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Dissertation

Hydrogel surfaces: development, characterization, and application in protein chips

to attain the academic degree of a
Doctor of Technical Sciences

Supervised by

Univ.Doz.Dipl.-Ing.Dr.Georg Haberhauer, MBA
E 164 – Institute of Chemical Technologies and Analytics

and

Dr. Claudia Preininger
Austrian Research Centers GmbH-ARC
Division of Biogenetics-Natural Resources
Department of Bio-Resources/Microbiology

Submitted to the
Faculty of Technical Chemistry
of Vienna University of Technology, Austria

Katarzyna Derwinska

0425492

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We must not forget that when radium was discovered no one knew that it would prove useful in hospitals. The work was one of pure science. And this is a proof that scientific work must not be considered from the point of view of the direct usefulness of it. It must be done for itself, for the beauty of science, and then there is always the chance that a scientific discovery may become like the radium a benefit for humanity.

Marie Curie (1867 - 1934)

My research and thesis would have not materialized if I did not have love, support, and encouragement from a number of people. Their words and suggestions often boosted my courage and determination to write this thesis, helped polish my written English, and finally helped me grow as a scientist and a human being.

Moim Rodzicom

Abstract

Highly reactive and functional polymers are crucial for strong binding of proteins to solid surfaces, especially in miniaturized highly parallel analysis systems, such as biochips. Because surface chemistry strongly influences molecular interactions and the bioactivity and target binding skills of the bound species, development of efficient surface chemistries for immobilization of proteins is of major importance.

3-dimensional (3D) hydrogels are the material of choice for protein immobilization: they offer the unique advantage of a quasi-liquid microenvironment that retains the protein´s structure and activity. Furthermore, hydrogels provide high immobilization capacity and low non-specific binding.

The thesis summarizes the work on 3D hydrogel surfaces based on polyurethane (PU) and poly(vinyl alcohol) (PVA) that were either used plain, activated with periodic acid, modified with amino-functional crosslinkers, such as hexamethylene diamine (HDA), poly(allylamine) (PAH) and adipic acid dihydrazide (ADA) or functionalized with additives, like chitosan. The hydrogel surfaces were characterized with regard to surface roughness, layer thickness, water content, hydrophilicity and surface topography and evaluated in direct, competitive and sandwich immunoassays with respect to signal-to-noise ratio, fluorescence background and data reliability (percentage of data available for data anlysis, % CV, etc.).
Results deriving from physical and biological characterization were correlated to better understand how the proteins and surfaces affect each other and as a consequence, allow a definition of surface requirements to immobilize the proteins in a more controllable way.
Moreover, the effect of scanning mode, PMT gain and spot circularity on the data quality and reliability of on-chip immunoassays using hydrogel surfaces was studied.

Kurzfassung

Reaktive, funktionelle Polymere sind maßgeblich für eine feste Bindung von Proteinen an Oberflächen und Substrate, vor allem in miniaturisierten Analysesystemen, die für den Hochdurchsatz bestimmt sind. Dabei hat die Oberflächenbeschaffenheit entscheidenden Einfluß auf die molekularen Interaktionen, die Bindungseigenschaften und die Proteinaktivität. Deshalb ist die Entwicklung von „Chipoberflächen nach Maß“ für die Anwendung in Proteinchips von großer Bedeutung.

3-dimensionale (3D) Hydrogele sind das Material der Wahl für Chipoberflächen zur Proteinimmobilisierung: Hydrogele bieten den einzigartigen Vorteil einer quasi-flüssigen Microumwelt, die die Proteinstruktur stabilisiert, und die Proteinaktivität aufrecht erhält. Außerdem zeigen Hydrogele ausgezeichnete Immobilisierungskapazität und geringe unspezifische Adsorption.

Die vorliegende Dissertation faßt die Entwicklung von Hydrogeloberflächen aus Polyurethan und Poly(vinyl alkohol) zusammen, die entweder als Reinmaterial, aktiviert mit Periodsäure, modifiziert mit amino-funktionellen Crosslinkern wie zB. Adipinsäuredihydrazid oder funktionalisiert mit Additiven wie Chitosan verwendet wurden. Mittels analytischer Methoden (Kontaktwinkelmessung, Profilometrie, AFM) wurden die Rauhheit, die Schichtdicke und das Quellverhalten der Hydrogeloberflächen ebenso bestimmt wie die Hydrophilie und Oberflächentopographie. Außerdem wurden die Hydrogeloberflächen in direkten, kompetitiven und Sandwich Immunoassays hinsichtlich Signal-Rausch Verhältnis, Fluoreszenzhintergrund und Datenreproduzierbarkeit bewertet. Die Ergebnisse aus physikalischer und biologischer Oberflächencharakterisierung wurden zueinander in Beziehung gesetzt mit dem Ziel, die Wechselwirkung zwischen Protein und Oberfläche besser zu verstehen und infolge Anforderungen an Chipoberflächen formulieren zu können, die es erlauben, Proteine in kontrollierbarer Weise zu immobilisieren.

