conjugated to BSA were diluted in printing buffer “cb” (1 $\times$ PBS (pH 7.2)/0.005% CHAPS, 0.01% BSA) to concentrations of 0.5 mg/mL each. The appropriate spotting conditions (composition of print buffer, humidity during spotting and antibody concentration) were established in previous research [19]. The proteins were arrayed on proprietary ARChip Epoxy slides using the Omnigrid contact spotter from GeneMachines (pin SMP3). The spot-to-spot distance was 350 μ m. Each of the probes was spotted in triplicates in 12 identical arrays at a relative humidity of 50%. To allow full immobilization of the probes the slides were kept at 4 $^{\circ}$ C for at least three days.

2.4. Internal Calibration Curve

S-100 protein was conjugated to BSA to improve immobilization on ARChip Epoxy. The conjugate was prepared by incubating 100 μ L S-100 (0.5 mg/mL Stock) with 1 mg EDC for 10 min on an orbital shaker. An additional 10 μ L BSA (5 mg/mL) were added and the reaction was allowed to proceed for 4 h. Zero to 218.7 ng/mL S-100-BSA-conjugate in buffer “cb” was then spotted onto the chip.

2.5. Chip Processing

Surface blocking was performed for 30 min in 1 $\times$ PBS (pH 7.2)/0.1% Tween 20 in order to remove any unbound probes and to deactivate reactive surface groups. Slides were washed twice in 1 $\times$ PBS (pH 7.2) and dried using compressed air. Assay buffer used consists of CRP free human serum and 0.1 M Tris (pH 7.4), 10 mM CaCl₂, 100 mM NaCl, 0.1% Tween in a ratio of 1:10. This corresponds to 20 μ L patient serum sample diluted with 180 μ L Tris buffer for the classical chip and 4 μ L patient serum sample diluted with 36 μ L Tris buffer for the miniaturized chip.

2.5.1. Classical Chip

The protocol of the “classical sepsis chip” has been introduced before [14]. Briefly, for one experiment a 4-slide set was mounted into the FastFrame (Whatman) to generate 4 $\times$ 12 array fields (7 mm $\times$ 7 mm each). This set accommodates 12 replicate measurements (four arrays with three replicate spots each) of nine point calibration standards and three “patient samples”, which are distributed on the slides taking into account the variation of production parameters. Each array was incubated with either 50 μ L diluted sample or a calibration standard for 2.5 h.

Calibration standards were prepared by serial dilution of mixes of analytes in CRP-free serum and diluted with assay buffer 1:10. For the so called “external calibration” nine spiked standards were applied. After washing three times with 1 $\times$ PBS (pH 7.2)/0.1% Tween-20, slides were incubated for 45 min with 50 μ L biotinylated antibody mixes with end concentrations of 1 μ g/mL each. After washing three times, slides were incubated with 4 μ g/mL Dy647 streptavidin for 45 min. All incubation steps were carried out on the orbital shaker (Stovall) at maximum speed at room temperature. Slides were washed and dried using compressed air and stored in the dark until scanning.

2.5.2. Miniaturized Chip

While the classical chip was processed in the FastFrame that provides wells that are 7 mm in length and width the low volume chip was arranged in silicon miniwells (GraceBio Laboratories, city, country) with incubation chambers of 4.5 mm in diameter. Those wells were small enough for a sample of 10 μ L to completely cover the wells harboring the arrays. 10 μ L patient serum sample or standard dilution series diluted 1:10 with assay buffer was incubated for 2.5 h in a humid chamber on the magnetic stirrer (Variom, Poly 15) at 450 rpm to allow sample mixing. The standard dilution series was spiked with appropriate amounts of antigen, biotinylated antibodies (1 μ g/mL) and strep.MPs. (4 μ g/mL). Detection occurred via labeling with a biotinylated antibody against Cy3/Cy5

and Cy3 labeled streptavidin (1 $\mu\text{g/mL}$ each). Slides were washed and dried using compressed air and stored in the dark until scanning.

2.6. Fluorescence Scanning

Fluorescence signals (λ_{ex} : 635 nm, λ_{em} : 670 nm) were recorded using the Genepix 4000B non-confocal scanner (Axon Instruments, Sunnyvale, CA, USA). Data were analyzed with the Genepix 6.0 software. The photomultiplier tube (PMT) voltage was held constant throughout the scans for each analyte to allow data comparison.

2.7. Data Analysis

The mean signal values were calculated from 12 replicates and were background corrected. Data that were out of the mean signal values $\pm$ the standard deviation (SD) were excluded. Standard curves were set up with GraphPad Prism 5.0 using the five parameter logistic fit.

The IC_{50} value (also reported as ED_{50}) is defined as the concentration halfway between the zero point and the upper asymptote when the value of the asymmetric factor is 1. The limit of detection (LOD) is defined as lowest concentration that can be detected and was calculated by the mean zero concentration + three standard deviations (SD) of the blank [20]. The limit of quantification (LOQ) was defined as the lowest analyte concentration that could be reliably and quantitatively determined and was calculated by the mean zero concentration + 10 standard deviations of the blank [20]. The inter-array variability refers to the variation between the arrays ($\text{CV}_{\text{inter}}\% = \text{SD}/\text{mean} \times 100$) and the intra-array variability to the variation within one array ($\text{CV}_{\text{intra}}\% = \text{SD of one triplicate}/\text{mean of one triplicate} \times 100$). Recoveries were calculated to identify the accuracy of the assay displaying the % relation between the calculated concentrations and the expected concentrations.

3. Results and Discussion

3.1. Comparison of Chip Formats: Classical versus Miniaturized

To prevent excessive blood withdrawal from newborns the sepsis chip was miniaturized by applying a fivefold reduced sample volume. The main challenge in the realization of the miniaturized protein chip was developing the appropriate incubation and stirring system to maintain good assay performance with respect to sensitivity, reproducibility and accuracy. IC_{50} values, CVs and LODs of the resulting calibration curves were defined as criteria for assay performance. The assay designs used in the classical and miniaturized chip are presented in Figure 1. Figure 1(a) shows the classical chip format: 20 μL of patient serum sample diluted with 180 μL assay buffer are incubated and analyte concentrations are detected by incubating biotinylated detection antibodies and Dy647-labeled streptavidin. Three incubation steps are necessary to accurately run the classical chip resulting in an entire incubation time of 4 h. Figure 1(b) presents the miniaturized chip format: only 4 μL of patient serum sample diluted with 36 μL assay buffer are required to accurately run the assay with a four-fold replication. Strep.MPs are implemented in the assay for two reasons: first, to stir the small volumes during sample incubation, and secondly, to act as a detection reagent when bound to the biotinylated secondary antibodies. In the second incubation step biotinylated antibody against Cy3/Cy5 and Cy3 labeled Streptavidin are added. Hence, only two incubation steps are necessary to accurately run the miniaturized chip resulting in an entire incubation time of 3 h and 15 min.

Figure 1. On-chip sandwich immunoassay steps for (a) the classical chip (b) the miniaturized chip and (c) the optimized miniaturized chip. Capture antibodies are immobilized equally providing same array format for the classical chip and the miniaturized chip. a(I) incubation of analytes (2.5 h), a(II) incubation of biotinylated detection antibodies (45 min), a(III) detection of bound antigens/antibodies via Dy647 Streptavidin incubation (45 min); b(I) Incubation of analytes, biotinylated antibodies and streptavidin conjugated magnetic particles in one step (2.5), b(II) detection via biotinylated antibody against Cy3/Cy5 and Cy3 labeled streptavidin; c(I) Incubation of analytes, biotinylated antibodies and Dy647 Streptavidin conjugated magnetic particles in one step (2.5 h).

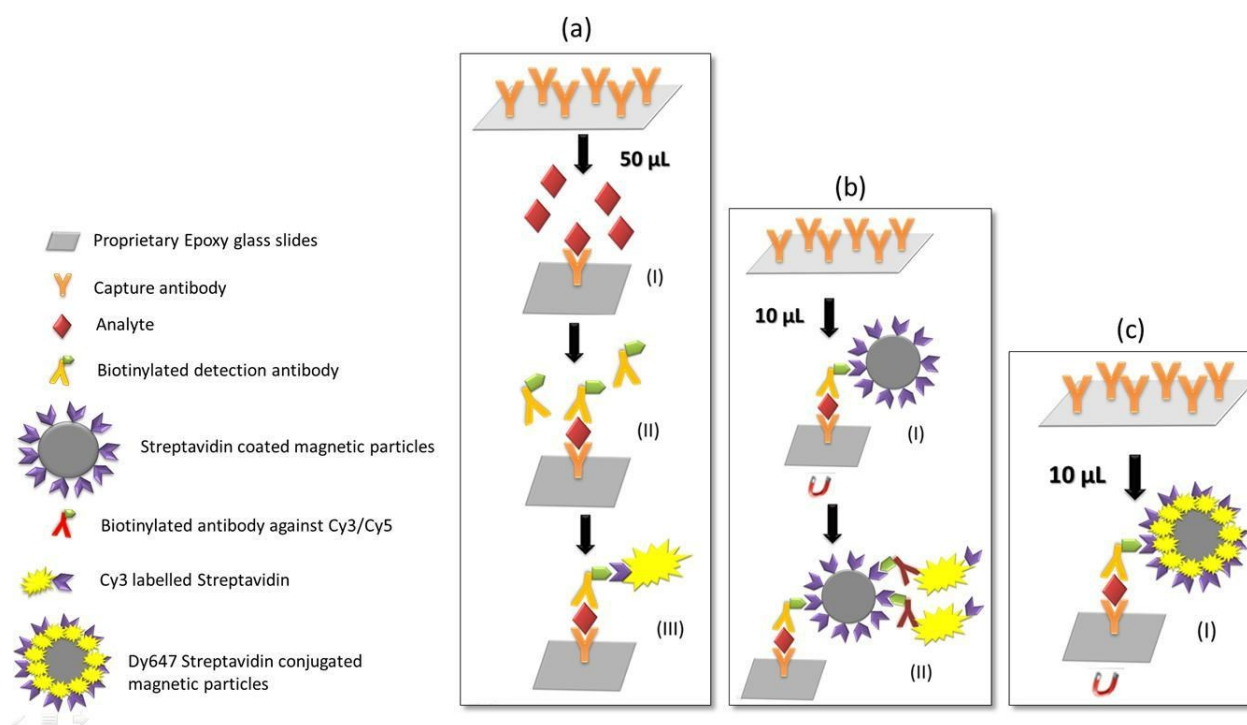
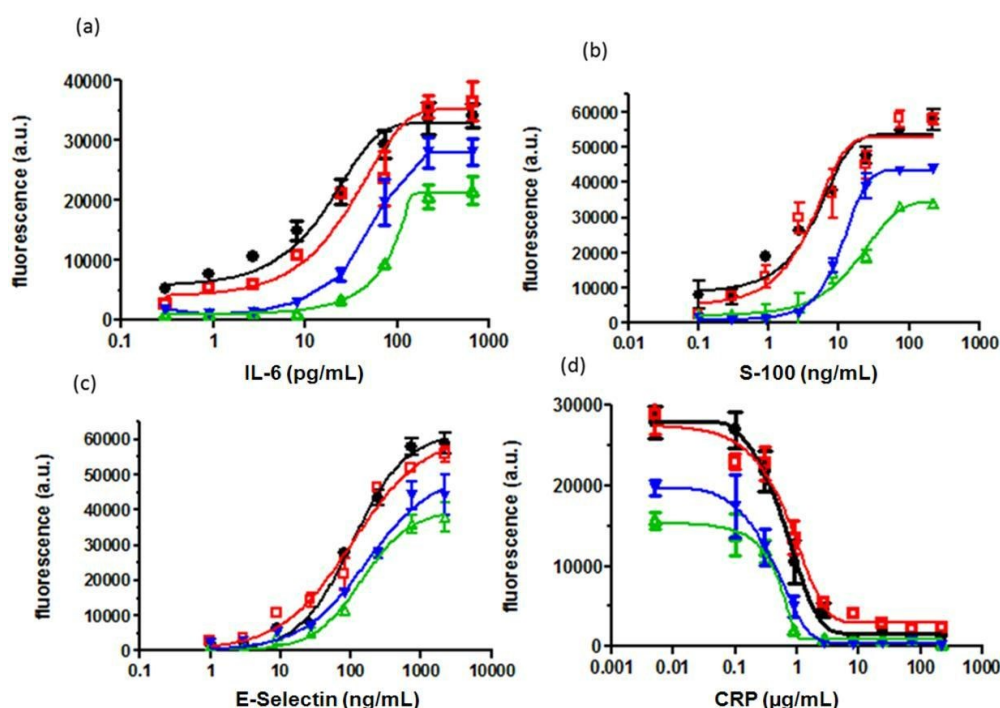


Figure 2 shows the standard curves obtained for S-100, IL-6, CRP and E-Selectin when comparing the assay performance of the classical chip (50 μL) with the miniaturized chip (10 μL without strep.MPs). The IC_{50} value was taken as evaluation parameter since it lies within the ascending slope providing the highest precision of the measurement and a good reference point for comparison of different treatments. For the classical chip the IC_{50} values for IL-6, S-100, E-Selectin and CRP are 46 pg/mL, 10 ng/mL, 170 ng/mL and 4.7 $\mu\text{g/mL}$ respectively, while for the miniaturized chip they were 82 pg/mL, 19 ng/mL, 202 ng/mL and 2.5 $\mu\text{g/mL}$. This demonstrates that reduction of the sample volume leads to losses in sensitivity. Obviously, sample volumes of 10 μL are too small to be properly mixed on the orbital shaker during incubation. Thus, strep.MPs were implemented as so-called stirring detection reagents in the assay to accelerate the antibody antigen kinetics. Acceleration of diffusion processes is a common issue and magnetic particles have been previously outlined as successful tool to provide sample mixing in microscale devices [21, 22]. In optical biochips, Heer *et al.* [23] reported up to a four-fold enhancement of fluorescent signals upon controlled movement of magnetic microspheres in the DNA hybridization buffer, while Smith *et al.* [18] used antibody-labeled magnetic particles to pre-concentrate the target via magnetic separation and integrate them as

a tracer in the immunoassay.

Oligonucleotide- [24] and antibody-labelled [25] magnetic particles were also used in magneto-resistive biochips. In our assay strep.MPs were implemented to firstly, achieve effective sample mixing and secondly, to measure antigen antibody binding events using fluorescence detection. Figure 2 shows the calibration curves obtained when adding strep.MPs to the test solution (10 μ L). The assay performance was significantly enhanced in terms of the assigned IC_{50} values (16.5 pg/mL, 4.3 ng/mL, 105 ng/mL and 4.4 μ g/mL for IL-6, S-100, E-Selectin and CRP).

Figure 2. Calibration curves for (a) IL-6 (b) S-100 (c) E-Selectin and (d) CRP. The black curve ($\bullet$) displays the 10 μ L assay and the red curve ($\square$) shows the 50 μ L assay when adding strep.MPs; the blue curve (∇) displays the classical 50 μ L assay and the green curve ($\blacktriangle$) displays the 10 μ L assay without addition of strep.MPs.



Nano-sized and micro-sized strep.MPs (130 nm, 500 nm and 1 μ m) were applied to the miniaturized assay to assess potential concerns about steric hindrance, but the assay performance was similar no matter what particle size was used.

Comparison of the miniaturized protein chip with and without strep.MPs shows that sensitivity is increased by a factor of 2 (for E-Selectin and CRP) to 4 (for IL-6 and S-100) through the addition of strep.MPs. In Section 3.2.1 this method will be further advanced in that fluorescent strep.MPs will be implemented as stirring detection reagent.

3.2. Optimization of the Miniaturized Chip

3.2.1. Reducing Assay Steps

As outlined in Figure 1 the classical 50 μ L chip (a) requires three separate incubation steps whereas the miniaturized bio-chip (b) requires only two incubation steps. In order to optimize the low volume chip the assay was performed in one step only (Figure 1(c)). This means that analytes, biotinylated

antibodies and streptavidin coated magnetic particles were incubated simultaneously on the chip. Sauer *et al.* [14] showed that in the classical 50 μL format the assay performance of an “all-in-one-step” assay was significantly deteriorated compared to the three step assay. However, this does not apply to the new low volume chip, since this protocol incorporates strep.MPs which strongly promote mixing of the solution and analyte binding. Still, the detection step (Figure 1(b-II)) of the miniaturized biochip is quite complex. Therefore, magnetic particles were conjugated with dye labeled streptavidin in order to completely replace the second incubation step. Figure 2 compares the assay performance of the optimized low volume assay (black curve) with the classical assay (red curve) showing that the standard deviations, the inflection point and the maximal signals of the curves are similar. This indicates that reducing the number of assay steps has no impact on the assay sensitivity and reproducibility of results. The new low volume assay accomplishes within 2.5 h providing an accurate “all-in-one-step” assay for point-of-care diagnostics of sepsis-relevant biomarkers.