Außerdem wurde der Einfluß des Scanmodus, der PMT Verstärkung und der Spotmorphologie auf die Datenqualität und Reproduzierbarkeit in on-chip Immunoassays auf Hydrogeloberflächen untersucht.

CONTENTS

1. Scope of the thesis

One of the main challenges for further development of protein chips is the strength of signals produced by the probe-target interaction, and the coupling process itself, the assessment of protein-surface linkage and stability, and functioning of the assemblies.

Proteins are complex due to their tremendous variety in structure. When bound to a solid surface, they often loose biological activity by dehydration, denaturation or oxidation. Furthermore, proteins show a strong tendency to adsorb non-specifically. When combined, all of these factors can compromise the performance of protein chips, and thus their sensitivity and specificity. As a result, the development of reproducible surface chemistries, good characterization techniques for coupling biologically significant proteins and a better understanding of the interactions between proteins and chip surfaces are of major importance for improving the assay performance of protein chips, and biosurfaces in general.

In the presented work we aim at giving new insights in the interactions between proteins and solid surfaces using several modified and unmodified hydrogel surfaces in direct, competitive and sandwich assay formats taking into consideration the assay performance (signal-to-noise ratio, fluorescence background, immobilization capacity, assay sensitivity, data reliability) as well as physical parameters of the hydrogel surface (thickness, roughness, water content, hydrophilicity, wettability, surface topography etc.). Results of these studies are presented in "Effect of surface parameters on the performance of protein-arrayed hydrogel chips: a comprehensive study" (submitted to Langmuir, IF 3.71) and "A comparative analysis of polyurethane hydrogel for immobilization of IgG on chips" (submitted to Anal. Chim. Acta, IF 2.76).

Moreover, the impact of adsorption, covalent random and covalent site-specific protein immobilization on assay sensitivity and reproducibility is investigated and summarized in "Comparison of adsorption, covalent/non-oriented and covalent/site-specific IgG immobilization on poly(vinyl acohol) surfaces " (to be submitted to Anal. Biochem., IF 2.67).

Questions like: What quality measures can be introduced in protein chips to significantly improve data reliability? and How do scanning mode, PMT gain and spot circularity affect data reproducibility in protein chips? are answered in "Reproducibility of hydrogel slides in on-chip immunoassays with respect to scanning mode, spot circularity and data filtering" (submitted to Anal. Biochem.)

2. Protein chips

The concept of microarrays can be tracked back 25 years and originates from the southern blot that was introduced by Ed Southern who in 1975 fixed DNA onto a solid support that was used to attract complementary DNA (this process is called southern blot) [1]. The first arrays not yet microarrays were created in the late eighties and were called macroarrays [2]. They were produced by using a membrane-type material (e.g. nylon) for spotting a variety of DNA probes on, with spot sizes of about 300 microns, which limited the density of the spots to about 2000 probes. The macroarrays were applied for screening of DNA clone libraries, PCR products or oligonucleotides and typically used with radioactively-labelled targets. However, the modern microarray concept was presented only in 1991 by Fodor, who showed the first DNA microarray on a glass substrate [3]. The DNA microarray was further developed to a highly complex assay for gene expression by Schena, Brown and Davis [4] in 1995, who not only presented the concept of a gene biochip based on cDNA, but also the technical consideration of a microarray production.

As a promising high throughput technology gene chips were used in applications from gene expression to analytics and as a result, the use of microarrays in basic and applied research is growing at an extraordinary rate.

Despite the short history of DNA microarrays DNA arrays were implemented in a huge field of applications starting form the simple genome libraries in (1991) to the whole human genome array that was developed in 2004. Already in 1995 the quantitative monitoring of gene expression patterns with a complementary DNA microarray was presented [5] and one year later commercialized by Affymetrix [6]. The year 1997 brought a boost in microarray publications including the wide expression monitoring in yeast [7]. Implementing gene chips to almost all conceivable fields generated the need for more profound research not only on the genome scale but also on the protein level (summarized in many reviews [8-15]).

Protein microarray technology became the tool of choice that very well filled the need for simultaneous, high throughput multi-parametric analysis of a biologic sample, and likewise DNA microarrays is originating from dot blot techniques. However, when switching to microarrays the move was done from measuring a single protein at the time to parallel high throughput detection. The analysis of proteins is not as simple as that for DNA or cDNA because of major differences in structure, diversity and sensitivity for outer conditions [22]:

- In contrary to DNA made from 4 nucleotides providing a hydrophilic nature and negative surface charge, proteins are made of 20 amino acids and are not homogeneously charged

- The amino acids are chemically diverse and as a building material for proteins give tremendous diversity in structure, charge and binding properties
- Unlike DNA built as a helix proteins have 4 basic protein structures going from the primary structure which is the chain of amino acids, through secondary structure represented by α helix and β sheet, tertiary structure built of the elements of secondary structure which usually folded into a compact shape using a variety of loops and turns and finally quaternary structure which is the effect of interaction between several chains of peptide bonds.

Before microarray development many techniques were used for identifying proteins starting from blotting, protein electrophoresis, protein chromatography, mass spectrometry, x-ray crystallography, and biomolecular NMR. However, most common techniques used for protein characterization and quantification were: western blot (also called immunoblot) and ELISA (enzyme-linked immunosorbent assay). Western blot is a method widely used in molecular biology to detect protein in a given sample based on gel electrophoresis separation. ELISA is known as highly sensitive and reliable method for protein quantification and therefore is still a widely used and well-established technique. Immunodetection using ELISA requires a separate well for each sample of interest, moreover the immunoassay is laborious and time consuming. The disadvantage of enzyme-linked immunosorbent assay is the limited dynamic range that often requires repeating the test with further dilution of specimen. Due to the limitations of gel separation technology and ELISA the development of protein microarrays was an attractive alternative. In the late eighties Ekins [17-19] presented the theoretical background for protein based ligand binding assays. His idea of protein assays was based on antibodies, which not only were able to simultaneously screen the complex analyte, but also were more sensitive and fleet than conventional methods with much less sample consumption as the screening was performed in the microscopic scale of a glass slide. The early protein microarrays were trying to convert the classical immunoassays made in 96-well microtiter plate format (ELISA) onto plane – chip format that allowed simultaneous detection of multiple analytes at multiple array addresses within a single well [20, 21]. This approach was a logical progression toward a smaller assay format allowing multiple analytes to be detected. As reported in literature [20] "ELISA on the chip" showed similar assay sensitivity and detection ranges than classical ELISA.

The main advantages of protein microarrays over ELISA or gel electrophoresis followed by mass spectrometry are the miniaturized assay format, the ability for high throughput detection and quantification as well as low consumption of reagents. Technical aspects and applications of protein microarrays are reviewed [22-27].

2.1 Principles

Biochips are ordered arrays of biomolecular probes immobilized onto solid supports for highly parallel, miniaturized, and functionally integrated analysis systems. In protein arrays probes like antibodies, enzymes or peptides are immobilised onto a support which can be a glass slide, a set of particles in a solution or a silicon waver. The protein immobilized on the support (capture protein) is exposed to the analyte. The interactions between the proteins can be monitored with optical techniques like flourescence, or by radioactivity measurement, or electrochemistry as well as piezoelectric detection methods. Protein arrays currently described in literature fall into two main categories:

- Protein function arrays
- Protein detection arrays

In protein function arrays spotted probes are the representation of proteins derived from a specimen under research, and are studying activity of native proteins. Detection arrays provide smaller probe sets and consist of spotted ligands (e.g. antibodies) that are highly specific for the proteins to be detected. These microarrays are widely used as analytical tool in screening biological solutions.

There are two main types of protein detection arrays [28,29]:

- Direct label
- Sandwich

The choice of optimal protein array format depends on the particular application in the chosen research field as well as on the ability of labelling the proteins. The two main formats that are actually used either measure the proteins that are labelled and allow detection based on capturing the labelled sample by spotted antibodies or are based on the pairs of corresponding capture and labelled antibodies for protein detection.