3.2.2. Optimizing Incubation Time

To guarantee a fast and effective treatment of the disease, a rapid diagnosis and thus short assay times are essential. However, in order to achieve short assay times, a compromise must be made between incubation time, assay sensitivity and data reproducibility. In order to determine the lowest incubation time necessary to perform a sensitive and accurate chip analysis three different incubation times were tested and evaluated using IC_{50} , CV and LOD as measures for the assay performance. While for the detection of IL-6, S-100 and E-Selectin sandwich immunoassays were performed, a binding inhibition test was done for CRP. Figure 3 shows the calibration curves for IL-6, S-100, E-Selectin and CRP obtained with the low volume chip described in Section 3.2.1 after 1.5 h, 2 h and 2.5 h “all-in-one” incubation.

Figure 3. Calibration curves for (a) IL-6 (b) S-100 (c) E-Selectin and (d) CRP in human serum 1:10 diluted with assay buffer after 2.5 h (●), 2 h (◻) and 1.5 h (▲) processing time.

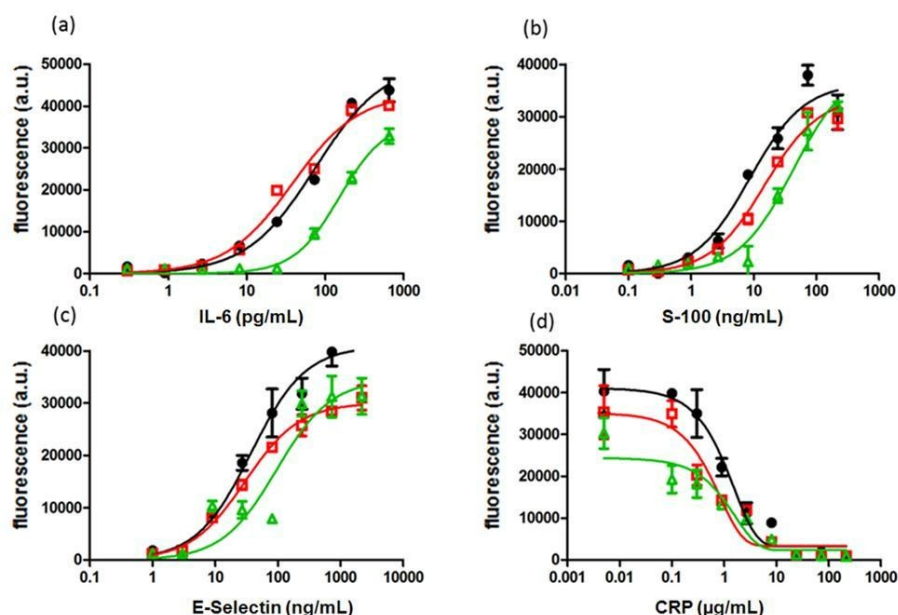


Figure 3 indicates that the effect of incubation time on the calibration curves is similar for all analyzed biomarkers. A decrease of incubation time provokes a loss in sensitivity which can be seen from the shift of calibration curve to higher analyte concentrations, reduced fluorescence signals and increased IC₅₀ values. IC₅₀ values are listed in Table 2 together with LODs and CVs. While sensitivity expressed by LOD, LOQ and IC₅₀ is similar for IL-6 and E-Selectin after 2 h and 2.5 h incubation, there are significant differences observed for S-100 and CRP. Sensitivity was drastically reduced for all tested biomarkers, if incubated for only 1.5 h. Interestingly, reproducibility of data was not affected and remained the same (CV < 14%) regardless what incubation time or analyte was used.

Table 2. Assay parameters for IL-6, S-100, E-Selectin and CRP obtained after 2.5 h, 2 h and 1.5 h incubation.

Analyte	IL-6 (pg/mL)			S-100 (ng/mL)			E-Selectin (ng/mL)			CRP (µg/mL)		
Time	2.5 h	2 h	1.5 h	2.5 h	2 h	1.5 h	2.5 h	2 h	1.5 h	2.5 h	2 h	1.5 h
LOD	1.1	1.2	149.0	0.7	1.8	5.0	4.0	6.3	30.1	0.10	0.27	0.48
LOQ	6.4	7.0	151.5	1.6	7.1	14.7	12.6	11.4	41.7	0.22	0.42	0.87
IC ₅₀	68	70	152	9	15	43	31	30	96	30	64	138
CV (%)	11	10	14	10	8	14	11	12	11	10	11	13

In summary, a 2 h incubation step is most likely sufficient for the diagnosis of neonatal sepsis. While assay sensitivity remains unchanged for IL-6 and E-Selectin, assay sensitivity is slightly decreased for S-100 and for CRP. For diagnostic purposes the right balance between incubation time and sensitivity has to be specified individually according to the clinical requirements.

3.3. Quantification of “Patient” Samples

The optimized low volume protein chip as described in Section 3.2 was tested for quantification of “patient” samples and evaluated in a recovery experiment. The assay performance was assessed by recovery values calculated as the relation of interpolated and expected concentrations. Recoveries that lie within 70 and 130% are considered as acceptable in clinical diagnosis indicating reasonable accuracy of the assay [26]. Three “patient” samples with known clinically relevant concentrations of IL-6, S-100, E-Selectin and CRP were applied additionally to the standard dilution series. The concentrations of all “patient” samples were chosen to comply with real patient serum samples. Each analyte was tested in 3 different concentrations ranging from low to high clinically relevant values to cover the whole notable range.

Mean intra-array variations of 11.8%, 10.3%, 12.4% and 13.3% for IL-6, S-100, E-Selectin and CRP respectively are obtained. Average inter-array variability of the four tested markers was 11.7% which indicates a good chip-to-chip reproducibility. Figures of merit are summarized in Table 3. High accuracy (within 70 to 130%) is obtained for concentrations within the ascending part of the slope whereas recoveries obtained for especially low or high concentrations are out of the clinically acceptable range.

Table 3. Recoveries, LODs, LOQs and inter- and intra-array variability for the optimized low volume assay.

Biomarker	Conc _{exp}	Conc _{calc}	Recovery (%)	LOD	LOQ	CV _{inter} (%)	CV _{intra} (%)
IL-6 (pg/mL)	1.50	1.2	79	1.2	6.5	10.7	11.8
	7.5	6.6	88				
	37.5	64.5	172				
S-100 (ng/mL)	0.50	- (*)	-	0.89	5.3	10.0	10.3
	2.5	1.7	69				
	12.5	11.7	94				
E-Selectin (ng/mL)	4.50	4.2	92	2.7	4.5	12.2	12.4
	22.5	16.8	75				
	112.5	103.0	92				
CRP (µg/mL)	0.5	1.0	200	0.13	0.32	14.0	13.3
	2.5	2.6	102				
	12.5	- (*)	-				

(*) out of the range of the calibration curve.

3.4. Internal Calibration Curve

For calibration of the protein microarray and quantification of the unknown sample a standard dilution series was prepared for each assay (external calibration). To do so, nine spiked antigen mixes replicated four times were applied to 36 wells. This leaves 12 wells for patient samples (e.g., three samples, four replicates) when using a set of four slides. To reduce the effort and further simplify the protein-chip the calibration was directly integrated on the chip (internal calibration). Several attempts to provide a one patient, one biochip format have been made by immobilization of the protein [27] or the detection components [28] in increasing concentrations. In our approach S-100 was conjugated to the carrier protein BSA and subsequently immobilized in increasing concentrations on ARChip Epoxy forming an internal calibration. The array of the immobilized BSA-S-100 conjugate and specific capture antibodies was then reacted with the unknown sample, biotinylated secondary antibodies and 500 nm Dy647-strep.MPs as schematically shown in Figure 2(c). Chip performance of the internal and external calibration curve was compared. As can be seen from Figure 4, both internal and external calibration curves are very similar in terms of shape, measurement range and sensitivity as defined by the slope of the curve which demonstrates the suitability of the approach. Nevertheless an internal standard curve is advantageous over an external curve as it needs less reagents and shorter handling time and allows faster availability of test results: while quantification via an external calibration curve requires nine wells per slide and one well for the sample of interest, integration of an internal standard curve reduces the number of wells per slide to only one well, the sample well.

Figure 4(a) compares the curves obtained for the internal and external calibration, and Figure 5 shows a typical fluorescence image of processed calibration spots. The image demonstrates the high quality of the arrayed spots which is depicted by the regular spot morphology, the concise shape and the even distribution within the spot. This is also reflected in the good reproducibility of biomarker measurement as can be seen from Table 3: the coefficient of variation is $\leq 14\%$.

Figure 4. Calibration curves (a) and Scatter plot (b) for the internal (•) and external chip calibration (■). Internal and external calibration curves cover a very similar measurement range and most fluorescence intensity values cluster within the 95% confidence band (grey dotted lines) of the fit line. The coefficient of determination (R^2) was 0.968.

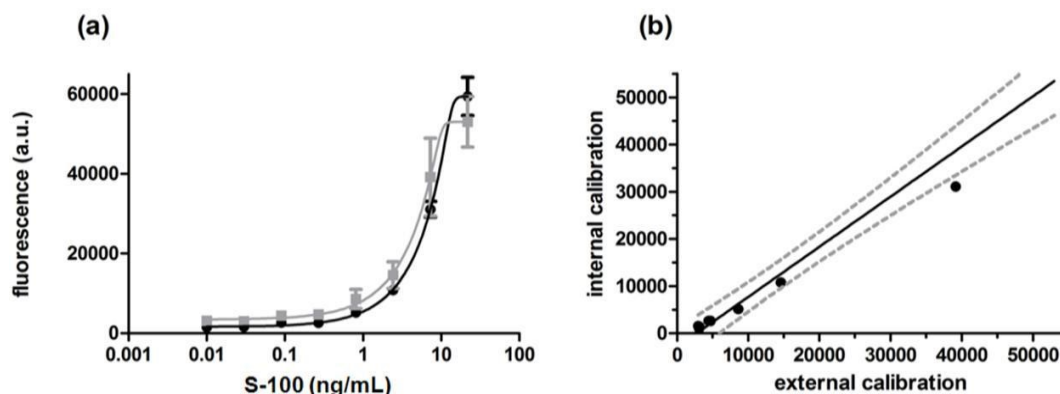
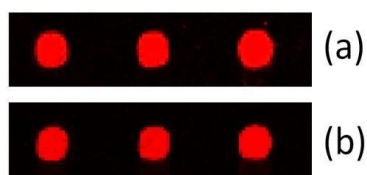


Figure 5. Processed chip showing spots obtained for the external (a) and internal (b) calibration at a concentration of 8.1 ng/mL S-100.



The calibration curves are almost identical both in terms of shape and maximal signal intensities which is in accordance with the assay parameters obtained (internal calibration: LOD 0.239 ng/mL, LOQ 1.152 ng/mL, IC_{50} 3.38 ng/mL, CV_{inter} 12%; external calibration: LOD 0.241 ng/mL, LOQ 0.976 ng/mL, IC_{50} 2.84 ng/mL, CV_{inter} 13%). Figure 4(b) depicts the correlation and precision between internal and external calibration. The fluorescence signals obtained for the external calibration are plotted against the fluorescence signals from the internal calibration. A strong linear correlation (coefficient of determination (R^2) of 0.968) was observed: most values cluster within the 95% confidence band (grey dotted lines) of the fit line. The significance of the linear correlation is confirmed by a p-value below 0.01%. The mean intra-array variation was 10.4% for the external and 10.8% for the internal calibration.

Additionally to the standard dilution series “patient” samples with known analyte concentrations (2.5 ng/mL and 12.5 ng/mL) were processed on the chip to evaluate the accuracy of the calibration curves calculated from the %-relation between interpolated concentrations and expected concentrations. The recoveries obtained were 70% and 103% when interpolating to the external calibration and 70% and 83% when interpolating to the internal calibration. The recoveries are within 70–130% which is appropriate for clinical purposes.

4. Conclusions

We have presented herein a protein chip that fulfills the requirements for point-of-care diagnostics in terms of miniaturization, assay time and internal calibration. A sample volume of 40 μ L which

corresponds to 4 μ L patient serum sample diluted 1:10 with assay buffer is sufficient to quantify sepsis associated serum proteins. The optimized low volume chip is especially attractive for diagnosis in neonates since the reduced sample volume minimizes stress and pain caused through excessive blood withdrawal. Sample mixing to overcome diffusion limitations is ensured through the addition of streptavidin coated magnetic particles (strep.MPs) acting as both stirring and detection components. Fluorescence labeling of the streptavidin coated magnetic particles further accelerates and simplifies the assay assembly resulting in a single step assay that is accomplished within 2.5 h. We demonstrated that for some analytes (exemplarily shown for IL-6 and E-Selectin) an incubation time of 2 h is sufficient to allow sensitive and accurate quantification. For some analytes (exemplarily S-100 and CRP) shortening of the incubation time to 2 h accounts for decreased sensitivity calling for variation of the incubation time according to the requirements with respect to assay sensitivity and assay time. The establishment of an internal calibration makes the protein chip even more attractive for point-of-care applications since it further simplifies the device in terms of quality control and handling. The low volume protein-chip allows accurate determination of the sepsis biomarkers with good reproducibility (CV < 14%). Recoveries are within the clinically acceptable range (70–130%).

Acknowledgments

The authors thank the Austrian Research Promotion Agency (FFG) for financial support of the MNT Era Net Project “Valipro” (No. 823977) within the Austrian NANO Initiative Programme Action

Line “Transnational Cooperative RTD Projects”.

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Publication 2

Four assay designs and on-chip calibration: Gadgets for a sepsis protein array

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ANALYTICAL CHEMISTRY, SUBMITTED IN JANUARY 2014

P. Buchegger carried out all microarray experiments and data evaluation. C. Preininger contributed to data interpretation, planned the concept and supervised the work.

Four assay designs and on-chip calibration: Gadgets for a sepsis protein array

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KEYWORDS: Protein array, Sepsis, Internal Calibration, Phosphocholine, High density labeling

Abstract

A protein microarray for the early-stage diagnosis of sepsis that allows the simultaneous detection of C-reactive protein (CRP) (2 – 200 $\mu\text{g/mL}$), Procalcitonin (PCT) (0.2 – 50 ng/mL) and Interleukin 6 (IL-6) (2 – 2000 pg/mL) has been developed. To enable the parallel detection of the differently abundant analytes, the low binding affinity between CRP and phosphocholine is exploited in a “low-sensitive” sandwich assay for CRP. The calibration is integrated directly on the chip resulting in a “one patient – one array” format, to provide a user-friendly and rapid diagnostic tool. Four different assay designs are introduced: (I) the classical assay that works with biotin-streptavidin chemistry, (II) the rapid assay that is performed in a single detection step and two ultra-sensitive assay designs accomplished either by (III) an enzymatic or (IV) an antibody mediated amplification resulting in high density labeling. The assay designs were evaluated by the repetitive measurement of low, medium and high concentration levels of commercially available certified control sera. The precision was similar across all assay designs (CV_{intra} : 8 – 14 %, CV_{inter} : 18 – 34 %), while the sensitivity (LODs) increased by one order of magnitude for the ultra-sensitive assays (III, IV) and the accuracy was analyte dependent but best for the classical (I) and the antibody amplified (IV) assays.

Introduction

Microarrays constitute a valuable analytical tool for multiplex and high-throughput analysis and are widely used in genomics ¹ and proteomics ². During the last decades, protein chips have found increasingly acceptance for diagnostic applications due to the sweeping advantages over conventional bioanalysis systems in terms of miniaturization, parallelization and sensitivity ³.

Sepsis is a systemic body infection triggered by pathogens like bacteria, viruses or fungi and has a population incidence of 0.5 to 1.5 in 1000 ⁴. Despite the low sensitivity and delayed availability of test results, blood cultures still constitute the gold standard in the diagnosis of sepsis ⁵. If diagnosed in an early stage, sepsis can be cured by the timely administration of antibiotics. Therefore, diagnosis asks for rapid and reliable diagnostic tools.

Herein we present a customer-friendly, simple and rapid diagnostic tool for the early and specific diagnosis of sepsis based on the simultaneous quantification of the protein biomarkers ⁶ C-reactive protein (CRP), Procalcitonin (PCT) and Interleukin 6 (IL-6).

CRP is an acute-phase protein that gets augmentedly expressed in the course of various inflammatory conditions. The serum levels of healthy individuals are below 10 µg/mL but CRP levels approach 300 µg/mL in response to severe infections ⁷.

PCT is a more specific marker for bacterial infections ⁸. The physiological values are below 0.1 ng/mL and as PCT levels rise with the severity of sepsis, serum levels may advance to 100 ng/mL in critically ill patients ⁹.

IL-6 is a pro-inflammatory cytokine and its serum levels increase from 10 pg/mL in healthy persons to more than 1000 pg/mL in septic patients ¹⁰. Due to the complexity of the disease, only the combinatorial use of the biomarkers allows the specific and sensitive diagnosis of sepsis.

CRP exceeds the levels of PCT and IL-6 by three and six orders of magnitude. It consists of 5 identical subunits of approximately 23 kDa each. The subunits are non-covalently linked and each subunit has two binding sites for Ca^{2+} and one binding site for phosphocholine.

Phosphocholine binds to CRP with low affinity ($K_D = 5 \mu\text{M}$) at a physiological Ca^{2+} concentration (10 mM)¹¹ whereas antibodies usually have K_D s in the lower nM range. The binding between CRP and phosphocholine is used for the specific CRP detection in various assay formats like turbidity measurements¹² or ELISA tests¹³. Herein, we exploit the low binding affinity of phosphocholine for the development of a “low-sensitive” sandwich assay to cope with the high abundance of CRP in serum and hence enable the simultaneous detection of CRP, PCT and IL-6.

To provide a customer-friendly and simple diagnostic tool, the multiplex protein array works in a “one patient – one array” assay format that integrates the calibration necessary for biomarker quantification directly on the chip. This reduces the effort of preparing a so-called external calibration as is typically used in ELISAs but also compensates for inter-array deviations that occur during the various processing steps. The integrated (internal) calibration is realized by immobilizing detection antibodies in increasing amounts.

A major drawback of many immunoassays is their lack of accuracy especially in the lower concentration ranges of low abundance proteins. Much effort has recently been made to develop methods for signal amplification in order to improve the overall assay sensitivity. The most promising techniques constitute the loop mediated isothermal amplification^{14, 15}, the tyramide signal amplification¹⁶ and the antibody-mediated signal amplification¹⁷. We herein adapted the last two amplification methods for enhanced immuno-sensing.

The following sections will demonstrate that our multiplex protein array allows the simultaneous quantification of CRP, PCT and IL-6 in a self-calibrating manner, hence is suitable for the early and reliable diagnosis of sepsis. Four different assay designs will be evaluated in terms of sensitivity (LODs), precision (CVs) and accuracy (recoveries): (I) the classical biotin-streptavidin assay, (II) the rapid assay using directly labeled antibodies, (III) the enzymatic amplification assay and (IV) the antibody amplified assay.

Experimental Section

Materials. Proprietary ARChip Epoxy glass slides served as substrates for microarray printing. CRP-free human serum, mouse anti-human PCT (MAb42), biotinylated mouse anti-human PCT (MAb16B5) as well as mouse anti-human CRP (MAbC7) were from Hytest (Finland). Rat anti-IL-6 (MQ2-13A5), biotinylated rat anti-human IL-6 (MQ2-39C3) and human IL-6 were purchased from eBioscience (USA). Human CRP was from Meridian Life Science (USA) and human PCT was obtained from ProSpec-Tany Techno Gene Ltd (Israel). Rabbit anti-human IL-6 (4H16L21) was from Life Technologies (USA). Dy647-Streptavidin as well as Dy647 labeling of the detection antibodies was from Dyomics (Germany). Anti-rat IgG (H+L) CF-647, anti-mouse IgG (H+L) CF-647, Tween 20, sodium deoxycholate, cytidin-5'-diphosphocholine sodium salt dehydrate, bovine serum albumin (BSA), Streptavidin-HRP polymer, carbonate-bicarbonate buffer capsules (pH 9.6) and glycerol (99 %) were obtained from Sigma (USA). 4-Aminophenylphosphorylcholine was purchased from Santa Cruz Biotechnology Inc. (USA). EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) was from Fluka Biochemicals (USA). DST (disuccinimidyl tartrate), BS³ (bis[sulfosuccinimidyl] suberate) and BS(PEG)5 (bis[sulfosuccinimidyl] containing 5 polyethylene glycol spacers), sheep anti-human PCT (PA1-75361) as well as MES buffered saline packs (pH 4.7) were from Thermo Fisher Scientific Inc. (USA). PBS (phosphate buffered saline, pH 7.2) was from Gibco (Invitrogen, USA). TRIS (2-Amino-2-hydroxymethyl-propane-1,3-diol) was from Merck KGaA (Germany) and Alexa Fluor® 647 tyramide was from Invitrogen Molecular Probes® (USA).

The low (15.4 µg/mL) and medium (70.6 µg/mL) level serum control samples for CRP (CRP liquid L-1, CRP liquid L-2) were from Seronorm (Norway). The high (199.3 µg/mL) serum control samples for CRP (Invitrol CRP high) and all serum control samples (0.75 ng/mL, 3.7 and 17.25 ng/mL) for PCT (Invitrol Procalcitonin) were purchased from invicon diagnostic concepts

GmbH (Germany). The IL-6 (10.8 pg/mL, 106.6 pg/mL and 341.6 pg/mL) control sera (CY5006) were from Randox Laboratories Limited (UK).

Conjugation of phosphocholine to BSA (PC/BSA). Two different phosphocholine derivatives (aminophenylphosphorylcholine and cytidin-5'-diphosphocholine) and four different crosslinkers (EDC, BS³, DST, BS(PEG)₅) were tested for the preparation of PC/BSA conjugates:

(1) EDC + cytidin-5'-diphosphocholine and (2) EDC + aminophenylphosphorylcholine: 2 mg PC, 2 mg BSA and 1 mg EDC were dissolved in 0.8 mL 0.1 M MES buffer (pH 4.5) and incubated for 3 hours.

(3) BS³ + aminophenylphosphorylcholine: 2 mg BSA and 2 mg PC were dissolved in 0.8 mL 100 mM Carbonate/Bicarbonate buffer (pH 7.2). 8 µL of a 50 mM BS³ solution were added and the reaction was incubated for 30 minutes. To quench the reaction 20 µL quenching buffer (1 M Tris (pH 7.5)) were added and incubated for 15 minutes on room temperature.

(4) DST + aminophenylphosphorylcholine: 2 mg BSA and 2 mg PC were dissolved in 0.8 mL 100 mM carbonate/bicarbonate buffer (pH 7.2). 4 µL of a 100 mM DST solution were added and the reaction was allowed to proceed for 30 minutes and quenched by the addition of 20 µL quenching buffer.

(5) BS(PEG)₅ + aminophenylphosphorylcholine: 2 mg BSA and 2 mg PC were dissolved in 0.8 mL 100 mM carbonate/bicarbonate buffer (pH 7.2). 4 µL of a 100 mM BS(PEG)₅ solution were added and the reaction was incubated for 30 minutes and quenched by the addition of 20 µL quenching buffer.

The conjugates were used for spotting without further purification.

Microarray fabrication. To integrate the calibration directly on the chip, the respective detection antibodies (biotinylated or fluorophore labeled) in 1x PBS/0.01 % sodium deoxycholate were immobilized in increasing concentrations besides the capture antibodies: 0 –

72 µg/mL IL-6, 0 – 160 µg/mL CRP and 0 – 450 µg/mL PCT. The capture antibodies for IL-6 and PCT were diluted in 1x PBS/0.01 % sodium deoxycholate to end concentrations of 0.4 mg/mL and 0.5 mg/mL respectively. The PC/BSA conjugate serves as capture molecule for CRP and was diluted in 1x PBS/5 % Glycerol to an end concentration of 1 mg/mL. Microarray printing was carried out using the NanoPrint™ Protein microarrayer from ArrayIt (USA). The capture molecules were spotted in triplicates, while for the internal calibration single spots were printed. 12 replicate arrays were printed on the slides at a relative humidity of 55 % using one pin (SMP3).

Microarray processing. All incubation steps were carried out on a rotational shaker. Between the various assay steps, the slides were washed with 1x PBS/0.1 % Tween 20. The last washing step was carried out in 1x PBS. Surface blocking was performed for 30 min in 1x PBS/0.1 % Tween 20 in order to deactivate reactive surface groups. The slides were washed in 1x PBS, dried using compressed air and mounted into multi-well hybridization cassettes from ArrayIt. An external calibration curve was generated for comparison of the internal calibration regarding curve shape and maximal signal intensities. Therefore, a mixture containing 1907 pg/mL IL-6, 244 µg/mL CRP and 85 ng/mL PCT was spiked into CRP-free human serum and subsequently diluted in 2.5-fold steps to obtain 9 spiked standards. The so-prepared standard dilution samples of the external calibration curve as well as the commercial serum control samples were diluted 1:2 in assay buffer (0.1 M Tris (pH 7.4) + 10 mM CaCl₂, 100 mM NaCl, 0.1 % Tween 20) and 50 µL were incubated for 2.5 h. After washing, four different detection schemes were applied depending on the requirements regarding assay time and assay sensitivity (see Figure 2): (I) classical assay: Incubation of the biotinylated detection antibodies at 1 µg/mL each for 45 min followed by the incubation of Dy647-Streptavidin at 2 µg/mL for 45 min; (II) rapid assay: Incubation of Dy647-antibodies at 1 µg/mL each for 45 min; (III) enzymatically amplified assay: Incubation of biotinylated antibodies at 1 µg/mL each for 45 min followed by the incubation of

Dy647- Streptavidin and HRP-Streptavidin at 1 µg/mL each for 45 min and subsequent Alexa Fluor® 647-tyramide incubation in assay buffer containing 0.015 % H₂O₂ for 7 min; (IV) antibody-amplified assay: Incubation of Dy647-antibodies at 1 µg/mL each for 45 min and subsequent incubation of anti-rat IgG CF-647 and anti-mouse IgG CF-647 at 2 µg/mL each for 45 min. All slides were dried using compressed air and stored in the dark until scanning.

Data analysis. Fluorescence measurements ($\lambda_{\text{ex}}=635\text{nm}$, $\lambda_{\text{em}}=670\text{ nm}$) were performed using the Genepix 4000B non-confocal scanner (Axon Instruments, USA). For data comparison, the photomultiplier tube (PMT) gain was kept constant throughout the scans for each analyte. The fluorescence signals were aligned with the Genepix 6.0 software and background corrected signal intensities were used for data analysis. The internal calibration curves were set up using GraphPad Prism 5.0. The coefficient of variation (CV) reveals the reproducibility of the assay since it is calculated from the standard deviation (SD) x 100 / mean. The inter-array CV (CV_{inter}) expresses the signal deviation between the arrays and is calculated from 12 replicate arrays. The intra-array CV (CV_{intra}) expresses the deviation between the spotted triplicates within one array. The limit of detection (LOD) is defined as lowest concentration that can be reliably detected. The LODs of the internal calibration curves were calculated from the mean values of 12 replicate arrays and the corresponding standard deviations (SD) using the following equations: $\text{LOD: blank signal} + 3 \times \text{SD}$ ¹⁸. The signals from the control serum samples were interpolated to the respective internal calibration curve to obtain the calculated concentrations. Recoveries serve as parameter to evaluate the accuracy of the internal calibration curve to the samples. The values are calculated by the following equation to get the % - relation between the calculated and the expected concentrations: $\text{Recovery (\%)} = \text{Calculated concentration} / \text{expected concentration} \times 100$. For clinical purpose the recovery values have to be within 70 – 130 %.

Results and discussion

Low-sensitive sandwich assay for the detection of CRP. Since the pathological levels of CRP might approach concentrations up to 300 $\mu\text{g/mL}$, the high CRP concentrations constitute a big hurdle for the simultaneous quantification with low abundance proteins. In other publications, the different analyte abundances are addressed by the consecutive measurement of single biomarkers^{19, 20}. For the multiplex quantification of CRP in diluted serum, a classical sandwich immunoassay has proven to be too sensitive since the antibody binding sites are saturated even at low CRP levels (data not shown). In a previously reported assay set-up, two different on-chip assay formats, competitive and sandwich immunoassays, were applied to account for the various clinically relevant concentration ranges²¹. The competitive immunoassay is less sensitive and the dynamic range can be adjusted by increasing or decreasing the concentration of the detection antibody. Although the combination of competitive and sandwich assays is especially suitable for the parallel detection of the different analytes, it can't be utilized in this work as the on-chip calibration is not compatible with competitive assay formats. To address the needs of a low sensitive sandwich assay, the low but specific binding affinity between phosphocholine and CRP is exploited. Phosphocholine serves as capture molecule in the sandwich immunoassay. In order to support the immobilization of the low-molecular weight molecule, phosphocholine is coupled to a large carrier protein. Baltzer et. al tested various polypeptides for phosphocholine conjugation and demonstrated that the binding affinity of CRP to the phosphocholine conjugates depends on the chemical makeup of the carrier peptides and that the binding of proteins to small organic ligands is supported by weak non-covalent interactions like hydrophobic or electrostatic interactions²². Based on these findings, we tested four different crosslinkers (EDC, DST, BS³, BS(PEG)₅) with varying spacer lengths as well as two different phosphocholine derivatives (cytidin-5'-diphosphocholine and aminophenylphosphocholine) for the conjugation to BSA in order to modulate the binding

behavior of CRP to the immobilized PC/BSA conjugates. The homobifunctional crosslinkers DST, BS³ and BS(PEG)₅ have varying spacer lengths: 6.4 Å, 11.4 Å and 21.7 Å respectively and contain N-hydroxysuccinimide ester groups that react efficiently with primary amino groups under formation of stable amide bonds. EDC is a heterofunctional zero-length crosslinker that links carboxyl groups with amino groups.

Although the binding affinity between phosphocholine and CRP is three orders of magnitudes lower than with conventional antibody, the assay is still too sensitive for the detection of CRP in the clinically relevant range in combination with PCT and IL-6 no matter what kind and length of crosslinker or phosphocholine derivate was used. Figure 1 exemplarily depicts the calibration curve (black circles) obtained for the PC/BSA conjugates prepared by EDC coupling of the amino-substituted phosphocholine derivate. For all conjugates, a similar trend is observed: For high concentrations (> 10 µg/mL) a hook effect caused by antigen excess is observed resulting in a false negative signal (red encircled signals). In order to shift the dynamic range and to strike the clinically relevant CRP range, we induced an artificial dilution of the CRP sample by the addition of free phosphocholine to the assay buffer. The free phosphocholine molecules bind to the CRP molecules in the sample, thus prevent them from binding to the immobilized PC/BSA conjugates. We tested different amounts of cytidin-5'-diphosphocholine ranging from 1 mM to 100 mM. Figure 1 shows that the dynamic range can be tuned by modifying the amount of free phosphocholine. For example, the addition of 10 mM phosphocholine allows reliable quantification between 0.1 µg/mL and 10 µg/mL CRP, whereas the presence of 50 mM phosphocholine shifts the dynamic range to higher concentrations (2 - 200 µg/mL). The combination of supplementing the assay buffer with 50 mM phosphocholine and spotting the amino substituted phosphocholine conjugated to BSA via EDC chemistry results in a robust sandwich assay that can be used for the quantification of CRP in the clinically relevant range.

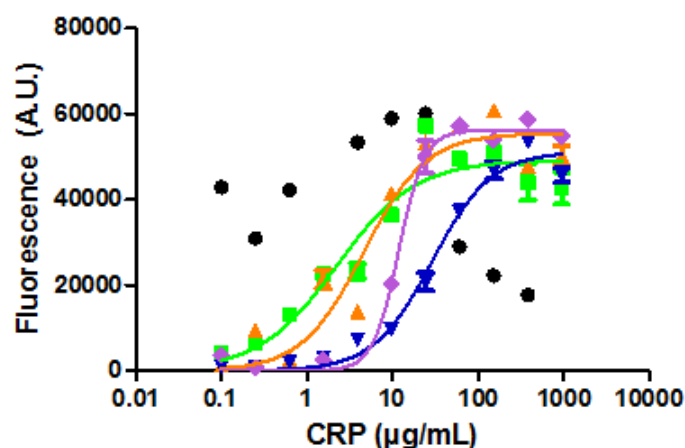


Figure 29. Calibration curves obtained for CRP in the presence of 0 mM (= control •), 1 mM (■), 10 mM (▲), 50 mM (▼) and 100 mM phosphocholine (◆)

Different assay designs including on-chip calibration. Diagnosis asks for rapid and easy to accomplish diagnostic tools. In order to reduce the effort of externally preparing a standard dilution series for analyte quantification, the calibration is integrated on the chip. Several attempts to provide a self-calibrating analytical tool are reported in literature. Jung et. al developed a self-calibrating assay based on tagged internal standards for CRP detection in human blood samples. In this assay set-up, fluorophore labeled CRP molecules compete with CRP that originates from the serum sample for binding to an antibody array²³. A similar approach is described in²⁴ where the intrinsic luminescence of magnetic particles is exploited as internal standard. In²⁵ and²⁶ increasing amounts of the target molecules are immobilized besides capture molecules to form an internal calibration. We used a similar approach to develop a self-calibrating protein array reported in²⁷. For quantification of S-100, the protein was conjugated to BSA and subsequently immobilized in increasing concentrations. Good correlations ($R^2 > 0.968$) between the internal and the external calibration curves were obtained. In order to provide a cost-effective diagnostic tool, this approach is further enhanced in a cost-cutting manner: detection antibodies are immobilized in increasing amounts besides the capture molecules. After incubation with the patient serum sample, the biomarker binding is detected according to the

four different assay designs. Figure 2 schematically depicts the applied designs: (I) The classical assay exploits biotin-streptavidin chemistry for the verification of analyte binding. (II) In order to reduce the assay time, the biotin-streptavidin detection step was omitted and fluorophore labeled antibodies were used instead. Additionally, two ultra-sensitive assay designs were employed based on (III) enzymatic amplification and (IV) two fluorophore labeled antibodies.