2.1.1 Direct label assays

The direct label method is derived from DNA microarrays and used mostly for expression studies on the protein level as well as in analytical assays in the competitive assay format. Thereby, labelled sample protein is applied to single antibodies bound to the chip to screen for analyte proteins

in the sample. The same approach can be used for analyte antibodies labelled for direct detection on protein arrays (see Fig 1 a). This method relies on the proteins that can be labelled beforehand and gives the possibility of easy detection of any protein in the sample that can interact with spotted antibodies and associated elements. This technique is widely used for examination of targets that are hard to characterize like cell signaling proteins in the comparative and qualitative way. Alternatively, in some applications, antibodies can be used to detect binding events. One common use is for antibody screening.

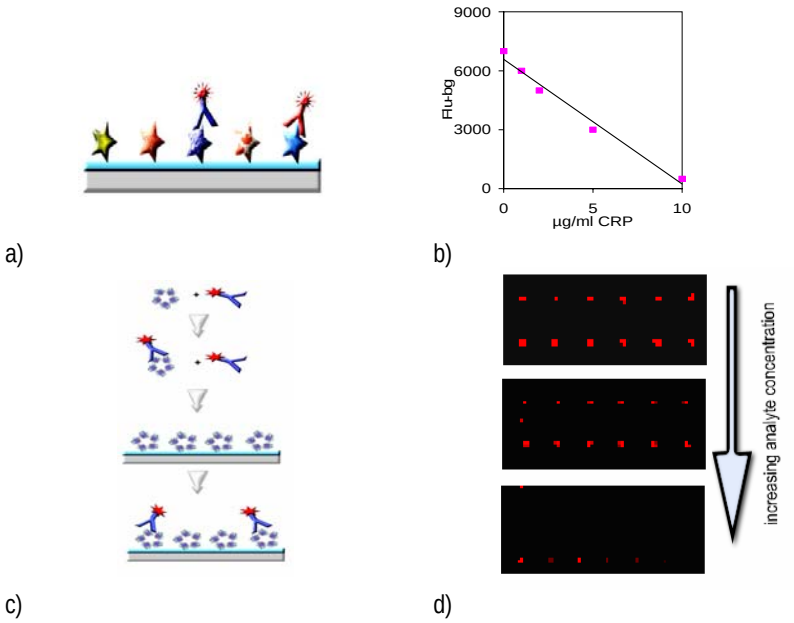

Figure 1 Principle of protein detection arrays a) direct label and b) calibration curve for the competitive assay, c) competitive assay principle and d) scans of the assay.

Label-based arrays have been used for protein profiling in biological samples [30-35]. In principle, only one antibody is used for one target, and if antibodies don't interfere with each other this type of array can consist of numerous antibodies allowing multiplex screening. Direct assays allow incubating of two different samples on the same array in the way that usually the control sample and test sample just labelled with two different fluorophores are incubated together. This type of assay is called competitive, as competition between proteins from both samples occurs when reaching the targeted probe. The competitive assay is advantageous over the non-competitive in measuring multianalytes with different protein concentrations within a single experiment as long as the control protein is of similar concentration without need of sample dilution [36]. When using two labels the concentration ratio between test and control samples can be estimated from the fluorescence signal ratio of a single spot. As a consequence, no additional calibration is needed. For this reason two colour assays are widely used in protein profiling studies, especially where pairs of antibodies for certain proteins are not developed yet.

The idea of competition can be also used for the analytical application of direct label assays where instead of using two labels the labelled protein is mixed with the sample and incubated onto the slide as shown in Fig 1 c. The protein of interest is spotted onto the slide surface. The sample is mixed with the

labelled antibody and preincubated. During this time the labelled antibody reacts with the analyte and the more analyte is in the sample, the less free labelled antibody is left to bind to the protein immobilized on the slide. The second step of the assay is to process the slide with the preincubated mixture. From the fluorescence signals the analyte concentration in the sample can be calculated based on the calibration curve done with standard concentrations of protein. The more concentrated the protein in the sample the lower are the read signals. An example of respective scans and calibration curve is shown in Fig 1d and b respectively.

2.1.2 Sandwich assay

Sandwich assays are based on the ELISA principle which uses bonding of protein of interest by both: capture ligand (capture antibody) and detection ligand (labelled protein). The detection ligand binds to the array only if the target protein is bound. The enzyme linked immnosorbent assay (ELISA) is a fundamental tool in clinical immunology.