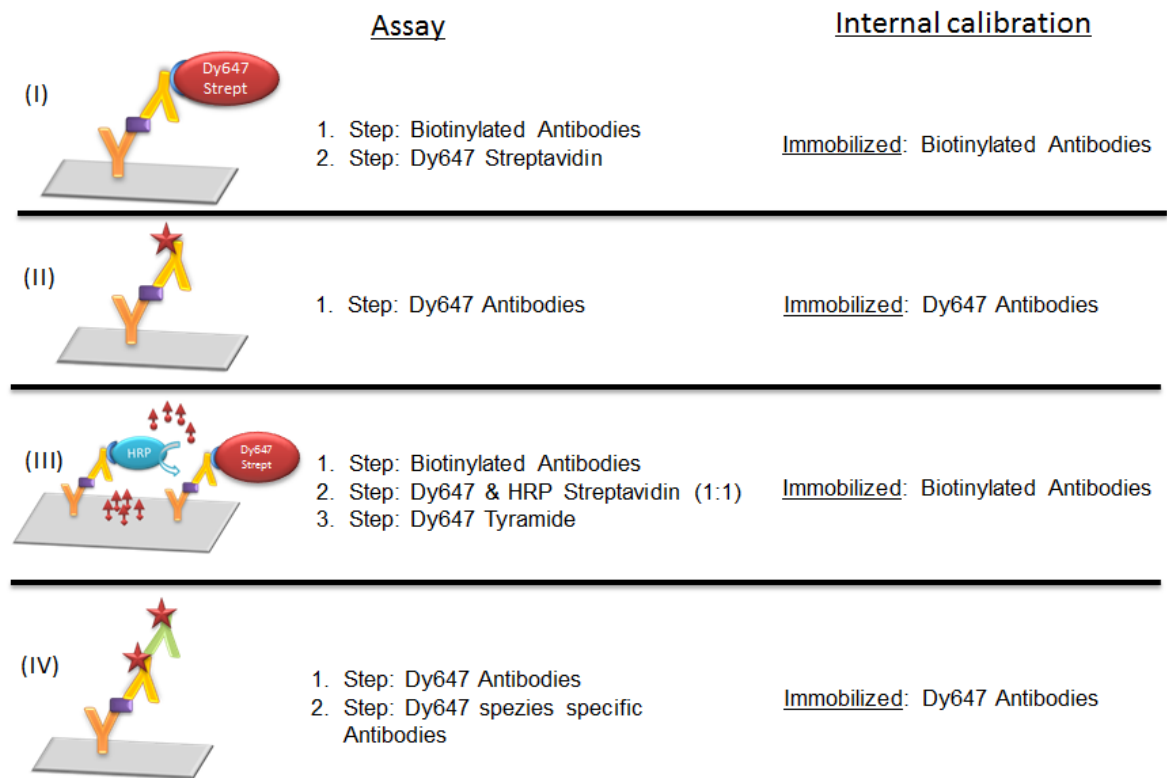


Figure 30. Schematic illustration of the assay designs I to IV.

For the classical assay (I), biotinylated detection antibodies are immobilized in increasing concentrations besides the capture molecules to form an internal calibration. To determine the accuracy of the on-chip calibration, the resulting curves were compared to the respective external calibration curves in terms of curve shape and congruence. Therefore, the externally prepared standard dilution series was incubated followed by the incubation of biotinylated antibodies. Verification of the internal and the external calibration curves was performed via fluorophore

labeled streptavidin. For the calibration curves, coefficients of correlation (R^2) > 0.986 were obtained.

In order to accelerate the assay, a one detection-step assay (II) was performed. Therefore, the detection antibodies were directly labeled with a fluorescent dye, hence merging the two detection steps of the classical biotin-streptavidin assay. To accomplish an internal calibration for the rapid assay, the biotinylated antibodies were substituted by immobilized fluorophore labeled antibodies. Again, the internal and external calibration curves match in terms of curve shape and maximal signal intensities (R^2 > 0.989) ensuring accurate quantification of unknown samples.

To address the concerns regarding assay sensitivity, two different approaches were applied: on the one hand (III) an enzymatic amplification step was included and on the other hand (IV) two fluorophore labeled antibodies were implemented to create high density labeling. The enzymatic signal amplification method (III) is accomplished by the incubation of the biotinylated detection antibodies, followed by HRP-streptavidin incubation and subsequent fluorophore labeled tyramide incubation. The enzyme HRP converts the tyramide to the short-lived but highly reactive tyramide radical. The radicals bind to phenol moieties of neighbored protein tyrosine residues, thus increase the local density of fluorophores. This amplification method proved to be highly sensitive but compatibility problems between the internal and external calibration curves emerged (data not shown). For all analytes, the external calibration curves were down-shifted to lower concentrations and the sensitivity increased by a factor of 10. Those results were dissent from the internal calibration. The curve shapes of the internal calibration significantly flattened resulting in decreased sensitivity and incongruence between the internal and external calibration. This phenomenon is probably due to quenching effects that occur in response to the high density of fluorophores. To reduce the quenching effects, the number of fluorophores was decreased by the simultaneous application of two different streptavidin variants, namely fluorophore labeled streptavidin and HRP labeled streptavidin.

Both streptavidin variants compete for the accessible binding sites resulting in decreased amounts of bound HRP available for converting fluorophore labeled tyramide. Different ratios of the two streptavidin variants were tested: 3:1, 2:1, 1:1, 1:2, 1:3. We proved that equal amounts of both streptavidin modifications manage the balance between maximum sensitivity and minimum quenching. With this three-step assay design, highly congruent calibration curves ($R^2 > 0.989$) with significantly enhanced sensitivities were obtained.

The antibody-mediated signal amplification method (IV) constitutes an elegant way to enhance the assay sensitivity in a two-step detection setup. The number of fluorophores is increased by the consecutive incubation of two fluorophore labeled antibodies. The primary detection antibody is target-specific and the secondary antibody is directed against the first antibody. This assay set-up requires sandwich antibody pairs with distinct species specificities. Since the antibody pairs for PCT and IL-6 were raised in the same species, we changed the capture antibodies for PCT and IL-6. To accomplish an internal calibration, fluorophore labeled detection antibodies were immobilized in increasing concentrations. Again, good correlation between internal and external calibration curves ($R^2 > 0.991$) was obtained. Nevertheless, the big hurdle in the development of an antibody amplified assay is to find well-performing species distinct antibody sandwich pairs.

In the following sections, the four assay designs will be evaluated by the repetitive measurement of certified reference materials. For each analyte, three concentration levels (low, medium, high) were measured: 15.4 $\mu\text{g/mL}$, 70.6 $\mu\text{g/mL}$ and 199.3 $\mu\text{g/mL}$ for CRP, 0.75 ng/mL , 3.7 and 17.25 ng/mL for PCT and 10.8 pg/mL , 106.6 pg/mL and 341.6 pg/mL for IL-6. LODs served as evaluation parameter for the assay sensitivity and the CVs served as parameter for the precision. The accuracy constitutes the key feature of a diagnostic assay²⁸ and was assessed by the obtained recoveries.

Assay Sensitivity. Figure 3 shows the internal calibration curves obtained for (a) CRP, (b) PCT and (c) IL-6 when applying the four different assay designs for comparison. The curves for the classical (I) and the rapid (II) assays are almost identical and shifted to higher analyte concentrations compared to the likewise congruent signal amplified (III, IV) curves. The LODs for the classical (I) and the rapid (II) assays were very similar: 12.8 $\mu\text{g/mL}$ and 15.3 $\mu\text{g/mL}$ for CRP, 1.6 ng/mL and 2.0 ng/mL for PCT and 19.1 pg/mL and 21.9 pg/mL for IL-6. High density labeling significantly increases the assay sensitivity as the detection limits decrease by one order of magnitude: 1.9 $\mu\text{g/mL}$ and 1.8 $\mu\text{g/mL}$ for CRP, 0.2 and 0.1 ng/mL for PCT and 2.6 and 2.8 pg/mL for IL-6. For the purpose of early-stage diagnosis, the signal amplified assays are preferable as they provide lower detection limits and allow the detection of even minor elevations of the biomarkers.

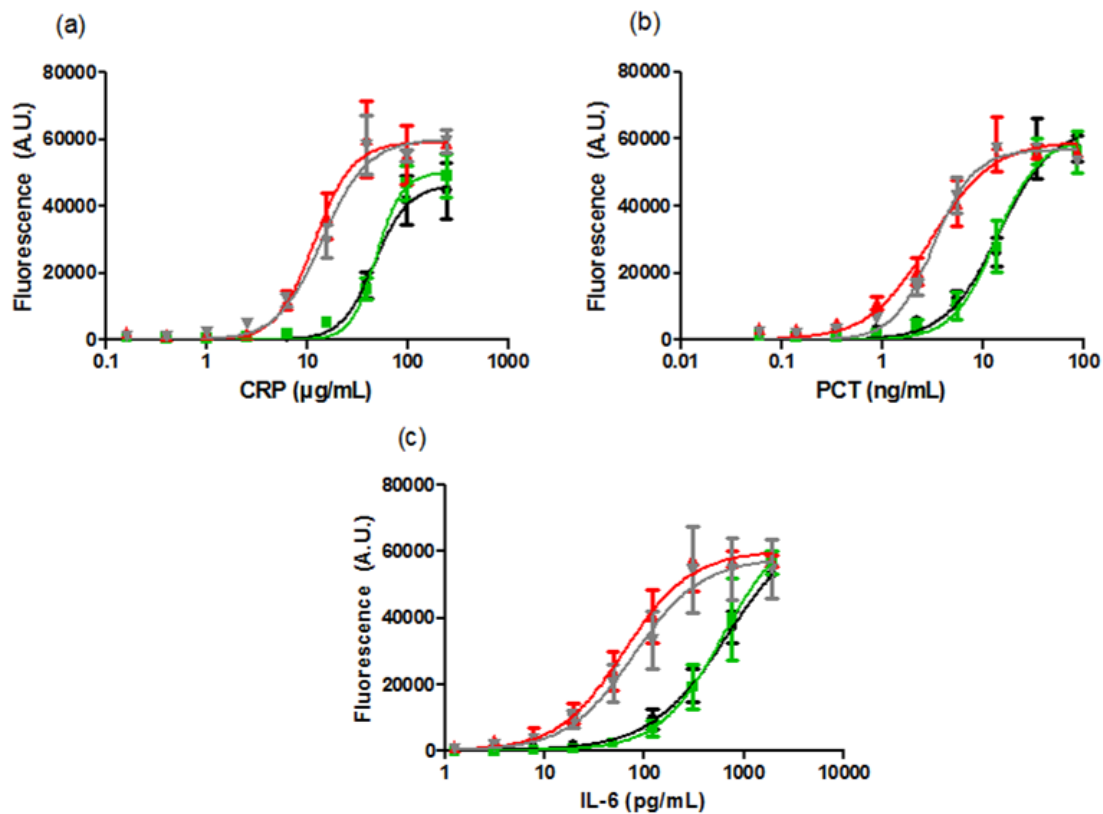


Figure 31. Internal calibration curves for (a) CRP, (b) PCT and (c) IL-6; Comparison of the four assay designs: classical ($\bullet$), rapid ($\blacksquare$), enzyme- ($\blacktriangle$) and antibody amplified ($\blacktriangledown$)

Assay Precision. Besides being highly sensitive, diagnostic tools need to be also robust and reliable. This means that measurements performed repeatedly under the same conditions shall produce the same results. The mean inter-array and intra-array CVs were calculated to assess the assay precision, hence the assay reproducibility. The inter-array precision is defined as the deviation of test results between repetitive measurements on distinct arrays whereas the intra-array precision defines the deviation of the signals derived from the same spots within one array. The weak inter-array reproducibility constitutes a common problem in bioanalytical methods, especially in immunoassays²⁹. We avoid reproducibility problems that arise with low inter-array CVs by integrating the calibration directly on the chip. Hence, with this assay format, which is a “one patient – one array” format, the intra-array reproducibility is crucial for precise repetitive measurements. Table 1 shows that the intra-array CVs are very similar for all assay designs (8 – 14 %) and significantly smaller than the inter-array CVs (18 – 34 %) showing that the deviations within one array are smaller than the deviations between different arrays.

Table 9: Assay parameter obtained for the four assay designs for the respective biomarker

Biomarker	Parameter	(I)	(II)	(III)	(IV)
CRP	LOD (µg/mL)	12.8	15.3	1.9	1.8
	CV_{inter} (%)	18	21	26	19
	CV_{intra} (%)	12	13	14	8
PCT	LOD (ng/mL)	1.6	2.0	0.2	0.1
	CV_{inter} (%)	19	29	18	18
	CV_{intra} (%)	9	11	11	11
IL-6	LOD (pg/mL)	19.1	21.9	2.6	2.8
	CV_{inter} (%)	32	34	22	29
	CV_{intra} (%)	10	13	11	11

I, II, III, IV refer to the respective assay design (see Figure 2)

Assay accuracy. Figure 4 shows the calculated recoveries obtained for (a) CRP, (b) PCT and (c) IL-6 for the respective assay design in box plots. The dashed line marks the 100 % recovery value and the dotted lines border the accepted range of 70 to 130 %.

The classical assay design (I) is a well-known and robust assay format and is therefore widely used in microarrays^{30, 31}. As shown in Figure 4, PCT and IL-6 received good mean recoveries that are within 89 % and 135 % for all three concentration levels confirming the well-established assay format. The concentrations of the low level serum controls are below the calculated LODs. Since the LOD is calculated from the blank signal and the corresponding standard deviation it is likely that the assay may yield reliable measurements below the calculated limit. In that case, the good recoveries for the low concentrations reassert the highly solid and stable classical assay. For CRP different results were obtained: mean recoveries of 110 %, 58 % and 152 % for the low, medium and high concentration levels were obtained. Accordingly, the classical assay design is especially suitable for the quantification of PCT, IL-6 and low levels of CRP but lacks accuracy for the higher CRP concentration ranges.

Despite the likewise assay performance of the classical (I) and the rapid (II) assays regarding sensitivity and reproducibility, the accuracy of the accelerated assay is significantly lower compared to the classical assay most notably for the PCT and IL-6 controls. Mean recoveries of 518 %, 191 % and 118 % and of 163 %, 80 % and 43 % are obtained for the PCT and the IL-6 serum levels. The poor precision of the lower concentrations can be explained by the fact that the lower serum levels have concentrations that are below the respective calculated LODs. Again, the situation is different for CRP: here, good mean recoveries that lie between 76 % and 96 % are yielded. To sum up, the rapid assay misses accuracy especially in the lower concentration ranges for PCT and IL-6 but yields highly accurate values for CRP across all tested concentration levels.

Except for PCT, the enzyme amplified assay (III) yields recoveries way above the accepted range. Additionally, the calculated values are widely spread showing besides poor accuracy also

low precision. More precisely, mean recoveries $> 289 \%$, $> 104 \%$ and $> 180 \%$ for CRP, PCT and IL-6 were obtained. The poor accuracy of the ultrasensitive assay is probably due to the simultaneous addition of two different streptavidin variants that compete for the available biotin binding sites. This competition reaction is likely to lack reproducibility which leads to widely spread data points and inaccurate measurements. Despite of the excellent results in the scope of assay sensitivity, the performance of the enzymatic signal amplified assay is not satisfying regarding accuracy and precision.

The antibody amplified assay (IV) yields very accurate and narrowly spread recoveries that lie within 72% and 128% for all analytes except for the lowest PCT and IL-6 concentrations. The poor accuracy in the lower concentration ranges is a prominent problem in analytical assays ³², ³³. Although the antibody amplified assay loses accuracy in the lower concentration ranges of IL-6 and PCT, this assay design constitutes a promising alternative as it allows the highly accurate determination of the raised concentration levels. Furthermore, the assay design is very sensitive and exhibits good reproducibility.

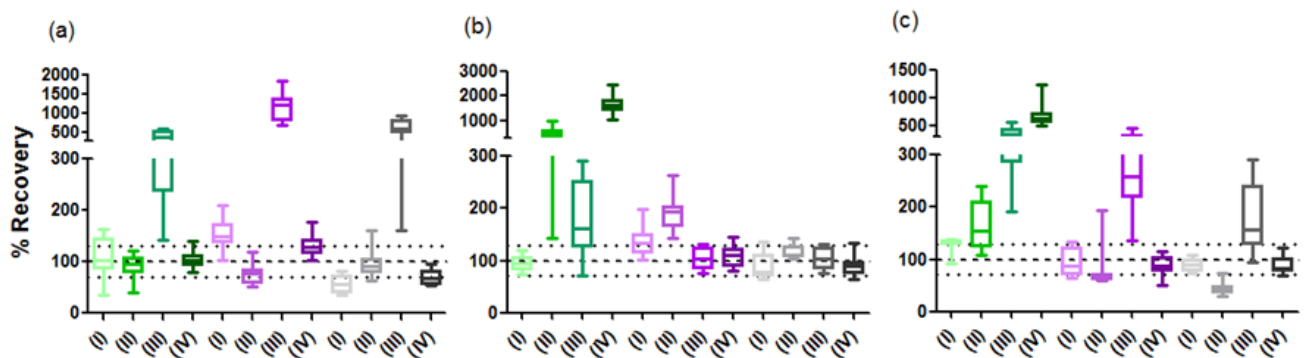


Figure 32. Box plots of the recoveries obtained for the four different assay designs (I, II, III, IV) for the low (green), medium (violet) and high (grey) (a) CRP, (b) PCT and (c) IL-6 control sera

Conclusion

We herein developed a protein microarray for the diagnosis of sepsis in a “one patient – one array” format by integrating the calibration directly on the chip. We fully met the challenge of measuring simultaneously the different abundances of CRP, PCT and IL-6 by the development of a low-sensitive sandwich assay for CRP using phosphocholine/BSA as capture probe. We introduced four different assay designs: (I) the classical biotin-streptavidin assay, (II) the rapid assay using fluorophore labeled antibodies, (III) the enzymatic amplification assay and (IV) the antibody amplified assay. The assay designs were then evaluated using certified control sera yielding the following results:

- Sensitivity: III, IV >> I, II
- Precision: I~II~III~IV
- Accuracy: IL-6: I > IV >> II, III
PCT: I > IV >> II, III
CRP: II, IV >> I, III

As the reproducibility was very similar across all assay designs, the sensitivity and accuracy were employed for the final assay assessment:

The classical assay (I) is very robust and stable and exhibits excellent recoveries except for medium and high CRP concentrations. Nevertheless, the assay sensitivity is decreased by a factor of 10 compared to the signal amplified assays (III, IV).

The rapid (II) assay is most suitable for the detection of CRP.

The enzymatic amplification assay (III) is highly sensitive. The LODs are decreased by one order of magnitude compared to the assays I and II. Nonetheless, the assay is inaccurate resulting in false-positive concentrations.

The antibody amplified (IV) assay works best with all three tested biomarkers and constitutes a promising alternative because of its excellent sensitivity.

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ACKNOWLEDGEMENTS

The authors thank the Austrian Research Promotion Agency (FFG) for financial support of the MNT Era Net Project “Valipro” (No. 823977) within the Austrian NANO Initiative Programme Action Line “Transnational Cooperative RTD Projects”.

Publication 3

Thermo-nanoimprinted biomimetic probe for LPS and LTA immunosensing

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ANALYTICAL CHEMISTRY, 2014,

P. Buchegger prepared the surface imprints, recorded the AFM images, carried out all sensor experiments and data evaluation. P.A.Lieberzeit and C. Preininger contributed to data interpretation, planned the concept and supervised the work.

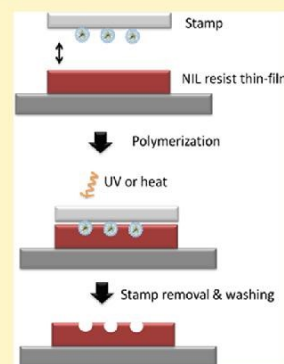
Thermo-Nanoimprinted Biomimetic Probe for LPS and LTA Immunosensing

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ABSTRACT: A complex prepolymerized film comprising monomers, cross-linkers, and initiator is usually used to create molecularly imprinted polymers. We herein exploit ready-to-use resist materials and link molecular surface imprinting with UV- and thermo-nanoimprinting techniques to create a sensor layer for the specific recognition of the bacterial surface markers lipopolysaccharide (LPS) and lipoteichoic acid (LTA). To account for the highly polar moieties of LPS and LTA, we evaluate different resist and stamp materials of distinct surface properties by AFM and molecularly imprinted sorbent assays. Thermo nanoimprinting of LPS and LTA micelles to Epon 1002F films exhibits excellent sensitivity of up to 13 times increased signals compared to those of the nonimprinted films and negligible cross-reaction with the tested nonspecific analyte. Additionally, the sensitivity and selectivity of the thermo nanoimprints is compared to conventional molecular surface imprints using a cocktail of acrylic monomers in QCM measurements.



LPS (lipopolysaccharide) and LTA (lipoteichoic acid) are surface markers specifically expressed on cell surfaces of Gram-negative and Gram-positive bacteria that for example, in the course of sepsis induce different inflammatory pathways leading to different cytokine release patterns.¹ From all occurring sepsis cases in intensive care units, 93% are caused by bacteria and can be effectively treated by the administration of antibiotics if diagnosed in an early stage.² To guide medication and therapy, the identification of the causal bacterial Gram-type is of tremendous importance. LPS and LTA can be exploited for the fast Gram-differentiation without inconvenient and time-consuming Gram staining. LPS (10 kDa) and LTA (10 kDa) are amphipathic molecules that consist of a hydrophobic lipid moiety that is responsible for anchoring the molecule into the cell membrane and a hydrophilic part that mainly consists of polysaccharides. Due to their hydrophobic and hydrophilic moieties, several LPS and LTA molecules form micellar aggregates in solution.³ We herein created nanocavities that are complementary to the respective surface markers in a polymer thin film. Therefore, we combine two prominent techniques: molecular surface imprinting and UV- and thermo nanoimprinting (hot embossing).⁴

Molecularly imprinted polymers (MIPs) are fabricated by the copolymerization of monomers and a template molecule. After elution of the template, a heterogeneous 3D network with complementary recognition sites that exhibit specific rebinding abilities is obtained. This so-called bulk polymerization approach works well for small organic molecules, but the imprinting of macromolecules (>1500 Da) implicates several obstacles caused by the increased complexity, entrapment, and diffusion limitation

of larger molecules into the dense 3D network.⁵ Therefore, much effort has been undertaken to develop methods to fabricate macromolecularly imprinted polymers. In addition to the bulk polymerization method,^{6–8} three different approaches are introduced in the literature, namely, particle,^{9–11} surface,^{12,13} and epitope^{14,15} polymerization. The surface imprinting approach constitutes the most widely used technique in the preparation of macromolecularly imprinted polymers.¹⁶ The sensitivity and selectivity of molecular imprints to the template is mainly realized through functional groups that exhibit weak and noncovalent inter-actions such as hydrogen bonds, van der Waals forces, ionic bonds, and hydrophobic interactions. The most common monomers and cross-linkers used for surface imprinting are acrylamide, methacrylic acid, aminophenylboronic acid, acrylic acid, and N-isopropylacrylamide. Frequently applied cross-linkers are bisacrylamides and ethylene glycol dimethacrylate (EGDMA), and polymerization of the respective monomer mixtures is triggered by UV exposure.¹⁶

UV- and thermo nanoimprinting/hot embossing constitutes a low-cost and high-throughput technique to create nanosized patterns in resist materials. To fabricate thermo-nanoimprinted surfaces, a stamp is pressed onto a thermoplastic polymer thin film that has been heated above its glass transition temperature (T_g) and allows therefore deformation of the film. Subsequent cooling below its T_g increases the resist's viscosity and thus freezes the nanostructures of the stamp.¹⁷ With UV-imprinting, a promising alternative to thermo imprinting has evolved since it allows imprinting at room temperature and under low pressure. UV-imprinting requires photocurable resist materials and UV permeable stamps such as quartz glass or PDMS molds.¹⁸

Received: October 25, 2013

Accepted: January 7, 2014

We herein fabricate surface nanoimprints of LPS and LTA using commercially available resist materials frequently applied in nanoimprint lithography (NIL), such as the two photo-curable resist materials ACOMO and Amonil MMS10 as well as the thermoplastic polymer Epon 1002F. ACOMO¹⁹ is a water-soluble acrylic resist, and the radical polymerization might be either thermally or UV initiated. Amonil is an organic/inorganic photoresist and has found applications in UV-based NIL.²⁰ The thermally curable Epon 1002F is derived from a liquid epoxy and bisphenol A. Due to its excellent optical properties and high biocompatibility, it has found applications in nanolithographic techniques,²¹ bioMEMS,²² and surface coatings.

Due to the amphiphilic character of LPS and LTA, different stamp materials are evaluated, particularly, glass coverslips, PVC coverslips, and soft PDMS. Lalo et al.²³ used soft lithography to fabricate MIPs in nanoscale. Therefore, the MIP precursor solution was transferred from a nanostructured PDMS stamp to the substrate via microtransfer molding. After photopolymerization, the stamp was removed leaving MIPs in the nanoscale that exhibited specific target recognition.

The resist layers are characterized by atomic force microscopy (AFM), and the rebinding ability of the surface imprints is evaluated in molecularly imprinted sorbent assays. Therefore, the imprinted cavities are exploited as plastic capture antibodies in a MIP-based sandwich-type immunoassay, and the specific analyte binding is detected via biotinylated secondary antibodies and subsequent fluorophore-labeled streptavidin binding.

Furthermore, the thermo nanoimprints are compared to conventional molecular surface imprints using a mixture of the monomers acrylamide, methacrylic acid, and methylmethacrylate. (1,2-Dihydroxyethylene)bisacrylamide (DHEBA) is used as cross-linker and azo-bis-(isobutyronitril) (AIBN) as radical starter. Since many surface-imprinted polymers are applied in QCM (quartz crystal microbalance) measurements,²⁴ the specific analyte binding is evaluated via the mass sensitive device that allows a simple and label-free analyte detection and comparison of the two methods.

MATERIALS AND METHODS

Materials. Epon 1002F was from Miller Stephenson Chemical, Co. (U.S.A.), Amonil MMS10 was from AMO GmbH (Germany) and ACOMO (acryloyl morpholine) as well as GENOCURE were from Rahn AG (U.S.A.). Sylgard 184 silicone elastomer and silicon agent were from Dow Corning Corporation (U.S.A.). Microscope slides (pure white, 1010412) and glass coverslips (borosilicate glass, 0101000) were from Marienfeld Superior (Germany). PVC coverslips were purchased from Ted Pella (U.S.A.). Isopropanol (99.5%), cyclopentanone, lipopolysaccharide from *Serratia marcescens* and lipoteichoic acid from *Bacillus subtilis* were from Sigma. Monoclonal biotinylated anti-LPS (aLPS, ABIN962298) and monoclonal biotinylated anti-LTA (aLTA, ABIN475642) were obtained from www.antibodies-online.com. Dy647 Streptavidin was from Dyomics (Germany). Phosphate-buffered saline (10× PBS) containing 10.59 mM potassium phosphate monobasic, 1.55 M sodium chloride, and 29.66 mM sodium phosphate dibasic (pH 7.2) was from Gibco (Life Technologies, U.S.A.). Ethanol, acetone, acrylamide, methacrylic acid, methylmethacrylate, and dihydroxyethylbisacrylamide (DHEBA) were purchased from Merck KGaA (Germany) in analytical or highest available synthetic grade.

Fabrication of the Surface Imprints on Glass Slides. Microscope slides were used as substrates and were cleaned in acetone, isopropanol, and H₂O for 10 min each in an ultrasonic bath and blown dry under a N₂ stream. To remove

residual solvent, the slides were baked at 200 °C for 30 min. The PDMS stamp was prepared by mixing the Sylgard 184 silicone elastomer and the curing agent in a ratio of 10:1. For degassing, the mixture was centrifuged for 2 min at 3000 rpm and cured at 90 °C for 30 min. Prior to inking, the stamps were rinsed in 70% ethanol and dried with N₂. The stamps were prepared by the incubation of 20 µL of 0.5 mg/mL LPS or LTA in 1× PBS (pH 7.2) for 45 min at RT. The stamps were dried under a N₂ stream and immediately used for imprinting. Fifty microliters of the respective coating material was pipetted on one end of the slide, and a homogeneous 30 µm wet coating was obtained after distribution with a film applicator (Erichsen). The stamps were placed on the coated slides with caution to exclude embedding of air bubbles. The non-imprinted reference slides were prepared equally using 1× PBS (pH 7.2) for inking. The films were prepared and cured according to the following procedure:

Resist Coatings. (I) Amonil MMS10 was centrifuged for 2 min at 3000 rpm and photocross-linked under a UV lamp (LOT, Swiss) for 10 min. (II) ACOMO was mixed with GENOCURE and chloroform at a mass ratio of 20:1:400 and cured under a UV lamp for 2 min. (III) Epon 1002F (10%) was diluted in cyclopentanone. The films were thermally cured on a heating plate at 95 °C for 10 min. After curing, the stamps were removed, the slides were washed in 1% SDS + 0.5 M NaOH for 1 h, and were rinsed three times in H₂O.

Conventional Acrylic Coatings. Acrylamide (1.3 mg), methacrylic acid (0.8 mg), methylmethacrylate (0.9 mg), and DHEBA (7.5 mg) were mixed in a molar ratio of 2:1:1:4 and dissolved in 300 µL of DMSO containing 1 mg of AIBN. The mixture was incubated in a water bath tempered at 70 °C until the gel point was reached. The prepolymerized coating was cured under a UV lamp overnight. The stamps were removed, and the imprinted micelles were removed by washing in water for 10 min.

Fabrication of the Surface Imprints on QCM Discs. Gold electrodes were screen-printed onto 10 MHz AT-cut quartz discs of 15.5 mm as described by Dickert et al.²⁵ Cut microscopic slides were used as stamps, cleaned with ethanol, and incubated with 5 µL of 0.5 mg/mL LPS or LTA in 1× PBS (pH 7.2) for 45 min to allow assembly of the micelles on the stamp surface. In order to avoid crystallization of the buffer, the excessive solution was spun off at 1000 rpm for 30 s. For surface imprinting, 5 µL of resist or acrylic material were spin coated onto each electrode at 2000 rpm. Then the stamps were placed on top of the coated electrodes and fixed with a clamp. The nonimprinted reference electrodes were prepared equally using 1× PBS (pH 7.2) for inking. The resist and acrylic films were cured and washed as described above.

Contact Angle Measurements. The contact angle of the surfaces was recorded using the KSV CAM 101 from KSV Instruments, Ltd. (Finland). Therefore, 4 µL of water was dropped on the respective surface, and the angle was measured. For statistical purposes, three different areas of the slide were measured, and the mean contact angle was calculated.

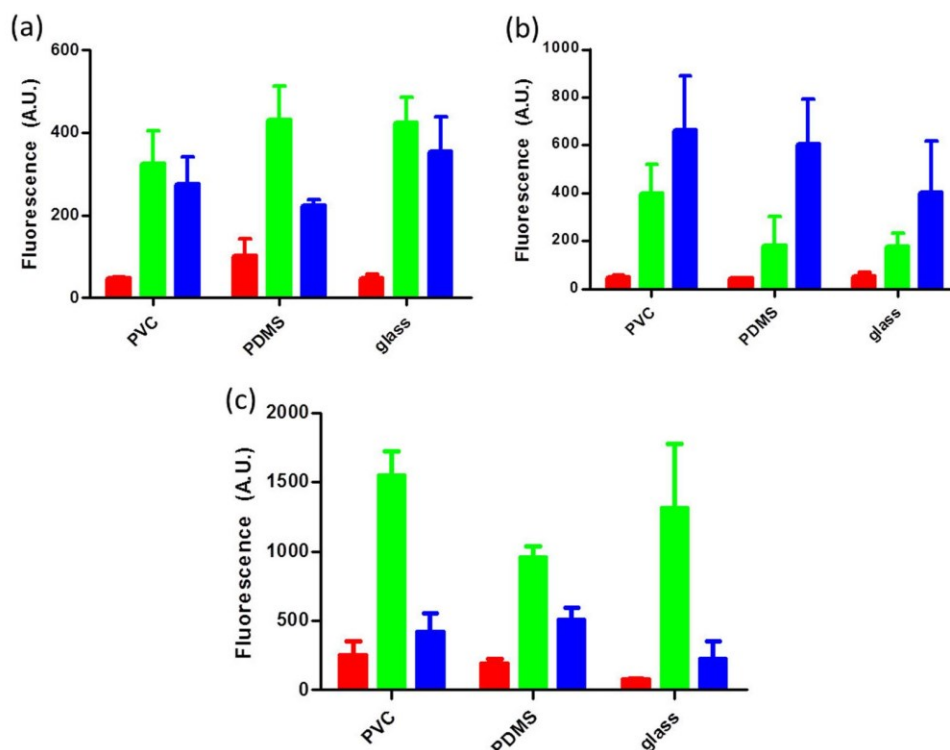


Figure 1. Fluorescence signals obtained for the respective LPS imprints in (a) Amonil, (b) ACOMO, and (c) Epon 1002F directly after the imprinting process on the imprinted sites (red bar), after incubation with LPS and detection via biotinylated antibody/Dy647 streptavidin on the imprinted sites (green bar), and the respective nonimprinted materials (blue bar).

AFM Scans. The imprinted coatings were scanned using atomic force microscopy (AFM, Nano Wizard II from JPK instruments) in the intermediate mode. An aluminum-coated silicon cantilever (NSC 35/AIBS) was used for scanning. The instrument parameters (scan rate, integral gain, proportional gain, frequency of oscillation) were adjusted separately for each sample. The scans were further analyzed using the AFM software provided by the instrument manufacturer (JPK Data Processing).