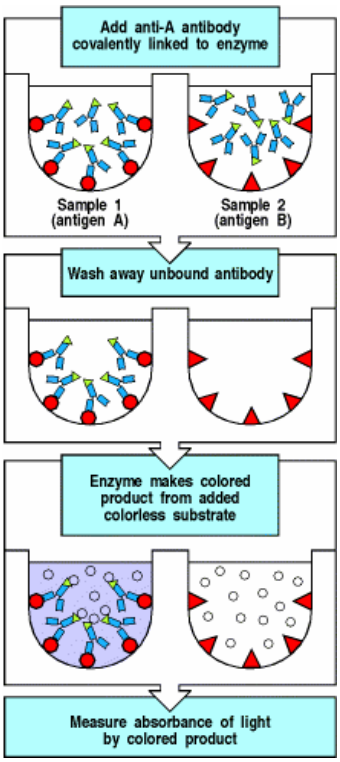

Figure 2 Principle of the enzyme-linked immunosorbent assay (ELISA).

From Immunobiology: The Immune System in Health and Disease - Sixth Edition

The principle of one of the types of ELISA is shown in Fig. 2. For detection of antigen A, purified antibody specific for antigen A is chemically linked to an enzyme, usually HRP (horseradish peroxidase). The antigens of interest are coated onto the surface of plastic wells to which they bind nonspecifically. The labeled antibody is then added to the wells and binding to antigen A causes the labeled antibody to be retained on the surface. Unbound labeled antibody is removed from all wells by washing, and bound antibody is detected by an enzyme-dependent color-change reaction usually with the use of chromogenic substrates like TMB (3,3',5,5'-tetra methyl benzidine) or ABTS (2,2"-azino-di-(3-ethylbenzthiazoline-6-sulfonate).

This test can be also performed with the use of two antibodies in the way that in the first step the antibody binds to the antigen, and in the second step peroxidase-labelled anti-human antibodies are added which react with the antibodies previously attached to the antigen.

There are many types of ELISA. Monospecific ELISA (enzyme immunoassays with a single antigen) provides a quantitative in-vitro assay for the detection of antibodies, whereas "Profile ELISA" reveals

a semiquantitative in-vitro assay for the detection of different antibodies in a single microplate. In "Pool ELISA" the solid phase is coated with an antigen mixture for the semiquantitative detection of antibodies whose specificity must be investigated subsequently by monospecific assays. Furthermore, there is the sandwich ELISA, used as prototype in microarrays which relies on specific antibodies coated onto the wells of the microtitre plate. After adding the sample, immobilized antibodies react with antigens present in the sample. Addition of enzyme-linked secondary antibody, which binds to the antigen, allows antigen detection by enzymatic conversion of a chromogenic substrate to a detectable form.

In Fig. 3 the principle of a sandwich immunoassay on microarray slides is shown: First, antibodies (capture antibodies, cAB) are immobilized on the chip surface and incubated with the sample solution containing the analyte to be detected. Secondly, labelled secondary antibody is applied to the chip which binds to the analyte bound to the capture antibody. The detector antibody is either modified with the label (fluorescent, isotope) or is biotinylated for detection with labeled streptavidin. The more analyte is bound to the cAB, the more labelled antibody will bind to the analyte resulting in a brighter fluorescence signal. The respective calibration curve is presented in Fig. 3. The sandwich approach can be used only, if the antibody pairs are developed and tested for cross-reactivity. If this is not the case, then other methods shall be used. For protein quantification a calibration curve of standard protein concentrations is needed. Sandwich immunoassays are widely used when there is a strong need for measuring very low concentrations of analytes, such as hormones, biomarkers, or growth factors. Using the ELISA approach it is very important to immobilize as much antibody as possible within a single spot. The development of sandwich immunoassays is more difficult than that of direct label assays, as the use of specific pairs of antibodies can cause cross reactivity due to the complex sample matrix. Sandwich immunoassays are more specific and more sensitive than direct label assays because two targeting antibodies are employed, allowing to screen low concentrated protein in a complicated matrix [37, 38].

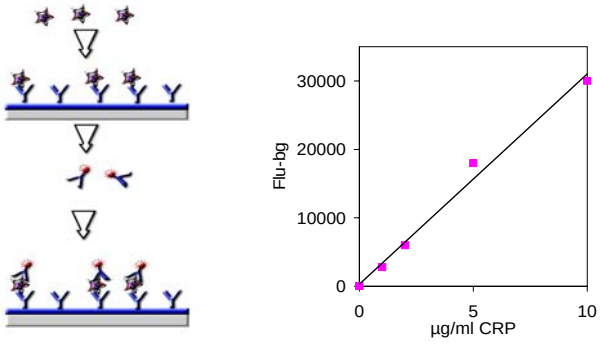

Figure 3 Principle of sandwich assay and the respective calibration curve

In conclusion, no matter the assay format of interest the antibodies need to be well characterized, especially for crossreactivity which is usually done by targeting different samples against antibodies. Table 1 [39] summarizes the different types of protein microarrays.