MIP-Based Sandwich Assay. The imprinted sites were incubated with 50 μL of LPS or LTA (0.5 mg/mL in $1\times$ PBS) for 2 h. In a second step, 2 $\mu\text{g/mL}$ biotinylated aLPS or aLTA and 2 $\mu\text{g/mL}$ Dy647 Streptavidin in $1\times$ PBS (pH 7.2) were preincubated for 20 min and were subsequently added to the imprinted sites for 45 min. The slides were washed twice in $1\times$ PBS (pH 7.2) and blown dry using compressed air.

Fluorescence Detection. The slides were stored in the dark until scanning. Fluorescence measurements ($\lambda_{\text{ex}} = 635$ nm, $\lambda_{\text{em}} = 670$ nm) were performed using the Genepix 4000B nonconfocal scanner (Axon Instruments). For data comparison, the PMT gain was kept constant throughout the scans. The mean fluorescence values were calculated from a grid containing 100 calculation points that was applied across the imprinted area. To identify nonspecific analyte and/or antibody binding, the mean fluorescence values from the PBS-imprinted sites (= nonimprinted) were calculated and served as a reference.

QCM Measurements. The respective quartzes were placed in a custom-made measuring cell ensuring that only one face of the respective device came in contact with sample solutions. A PDMS lid containing an inlet and an outlet tube

ensured airtight closure of the cell as well as tight electrical contact between the tin-plated copper contact electrodes of the cell and the gold electrodes of the quartzes. Self-made dual-channel oscillator circuits comprising the respective QCM channels as frequency-determining elements, respectively, were used to drive the sensors even under highly damped conditions. Two hundred microliters of the sample were injected, and frequency changes due to mass increase on the surface were measured with an Agilent 53131A frequency counter in stopped flow. Frequency data as a function of time was read out with a LabView routine via a GPIB-USB interface and analyzed using MS Excel.

RESULTS AND DISCUSSION

Characterization of the Resist Films. Since LPS and LTA are amphipathic molecules, they aggregate to micelles of approximately 20–30 nm diameter above the critical micellar concentration (CMC) in a physiological environment (pH 7.2).^{26,27} The CMC is directly proportional to the number of residues of the hydrophilic chain. Various LTAs from different bacterial species in PBS were investigated and revealed CMCs varying from 28 to 60 $\mu\text{g/mL}$.²⁸ For instance, LPS from *S. abortus equi* has an average molar mass of 5000–10000 g/mol and a CMC of 10 $\mu\text{g/mL}$, while LPS from *S. marcescens* with an average size of 20 000 g/mol has a CMC in the range of 11–25 $\mu\text{g/mL}$.²⁹ For surface imprinting, LPS and LTA were dissolved in $1\times$ PBS (pH 7.2) at a concentration of 0.5 mg/mL, which is above the CMC for both analytes. We tested three different resist materials, namely, Amonil, ACOMO and Epon 1002F, for their functionality in the fabrication of UV- and thermo-

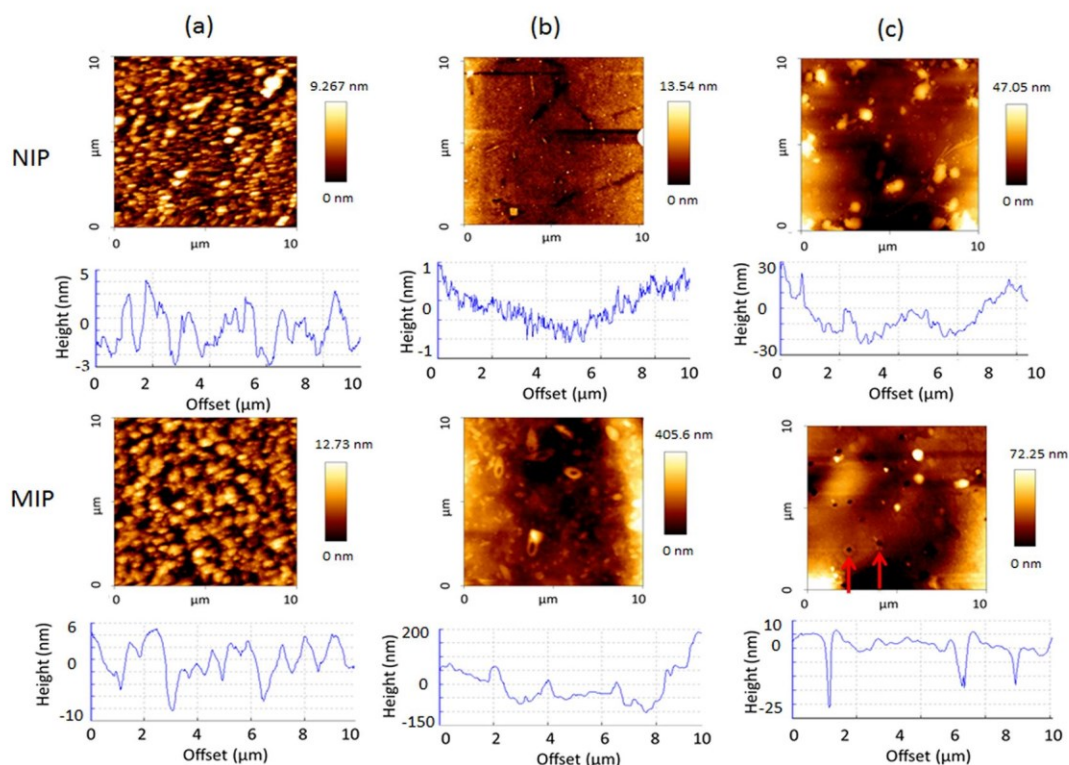


Figure 2. AFM images and selected cross sections of the nonimprinted (NIP) and the LPS imprinted (MIP) films for (a) Amonil, (b) ACOMO, and (c) Epon 1002F.

nanoinprinted biomimetic probes for the recognition of LPS and LTA. Due to the hydrophilicity of the template micelles, we first investigated the wettability of the resist films by contact angle measurements. All three resist layers exhibit hydrophilic properties: contact angles of $86^\circ \pm 0.3^\circ$, $38^\circ \pm 1^\circ$, and $65^\circ \pm 0.9^\circ$ for Amonil, ACOMO, and Epon 1002F, respectively, were obtained. Second, we tested stamps of different materials and wettability (expressed as contact angle): glass ($58 \pm 0.6^\circ$), PVC ($96.2 \pm 0.3^\circ$), and PDMS ($108.7 \pm 0.3^\circ$). On the basis of the wettability data, we expect that the micelles preferably adsorb on the hydrophilic glass stamps due to their hydrated polysaccharide shell, while they disaggregate facing (e.g., hydrophobic PDMS stamps) and form a layer with the lipid moieties oriented toward the stamp surface and the hydrophilic parts toward the solution. Due to the rather neutral wettability properties of the PVC stamp, we expect that the micelles retain their integrity upon adsorption.

To verify these assumptions, the specific rebinding ability of the nanocavities as well as nonspecific binding of the nonimprinted sites were evaluated as summarized in Figure 1. The first column depicts the autofluorescence after the imprinting process, which is low for all three materials regardless of the stamp used. The second and the third columns show the fluorescence signals obtained after incubation with 0.5 mg/mL LPS in PBS (pH 7.2) and detection via biotinylated aLPS and fluorophore-labeled streptavidin for the imprinted and the nonimprinted films, respectively.

For Amonil, the application of the glass and the PVC stamps yielded high nonspecific binding of the micelles and/or the antibody, and thus, no successful surface imprinting of the LPS micelles could be observed. In contrast, the PDMS stamp

that allows the imprinting of a LPS layer exhibiting decreased hydrophilicity yields 2 times higher fluorescence signals for the imprinted sites compared to the surrounding background and the nonimprinted sites. In this case, Amonil supports hydrophobic interactions that are beneficial for the recognition of the template layer.

For ACOMO, the fluorescence signals obtained for the nonimprinted sites are increased compared to the respective imprinted sites regardless of the stamp material used. This is probably due to nonspecific adsorption of the micelles since the resist film is highly polar.

The application of Epon 1002F yields significantly increased fluorescence signals for the imprinted sites compared to the respective nonimprinted sites for all stamp materials. Particularly, the signal increases by 73, 42, and 58% for the PVC, the PDMS, and the glass stamp, respectively. Clearly, with Epon 1002F, we managed to keep the balance between adjuvant template-resist attractions, such as hydrogen bonds, to ensure successful rebinding and reduced surface activity to prevent nonspecific adsorption of the micelles and/or the antibodies. For further evaluations, the PVC stamp was used for template adsorption and subsequent transfer to the thin films since it yielded the highest signals.

AFM Characterization of the Resist Films Using the PVC Stamp. AFM exhibits high resolution at the nanometer scale and hence was used to study the surface topography of the imprinted and nonimprinted resist films.

The Amonil surfaces are very flat and exhibit low roughness, approximately 7 nm for the molecularly imprinted (MIP) and 12 nm for the nonimprinted (NIP) reference films; in fact, the topography of the imprinted and the nonimprinted film is very similar. Despite the clearly different binding capabilities of the

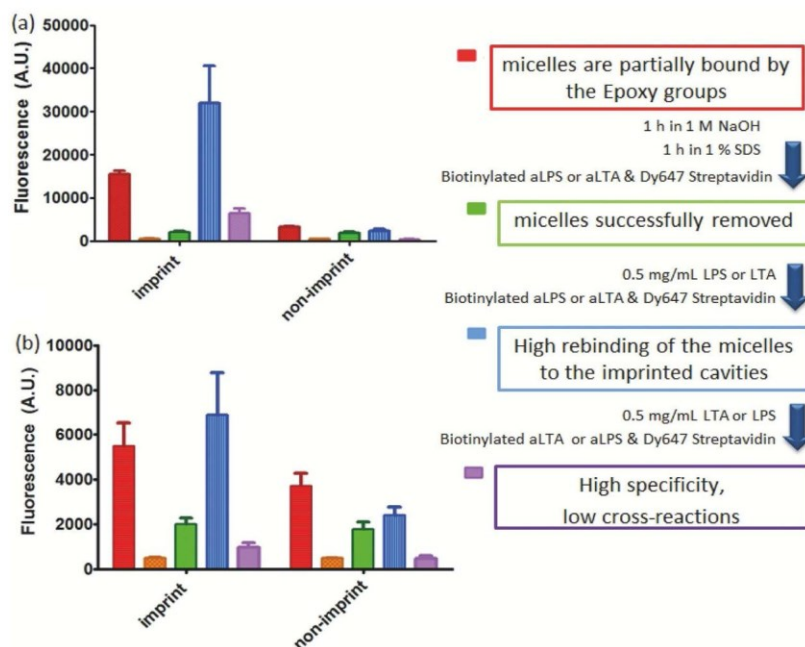


Figure 3. Fluorescence signals obtained for (a) LPS and (b) LTA imprinted to Epon 1002F for the different evaluation steps. Incubation of biotinylated aLPS or aLTA/Dy647 streptavidin directly after imprinting (red bar) and after washing (green bar), specific (blue bar) and nonspecific immunoassay signals (purple bar).

target molecule, identical topologies for MIP and NIP were also observed by S. Guillon et al.,³⁰ who concluded that the presence of a network of pores is essential to guarantee access of the target analyte to the imprinted sites. However, no nanocavities that might be attributed to micelles were observed in Amonil, indicating that the imprinted film most probably lacks such a porous network. This is in good accordance with our previous findings summarized in Figure 1, showing that the imprints fail to rebind the template micelles. In contrast, the imprinted and the nonimprinted ACMO films differ significantly in surface topography: The non-imprinted surface has a roughness of 2 nm and is thus extremely flat, whereas the imprinted surface accounts for an average roughness of 300 nm. Surface imprinting of the highly polar template micelles to the highly hydrophilic resist films apparently leads to elevated structures. Obviously, the surface imprinting process restructures the ACMO films and thereby modifies the surface properties, leading to decreased binding of the hydrated micelles compared to the nonstructured, non-imprinted layers shown in Figure 1.

The LPS thermo-nanoimprinted Epon 1002F surface topography exhibits distinct nanocavities with rough diameters of 10 to 25 nm and similar depths. The sizes of the nanocavities match the expected sizes of the micelles indicating that the Epon 1002F films were spatially successfully imprinted. Furthermore, these cavities are not present on the non-imprinted surfaces. Again, these observations are in good agreement with the results of the rebinding experiments depicted in Figure 1.

Elution of the Micelles. In the previous sections, we tested and evaluated three different resist layers to create biomimetic probes for the recognition of the bacterial surface marker LPS and LTA. We demonstrated using AFM and rebinding studies that Epon 1002F was the most appropriate resist material and PVC coverslips the most suitable stamp for this application

(Figure 2).

In order to analyze the orientation and localization of the micelles during the imprinting process in more detail, the contact angles of the PVC stamps were recorded during the imprinting. The pure PVC stamp exhibits a contact angle of 96°, but the contact angle decreases to 38° after inking with LPS. These findings prove the successful adsorption of the micelles and further shed light on the orientation of the micelles: as expected in PBS, the hydrophilic polysaccharide moieties face outward. After the imprinting process, the contact angle almost reverted to the initial values (90°) showing the entire transfer of the micelles to the resist film.

Epon 1002F contains epoxy groups that are able to covalently bind amino groups. Since LPS and LTA contain several amino sugars, it is likely that the micelles are covalently bound via the epoxy groups. After washing in 1× PBS (pH 7.2) containing 1% SDS, we incubated the layers with the specific biotinylated antibodies and subsequently verified antibody binding by incubation with fluorophore-labeled streptavidin. Figure 3 shows the fluorescence signals obtained for (a) LPS and (b) LTA. Compared to the respective nonimprinted sites, the signals increased by a factor of 2 and 5 for LTA and LPS, respectively, proving that the micelles are tightly bound. After excessive washing in 1 M NaOH and 1% SDS, aLPS or aLTA and fluorophore-labeled streptavidin were again incubated, and the signals decreased to the respective nonimprinted sites confirming the successful elution of the micelles.

Molecularly Imprinted Sorbent Assay. Besides applications in solid phase extraction,³¹ chromatography,³² and catalysis,³³ MIPs have found applications as antibody mimics in ELISA-type immunoassays. In 1993, Vlatakis et al. first used MIPs for the determination of theophylline and diazepam in human serum and introduced the term “molecularly imprinted sorbent assay” for assays using MIPs as recognition elements.³⁴

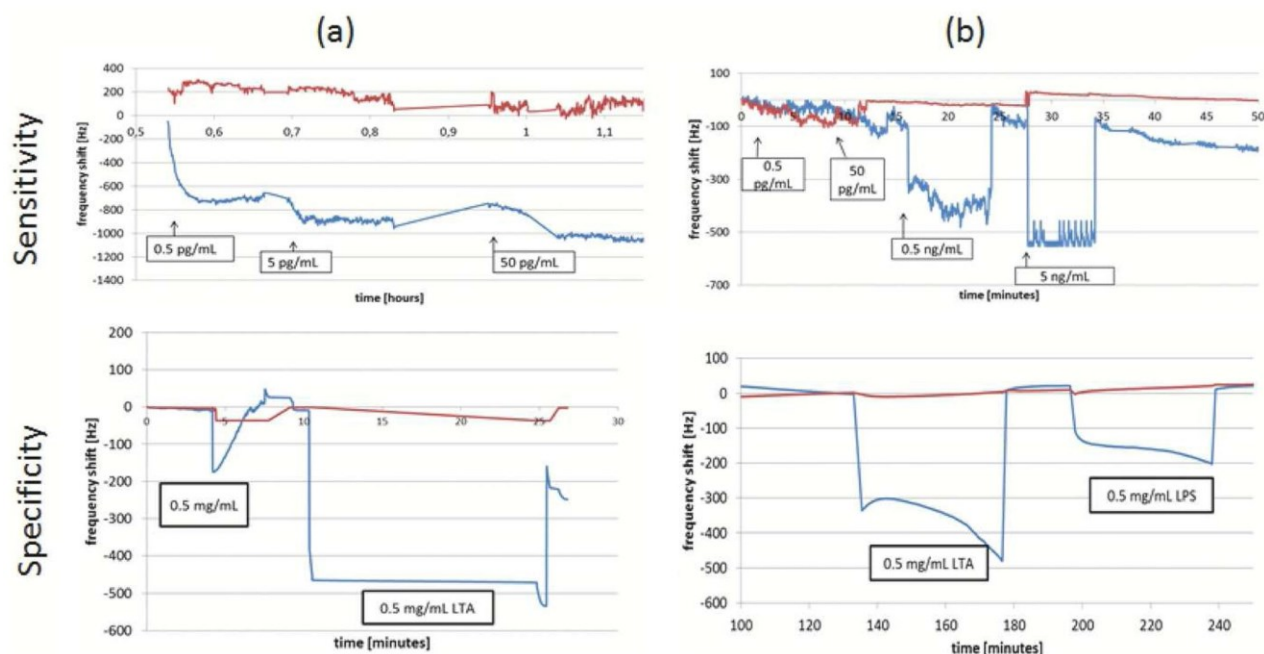


Figure 4. QCM response of the LTA imprinted (blue) and the nonimprinted (red) (a) Epon 1002F electrodes and the (b) acrylic polymer electrodes.

The plastic antibodies exhibit several advantages to their biological counterparts such as chemical and physical stability, low costs, and fast production. Since then, several researchers confirmed the superior properties of biomimetic probes in molecularly imprinted sorbent assays.^{35,36}

We herein exploited the thermo-nanoimprinted biomimetic probes using Epon 1002F for the detection of LPS and LTA in a MIP-based sandwich assay. Therefore, a nanocavity sensor layer was fabricated by surface imprinting of micellar aggregations of LPS or LTA to a Epon 1002F thin film. After completion of the imprinting process, the micelles were eluted by excessive washing using NaOH and SDS as described in the previous section. LPS or LTA at 0.5 mg/mL in 1× PBS (pH 7.2) was incubated, and specific binding of the micelles to the complementary nanocavities was verified by incubation of the specific biotinylated detection antibody and fluorophore-labeled streptavidin. The blue bars in Figure 3 depict the fluorescence signals obtained for (a) LPS and (b) LTA. Compared to the reference signal, the LPS-imprinted sites exhibit 13 times enhanced signals, while the LTA-imprinted sites result in a 3-fold signal enhancement, showing the excellent rebinding capabilities of the thermo-nanoimprinted biomimetic probes. These results indicate that the imprinting process of LPS is more efficient than that of LTA. However, as shown in the following section, the QCM measurements are very similar for both analytes, affirming that this difference is due to the quality of the purchased LTA antibody. Nonspecific binding of recognition elements constitutes a serious problem in the development of assays. Especially, cross-reactivities of the capture molecules to chemically or structurally similar analytes are common interferences leading to false-positive results.³⁷ Since the selectivity is a crucial evaluation parameter, we investigated this parameter for the imprinted Epon 1002F thin films by incubation of LTA on the LPS-imprinted sites and subsequent detection with aLTA and vice

versa for the LTA-imprinted sites. The violet bars in Figure 3 correspond to the resulting nonspecific fluorescence signals. For both analytes, the nonspecific signals are very similar to the signals of the respective nonimprinted sites showing that we fabricated highly specific thermo-nanoimprinted Epon 1002F films.

Epon 1002F Imprints versus Conventional Surface Imprints. MIPs are applied for the specific recognition of analytes in mass sensitive devices. QCM allows label-free and sensitive measurement of analyte binding accomplished by monitoring resonant frequency changes due to mass deposition on the piezoelectric material. MIP-based QCM sensors for the detection of small organic molecules, macromolecules, and even cells are reported in the literature.³⁸ We fabricated conventional molecular surface-imprinted polymers using a cocktail of functional acrylic monomers, a bisacrylamide as cross-linker, and the initiator. This mixture is incubated at elevated temperature until the gel point is reached in order to obtain a precursor polymer that is applied for further processing. The prepolymerization step is crucial to obtain the requisite viscosity for the application of a stable thin film. The template molecule is attached on the surface of a stamp and subsequently brought into contact with the prepolymerized thin film. The precursor polymer is fully cured by photopolymerization, and the stamp is removed leaving template-specific 2D cavities on the film surfaces. Polyacrylamides are among the most commonly used monomers for protein imprinting, which is due to their high selectivity accomplished through the resulting highly cross-linked polymer networks. Furthermore, the amino groups form hydrogen bonds with residues of the template molecules. The thermo-nanoimprinted Epon 1002F imprints are compared to the conventional acrylic molecular surface imprints in terms of sensitivity, selectivity, and their applicability for QCM measurements. To evaluate the sensitivity of the sensor layers, the lower limit of detection (LOD) was determined,

and the selectivity was again evaluated by cross-reactivity studies. Figure 4 summarizes the QCM results regarding sensitivity and selectivity obtained for (a) the thermo-nanoimprinted Epon 1002F films and (b) the conventionally surface-imprinted acrylic polymer films exemplarily for LTA. Similar results for LPS were obtained (data not shown). Generally speaking, appreciable concentration dependency and high rebinding properties are observed for both imprinted film materials. Assay sensitivity was determined by incubation of a serial dilution of LTA, and the LLOD was defined as the lowest concentration that leads to a detectable frequency shift. The thermo-nanoimprinted Epon 1002F electrode responds with an immediate frequency change of 700 Hz on the MIP electrode due to mass accumulation when injecting 0.5 pg/mL LTA. The NIP electrode did not respond. We receive a linear concentration dependency as the resonant frequency further decreases by 200 Hz each after incubating with 5 and 50 pg/mL LTA, respectively. For the conventional surface-imprinted acrylic electrodes, we observed a 1000× increase in the LLOD for the MIP electrode. For the imprinted acrylic QCM sensor, a detectable frequency shift of 400 Hz is observed after the injection of 0.5 ng/mL LTA. The frequency further decreases to 600 Hz after incubation of 5 ng/mL LTA. Again, no QCM response was observed for the nonimprinted electrode showing low surface activities for both polymer systems. Indeed, the astonishingly large effect of LTA-imprinted EPON resist material can be explained by considering the differences between the two materials (i.e., EPON and acrylic polymer): the overall sensor signal is irreversible in the first case and reversible in the second. Together with the very large sensor response at very low concentrations, affinity of the EPON must be substantially larger. The main difference between the two materials is the presence of epoxy groups in EPON. These can undergo covalent binding with amino groups present in LTA leading to irreversible binding. Such LTA molecules that are covalently bound to the sensor surface can then act as “micellation seeds” in a polymeric environment that has been optimized to bind such micelles both in terms of morphology and surface functionality. This obviously already leads to saturation of the polymer surface at very low analyte concentrations, as a further increase by 2 orders of magnitude yields only minor changes on the frequency signal.

The selectivity of the imprints was evaluated by exposing the imprinted and the nonimprinted electrodes to 0.5 mg/mL LPS. For both polymer systems, again no nonspecific binding to the nonimprinted surfaces occurred. For the thermo-nanoimprinted Epon 1002F electrode, a small response that self-recovers after a few minutes is obtained showing the high selectivity of the imprinted nanocavities. In contrast, the conventional surface imprints using the acrylic polymers are rather nonspecific since the incubation of LPS results in a distinct frequency shift of 200 Hz that could be recovered only by washing with 1× PBS (pH 7.2). Nevertheless, the reusability of the thermo nanoimprints is limited due to the rough washing conditions. The Epon 1002F resist material binds the micelles very tightly via the epoxy groups. In order to remove the micelles, the imprints are washed with 0.5 M NaOH containing 1% SDS. The alkaline washing conditions interfere with and disturb the QCM measurements. We tested the application of a pH-neutral washing buffer that contains SDS only, but the signal was not

recovered. Therefore, 0.5 M NaOH + 1% SDS were used for washing, and the surface was subsequently re-equilibrated by the repeated incubation of PBS for 10 min each. Nevertheless, even with this extended washing protocol, the surface recovery was limited to only a few cycles. In comparison, the imprints using the acrylic polymers are easily recovered by flushing the system two times with 1× PBS (pH 7.2).

CONCLUSIONS

The vast majority of molecular surface imprints are prepared using a complex and prepolymerized mixture of functional monomers, cross-linkers, and initiators. The so-created recognition sites must be specifically tailored in order to optimize the rebinding abilities by the challenging variation of the key components. For example, varying amounts of the cross-linker and different ratios of the functional monomers have to be tested. Additionally, much investigation to find the appropriate solvent has to be done, and a precursor polymer solution with increased viscosity has to be prepared to support the deposition of a stable film for molecular surface imprinting. When Epon 1002F is applied, a thermocurable ready-to-use resist material, time-consuming systematic testing of various component compositions to find out the most appropriate component mix can be completely avoided. No prepolymerization is necessary, and the target molecule can be easily and rapidly imprinted with high selectivity in a single step. The so-created LPS and LTA recognition sites show excellent selectivity with almost no cross-reaction to the tested nonspecific analytes. Remarkably, the nonspecific QCM response obtained after the incubation of LPS on the LTA-imprinted electrodes self-recovered after a few minutes. Furthermore, the plastic antibodies reveal LLODs of 0.5 pg/mL LPS or LTA, which is below the CMC of 11–26 µg/mL and 25–60 µg/mL for LPS and LTA, respectively, indicating that the cavities on the sensor layer influence and moreover promote the formation of micelles below the CMC. The thermo-nanoimprinted Epon 1002F films were successfully applied in molecularly imprinted sorbent assays and as recognition elements in QCM measurements. Though the usability of the system for QCM measurements is limited due to the harsh alkaline washing conditions that are necessary to remove the tightly bound micelles, the firm binding of the micelles is of critical importance in MIP-based immunoassays or for enrichment processes that include several incubation or washing steps.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank the Austrian Research Promotion Agency (FFG) for financial support of the MNT Era Net Project “Valipro” (No. 823977) within the Austrian NANO Initiative Programme Action Line “Transnational Cooperative RTD Projects”.

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4 Final conclusions

Sepsis is a systemic infection of the body and still constitutes a deadly threat in intensive care units. The gold standard in the diagnosis of sepsis involves the microbiological culturing of blood samples to identify the causative circulating pathogens. This standard method is nonsatisfactory regarding sensitivity and rapidity. Several biomarkers that are released in the course of sepsis have been identified to serve as reliable candidates for the early diagnosis including pro- and anti-inflammatory mediators. Due to the high complexity of the condition and the high dynamics of the biomarkers, only their combinatorial use gives adequate information to allow the precise and early-stage diagnosis of sepsis.

Protein microarrays show high potential to tremendously advance the field of medical diagnosis due to their outstanding properties regarding miniaturization, parallelization and high-throughput.

In this thesis a protein microarray was developed that allows the simultaneous detection of the biomarkers E-Selectin, S-100, CRP, PCT and IL-6 for the accurate and early diagnosis of sepsis.

Diagnosis asks for tools that are reliable in terms of accuracy and precision. Additionally, physicians and patients benefit from tools that harbor the following properties:

- Rapid accomplishment to ensure timely treatment
- Easy-to-accomplish to advance towards bedside diagnosis (point-of-care testing)
- Low sample consumption and
- Low-cost

We addressed the need for low volume assays by the development of a protein microarray that works with only 4 μL patient serum sample diluted with 36 μL assay buffer distributed to four arrays. Generally, reduction of the sample volume leads to losses in assay sensitivity due to diffusion limitations. In this thesis, a highly sensitive and rapid miniaturized assay was developed that exhibits up to four times enhanced LODs compared to the classical assay and performs in only 2.5 hours in a single-step-assay. This was accomplished by the addition of bi-

functional fluorophore labeled streptavidin coated magnetic particles that act as exceptional stirring and detection components. The reduction of the sample volume is especially beneficial in neonatal diagnosis since it minimizes stress caused by excessive blood withdrawal. Furthermore, assay miniaturization decreases sample consumption and in further consequence reduces the costs of the assay.

Despite the outstanding properties of protein microarrays, this analytical technique failed to successfully establish in clinical practice mainly due to the difficult handling procedures. To provide a customer-friendly tool, we integrated the calibration directly on the chip which avoids the effort of externally preparing a standard dilution series which significantly simplifies and accelerates the whole assay procedure. Therefore, the analytes were conjugated to the carrier protein BSA and immobilized in increasing concentrations besides the capture antibodies on the array. This approach was advanced in a cost-cutting manner by replacing the BSA/analyte conjugates by detection antibodies. High correlations between the internally generated calibration curves and the externally prepared calibration curves were obtained ($R^2 > 0.986$) allowing the accurate quantification of the biomarkers in a self-calibrating assay.

The chosen biomarker pool is very heterogeneous regarding analyte concentrations: CRP occurs in rather high serum concentrations ($\mu\text{g/mL}$), whereas PCT, E-Selectin and S-100 occur in the ng/mL and IL-6 in the pg/mL range. To allow the simultaneous detection of CRP with low abundance biomarkers, a competitive immunoassay which is less sensitive compared to sandwich immunoassays, was accomplished in the initial assay set up. Nevertheless, by implementing the calibration directly on the chip, the combinatorial use of the two assay formats was no longer feasible since the internal calibration is compatible only with sandwich immunoassays. Therefore, a low sensitive sandwich immunoassay for CRP was developed by exploiting the low but specific binding affinity between CRP and phosphocholine ($K_D = 5 \mu\text{M}$). This assay was still too sensitive for the quantification in the clinically relevant range, that's why we introduced an artificial dilution of CRP by the addition of free phosphocholine to the assay buffer. By adding different amounts of free phosphocholine, the dynamic range can be adjusted

and we demonstrated that the addition of 50 mM phosphocholine results in a calibration curve that strikes the clinically relevant range.

Protein microarrays often lack sensitivity in the lower concentration ranges of low abundance analytes such as cytokines that occur in the pg/mL range which prompted the development of sophisticated labeling strategies. In this thesis, the sensitivity problem was addressed by the generation of high-density labeling using either an enzyme or an antibody based amplification system. The enzyme horse radish peroxidase (HRP) converts fluorophore tyramide to the highly reactive tyramide radical which binds to neighbored amino acid residues which locally increases the density of fluorophores. The antibody amplification system uses the consecutive incubation of two fluorophore labeled antibodies to increase the density of fluorophores. In total, we introduced four assay designs: (I) the classical biotin-streptavidin assay, (II) the rapid assay using directly fluorophore labeled detection antibodies, (III) the enzyme amplification assay and (IV) the antibody amplification assay. The assay designs were evaluated in terms of sensitivity, accuracy and reproducibility using certified control sera for IL-6, PCT and CRP. Assay reproducibility was very similar across all assay designs. The assay sensitivity significantly increased by the application of high-density labeling (III, IV) as the LODs decreased by one order of magnitude compared to the classical (I) and the rapid (II) assay. The most accurate results for all three tested biomarkers were achieved for the robust classical assay (I) and the highly sensitive antibody amplified assay (IV).

Besides detection and quantification of the biomarker, the identification of Gram-type of the causative bacteria is important in the diagnosis of sepsis since it helps physicians to guide antibiotics medication. LPS (lipopolysaccharide) and LTA (lipoteichoic acid) are bacterial surface marker expressed specifically on the surfaces of Gram-negative and Gram-positive bacteria respectively and can be exploited for the fast Gram-differentiation. We fabricated a surface imprinted sensor layer for the specific recognition of LPS and LTA. Therefore, we applied the ready-to-use thermo-resist material Epon 1002F instead of the typically used complex mixture comprising of monomers, crosslinker and initiator for surface imprinting and

thus developed easy-to-accomplish and rapid surface imprints for the recognition of the bacterial surface marker. The sensitivity and specificity of the thermo nanoimprinted films were compared to conventionally prepared surface imprints using an acrylic polymer system in QCM measurements. The Epon 1002F polymer system exhibited excellent selectivity as the response of the non-specific analyte self-recovered after few minutes. Furthermore, the thermo nanoimprinted Epon 1002F films were highly sensitive in terms of LODs (0.5 pg/mL) that were three orders of magnitude lower compared to the acrylic polymer system (0.5 ng/mL). We fabricated highly sensitive and selective surface imprinted nano-cavities that are applied as antibody mimics in molecularly imprinted sorbent assays. Furthermore, the Epon 1002F polymer thin-films can be coinstantaneously exploited as substrates for the immobilization of capture antibodies.

The presented thesis outlined the development and validation of a highly sensitive and self-calibrating protein chip that combines the simultaneous quantification of a heterogeneous biomarker pool and the determination of the bacterial Gram-type via implemented surface imprints.

CONFERENCE CONTRIBUTIONS

µl volume protein biomarker chip for the early diagnosis of neonatal sepsis

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Neonatal sepsis has a prevalence of 1 to 8 in 1000 and is still a leading cause of death among newborns. Though symptoms are unspecific and occur late, early detection of sepsis in newborns is of tremendous importance enabling timely administration of antibiotics and avoidance of treatment in healthy newborns.

A protein biomarker chip for the diagnosis of sepsis allowing the parallel analysis of the sepsis associated serum proteins CRP, TNF alpha, Neopterin, IL-6, IL-8, PCT and IL-10 has been developed [1]. Specific antibodies are immobilized on ARChip Epoxy supported platforms [2] and verification of respective analytes is performed using biotinylated antibodies exploiting streptavidin-biotin chemistry and fluorescence detection. This chip is accomplished as an on-chip immunoassay permitting the performance of sandwich format immunoassays simultaneously with binding inhibition based assays and bypassing the large heterogeneity of analytes regarding concentration.

This chip is ready for validation and is further expanded with immobilized antibodies against serum biomarker proteins that are specific in the early diagnosis of sepsis in newborns providing a diagnostic protein chip for neonatal sepsis. LBP (Lipopolysaccharide binding protein), E-Selektin, IL-2sR α (soluble Interleukin 2 receptor alpha), IL-1ra (Interleukin 1 receptor antagonist), uPAR (urokinase receptor), Protein C and von Willebrand Factor have been identified to be sustainable biomarkers.

Because the probe material is very limited in neonatal diagnosis, it is of tremendous importance to advance the sepsis chip regarding sample volumes. While 50 µl sample volumes were used in previous set-ups, the new platform works with 5 µl to 10 µl sample volumes. In order to avoid errors like drying out or interspersing of samples and to maintain a high reproducibility and a low limit of detection the application of an adhesion film with mini-wells onto microarray platforms will be discussed.

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- [2] US patent, no. 7790472

Development of a quantitative sub 10 μ l volume biochip and a rapid lateral flow assay as diagnostic devices for neonatal sepsis

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Neonatal sepsis has a prevalence of 1 to 8 in 1000 and is still a leading cause of death among newborns. Since symptoms are unspecific and occur late, early and reliable detection of sepsis in newborns is of tremendous importance enabling timely administration of antibiotics.

In order to provide an accurate diagnostic tool, a protein chip allowing the parallel analysis of the sepsis associated serum proteins CRP, TNF alpha, Neopterin, IL-6, IL-8, PCT and IL-10 has been developed [1]. This biomarker panel was further expanded with the serum protein biomarkers S-100, E-Selectin and Protein C that occur in clinically relevant ranges of 36 – 640 pg/ml, 100 – 400 ng/ml and 20 – 450 ng/ml respectively. S-100 serves as marker for neuroinflammations whereas E-Selectin and Protein C are specific early biomarkers for a variety of inflammatory conditions. The applied on-chip assays include immobilization of specific antibodies for sandwich immunoassays and antigens for binding inhibition assays on ARChip Epoxy supported platforms [2]. Verification of respective analytes was performed using biotinylated antibodies exploiting streptavidin-biotin chemistry and subsequent fluorescence detection.

Whereas previous set-ups work with 50 μ l sample volumes applied to 7 mm x 7 mm wells this biochip works with a 5-fold reduced sample volume in much smaller fields (diameter 4.5 mm). Using the new low volume biochip only 1 μ l sample (diluted 1:10 with assay buffer) is necessary to accurately run the assay. This is especially beneficial to newborns as it prevents excessive blood withdrawal and consequently minimizes stress and pain.

Assay miniaturization however might provoke loss in sensitivity due to diffusion limitations. In order to maintain high assay sensitivity and reproducibility ferrofluids and streptavidin labeled magnetic nanoparticles were added to the sample to act as a micro-mixer and stir the small volumes during sample incubation. Alternatively, ultrasonic waves were used to allow a homogeneous distribution of the sample and improve the accessibility of the targets towards the immobilized probes.

Though the 10 μ l biochip provides accurate and rapid diagnosis of sepsis, chip analysis requires profound knowledge of laboratory techniques and expensive equipment. For this reason a multiplexed lateral flow assay for rapid but simpler point-of-care detection of serum proteins has been developed. Specific antibodies were immobilized on nitrocellulose membranes and gold conjugated antibodies were used for colorimetric detection providing a fast alternative for diagnosis of neonatal sepsis.

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Miniaturized protein microarray with internal calibration for diagnosis of neonatal sepsis

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Neonatal sepsis has a prevalence of 1 to 8 in 1000 and is still a leading cause of death among newborns. Since symptoms are unspecific and occur late, early and reliable detection of sepsis in newborns is of tremendous importance enabling timely administration of antibiotics. In order to provide a convenient diagnostic tool, a protein chip for the parallel analysis of the sepsis-associated serum proteins CRP, TNF alpha, Neopterin, IL-6, IL-8, PCT and IL-10 has been developed [1]. This biomarker panel was further expanded with the sepsis protein biomarkers S-100 and E-Selectin. The applied on-chip assays include immobilization of specific antibodies on ARChip Epoxy supported platforms. Verification of respective analytes is performed using biotinylated antibodies exploiting streptavidin-biotin chemistry and subsequent fluorescence detection. To minimize excessive blood withdrawal from newborns, the assay was miniaturized to work with only 4 µL patient serum samples diluted with 36 µL assay buffer and distributed to 4 replicate wells for repetitive measurements. Assay miniaturization provokes losses in sensitivity and reproducibility due to diffusion limitations. Therefore, streptavidin coated magnetic particles are added not only to stir the small sample volume but also to detect antibody antigen binding events. This set-up allows the quantification of the analytes in a “one - step” assay that accomplishes within 2.5 hours. Additionally, to supply a rapid “one patient – one chip” format, the protein chip copes without external calibration. We present a rapid self-calibrating protein chip that works with only 4 µL serum sample for accurate and repeatable (CV < 14 %) diagnosis of neonatal sepsis [2].

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Acknowledgements

This work is financially supported by the EraNet project Valipro (www.valipro.eu)

Development of surface imprinted artificial antibodies for the rapid differentiation of Gram-negative and Gram-positive bacteria

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Sepsis is a life-threatening systemic body infection caused by invasive microorganisms like bacteria, virus or fungi but most systemic infections occur in response to pathogenic bacteria. In the course of sepsis, Gram-positive and Gram-negative bacteria activate different inflammatory pathways that alter the cytokine profile. To ensure appropriate treatment of septic patients, rapid and early detection and identification of the causal microorganism class is of tremendous importance. Gram-negative and Gram-positive bacteria differ in their surface marker expression profile. The surface marker LPS (lipopolysaccharide) expressed by Gram-negative bacteria and LTA (lipoteichoic acid) expressed by Gram-positive bacteria can be exploited as specific marker for the fast Gram differentiation without the inconvenient and time-consuming preparation of blood cultures and subsequent Gram staining.

Surface imprinting techniques are applied to create nanocavities in a polymer thin-film for the specific recognition of LPS and LTA. Both bacterial marker contain a hydrophobic lipid tail and a hydrophilic polysaccharide portion. Due to their amphipathic character different stamp coatings as well as different polymer materials are evaluated to provide a repeatable imprinting protocol. Furthermore, the effect of site directed imprinting is tested. The surface imprinted nanocavities are exploited as artificial capture antibodies in a sandwich immunoassay and verification of bound bacterial surface markers is performed via biotinylated antibodies and fluorescence labeled streptavidin.

We provide a diagnostic tool for the fast differentiation of Gram-negative and Gram-positive bacteria as the causative microorganism to ensure adequate treatment of septic patients.

POINT-OF-CARE CHIP FOR DIAGNOSIS OF SEPSIS: Joining biomarker quantification and bacterial class identification

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Abstract: Sepsis still constitutes a common threat in intensive care units and arises from the body's response to an infection. Sepsis can be easily treated by the timely administration of antibiotics. Therefore, a point-of-care device for the early and reliable diagnosis of sepsis that combines immunoassay and surface imprinting techniques is developed. The serum protein biomarker CRP, PCT and IL-6 are quantified via an internal calibration which significantly accelerates and simplifies the assay. Additionally, nano-cavities complementary to the bacterial surface marker LPS and LTA created by surface imprinting that allow the differentiation of Gram-positive and Gram-negative bacteria are implemented. We provide a diagnostic tool that meets the requirements of point-of-care tools for the early diagnosis of sepsis and moreover supports guiding of antibiotic treatments by the determination of the bacterial Gram class.

Keywords: Sepsis, protein microarray, internal calibration, Gram differentiation, surface imprinting

Introduction

Sepsis is a life-threatening systemic body infection caused by microbial pathogens that can be easily treated by the timely administration of antibiotics. The serum proteins CRP (C-reactive protein), PCT (Procalcitonin) and IL-6 (Interleukin 6) represent a reliable biomarker pool that can be exploited for the early and reliable diagnosis of sepsis. The serum proteins occur in clinically relevant ranges of 1 – 500 µg/mL, 0.1 – 50 ng/mL and 1 – 1000 pg/mL respectively [1]. In the course of sepsis, Gram-positive and Gram-negative bacteria activate different inflammatory pathways that alter the cytokine profile [2]. To guide antibiotic treatment, early detection and identification of the causal microorganism class is of tremendous importance. Gram-negative and Gram-positive bacteria differ in their surface marker expression profile. The surface marker LPS (lipopolysaccharide) expressed by Gram-negative bacteria and LTA (lipoteichoic acid) expressed by Gram-positive bacteria can be exploited as specific marker for the fast Gram differentiation.

So far, the standard procedure in the diagnosis of sepsis is based on the cultivation of blood samples and subsequent identification of the microorganisms. This method severely lacks of sensitivity and promptness and diagnosis demands for rapid and accurate point-of-care devices.

Therefore, we developed a multiplexed protein microarray that allows the quantification of the sepsis-associated serum proteins and simultaneously allows the determination of the bacterial class of the causative pathogen. To meet the requirements of point-of-care devices and to provide a simple “one patient – one chip” device, we directly integrated the calibration on the chip. The internal calibration is only compatible with sandwich immunoassays. The large heterogeneity of the analytes regarding their concentrations requires a sandwich assay with low sensitivity for the quantification of CRP that occurs in rather high concentrations. CRP binds to phosphocholine with relatively low affinity ($K_D = 5 \mu\text{M}$) [3]. The low affinity between CRP and phosphocholine is exploited for the development of a low-sensitive sandwich assay by immobilizing phosphocholine as capture molecule for CRP.

In order to improve the overall assay sensitivity and the precision in the lower concentration ranges an enzyme-mediated signal amplification method that exploits the catalytic activity of the horse radish peroxidase (HRP) for turnover of multiple fluorophore labeled tyramide substrates to generate high density labeling is applied.

Additionally, the chip allows the fast differentiation of the bacterial classes by the detection of the respective Gram specific surface marker. Surface imprinting techniques are applied to create nano-cavities in a polymer thin-film that coinstantaneously serves as substrate for the immobilization of the capture molecules. The surface imprinted nano-cavities are exploited as artificial capture antibodies in a sandwich immunoassay.

We provide an accurate and fast point-of-care device that allows the simultaneous detection of the sepsis biomarker CRP, PCT and IL-6 that additionally allows the fast differentiation of bacterial classes by applying surface imprinting techniques.

Methods

A polymer thin-film is applied to glass slides that serves as substrate for surface imprinting of LTA and LPS and as substrate for the covalent immobilization of the capture antibodies for PCT and IL-6 or phosphocholine for CRP. The capture molecules necessary for the external calibration and the biotinylated detection antibodies exploited for the internal calibration are applied to the chip surface via contact spotting (NanoPrint, Arrayit).

In order to create specific surface imprinted nano-cavities with high rebinding affinities for LPS or LTA, cover slips are inked with the respective analyte and pressed on the polymer thin-film. After curing, the stamps are removed and washed excessively to remove residual analytes. The so created nano-cavities as well as the spotted capture molecules are used for quantification of the respective analytes. For all analytes a sandwich assay is performed by the incubation of 25 μ L serum diluted with 25 μ L assay buffer and followed by the incubation of the biotinylated capture antibodies. Then, HRP conjugated to Streptavidin is incubated and the tyramide amplification is performed by the incubation of Alex Fluor 647 labeled tyramide that gets converted to a short-lived radical that binds to neighbored protein residues resulting in a localized high density labeling.

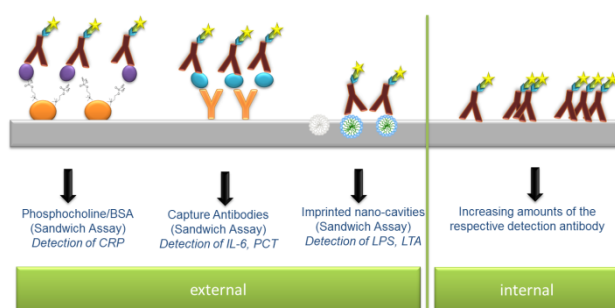


Figure 1: Schematic view of the on-chip assays

Fluorescence measurements (λ_{em} : 635 nm) are performed using a non-confocal scanner (Genepix, Axon Instruments) and background calibrated signals are used to set up 4-parameter fitted calibration curves (GraphPad Prism).

Results

Figure 2 shows the internal (green curves) and the external (black curves) calibration curves obtained for (a) IL-6, (b) PCT and (c) CRP. For all analytes the internal and the external curves match in terms of curve shape and maximal signal intensities. A coefficient of determination (R^2) of above 0.996 is obtained showing very strong correlations between the internal and external calibration. The significance is confirmed by a p-value below 0.0001 % for all 3 analytes. The curves match the clinically relevant ranges and LODs (limit of detection) of 4 pg/mL, 0.1 ng/mL and 1 μ g/mL for IL-6, PCT and CRP respectively are reached.

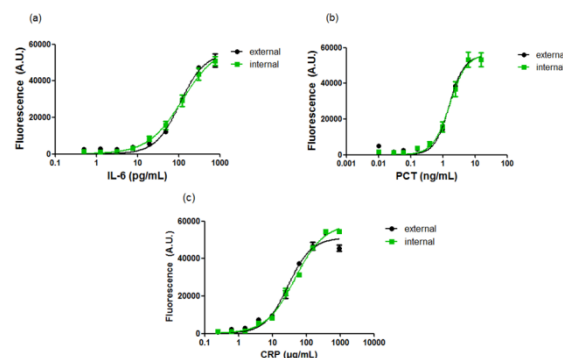


Figure 2: Internal and external calibration curves for (a) IL-6, (b) PCT and (c) CRP

The application of surface imprinting techniques for bacterial Gram classification results in complementary nano-cavities specific for LTA or LPS respectively. The fluorescence signals obtained after the assay when exploiting the imprinted nano-cavities as capture molecules are shown in Figure 3 for (a) LTA and (b) LPS. Only low signals are obtained for the non-imprinted reference sites and after incubation with the non-imprinted analyte (LTA for LPS imprinted sites and vice versa) to detect unspecific cross-reactions. When incubating with the imprinted analytes the signals increase 10-fold demonstrating the specific affinities of the imprints.

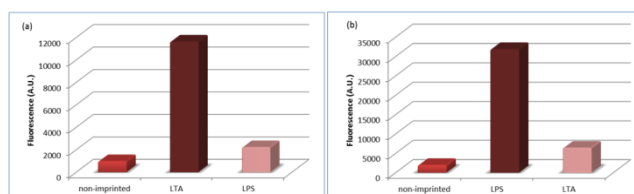


Figure 3: Fluorescence signals obtained for the non-imprinted sites and the (a) LTA and (b) LPS imprinted sites

Discussion

We developed a simple and fast diagnostic tool that allows the accurate and reliable diagnosis of sepsis. Through the application of the enzyme-mediated signal amplification step, the dynamic ranges of the assay strike the clinically relevant ranges of the serum biomarker CRP, PCT and IL-6. The key requirements of point-of-care devices are met by the implementation of the calibration directly on the chip. The internal calibration reduces the effort of preparing an external standard dilution series. Additionally, specific surface imprints with high affinities for LPS or LTA that allow the fast differentiation of Gram-positive and Gram-negative bacteria are implemented on the chip.

Acknowledgement

This work is financially supported by the EraNet project Valipro.

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ULTRASENSITIVE DETEKTION VON ENROFLOXACIN IN MILCH: ANWENDBARKEIT UNTERSCHIEDLICHER ASSAY FORMATE

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In der Lebensmittelanalytik, zB. bei Wareneingangs-, und Zollkontrollen werden oft schnelle on-site Screening Methoden benötigt. Biochips sind als robustes, einfaches und tragbares Analysewerkzeug dafür besonders gut geeignet. Die Oberflächenchemie des Chips hat dabei großen Einfluss auf die Sensitivität des Assays.

In der hier präsentierten Arbeit wurden drei Strategien zur Oberflächenmodifikation getestet, um einen on-chip Immunoassay für Enrofloxacin in Milch (Maximum Residue Limit = 100 µg/kg [1]) zu entwickeln:

- Immobilisierung von Enrofloxacin-Konjugaten mittels Trägerproteinen und Crosslinkern
- Chemische Modifizierung der Oberfläche (Poly-Lysin, Hydrazid, SAMs auf Gold)
- Herstellung von „Plastic Antibodies“ (Imprints in zB. Epon 1002F, ACMO)

Unterschiedliche Oberflächen ermöglichen den Einsatz unterschiedlicher Assay Formate: während Enrofloxacin-Konjugate als Sonden für den Binding-Inhibition Assay zum Einsatz kommen (Meßbereich 41–61 µg/L bei 1 mg/mL Enro-HRP), werden natürliche Antikörper und sogenannte „Plastic Antibodies“ zur Bestimmung mittels kompetitivem Assay verwendet. Alternativ dazu werden „Plastic Antibodies“ auch im direkten Assay eingesetzt, der aber deutlich weniger sensitiv ist. Die Robustheit des Assays wurde in Halbfett- und Vollmilch (zu 50% mit Assay Puffer verdünnt), auf mit Enrofloxacin-Konjugaten funktionalisierten Oberflächen erfolgreich getestet.

Um die Möglichkeiten des Biochips zur Gänze auszunutzen, wird ein Multiplex-Ansatz zum gleichzeitigen Nachweis verschiedener Antibiotika-Familien (zB: Flurquinolone, β-Lactame, Sulfonamide) verfolgt.

Referenzen:

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Curriculum Vitae

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