Table 1 Types of protein microarrays

Array type	Description
Antibody array	Polyclonal or monoclonal antibodies are arrayed onto the chip surface to detect and quantify specific proteins in a biological sample. An antibody array is effectively a parallel series of miniaturized immunoassays
Antigen or reverse array	The converse of antibody array, this chip has immobilized antigens that are used to detect and quantify antibodies in a biological sample
Functional array	Purified proteins are arrayed on the surface and used to detect and characterize protein-protein, protein-DNA, and protein-small molecules interactions
Capture array	Non protein molecules that interact with proteins are immobilized onto the surface. Such receptor molecules may be molecular imprinted polymers or oligonucleotide aptamers.
Solute arrays	The potential next generation of arrays is to have nanowells containing coded microspheres or bar-coded nanoparticles in solution

2.2 Applications

The use of protein biochips is based on the extraction and retention of targets from liquid media. Protein microarrays have the great potential to function in many array-type high-throughput applications including protein-protein and protein-drug interactions, protein localization, antigen-antibody interactions, enzyme-substrate, and receptor–ligand interactions. Up to now presented protein microarrays usually consist of antibodies, proteins or protein fragments, aptamers, peptides or carbohydrate elements that are arrayed on the substrate and used for screening and assessing patterns of interactions with samples containing distinct proteins or classes of proteins.

An overview of the types of protein microarrays was shown by Stoll (Fig. 4), where different types of protein capture microarrays and protein interaction arrays are presented [31]. Specific protein capture on microarrays can be performed in many different ways: affibodies (Figure 4 a) as well as aptamers (Figure 4 b) or antibodies (Figure 1 c, d) are widely applied as capture proteins. Detection of analytes bound to the immobilized proteins is mainly performed by direct labelling of the analyte (Figure 4 a–c) or antibody sandwich immunoassays (Figure 4 d).Such assays are successfully used in multiparametric diagnostics for both protein identification and protein quantification.

The second approach as presented in Fig. 4 is reverse screening and is based on immobilized cell lysates, which are a representation of proteins in cells at a distinct state. Captured proteins can be identified using mass spectrometry (e.g. SELDI) (Figure 4 e) or specific antibodies (Figure 4 f). The use of this type of protein arrays found application in diagnostics where patient sera are screened for presence or absence of proteins (Figure 4 f). There is also the possibility to immobilize tissue samples (Figure 4 g) or cells (Figure 4 h) which are then used for reverse screening approaches with antibody detection of specific markers.

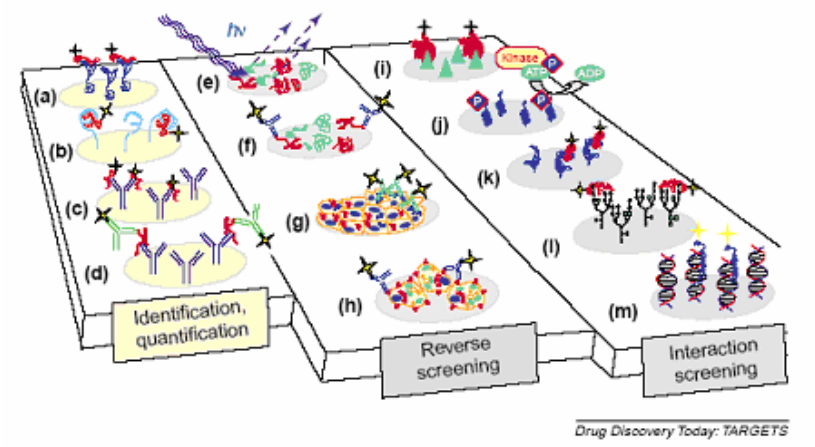

Figure 4. Types of protein microarrays

The third approach (Fig. 4) describes specific interaction microarrays for detection of receptor–ligand (e.g. small molecule drug candidates, phospholipids) interactions (Figure 4 i), enzyme–substrate interactions (Figure 4 j), protein–protein interactions (Figure 4 k), protein–oligosaccharide interactions and protein–DNA interactions (Figure 4 m). One important application field is the identification of direct 1:1 interaction partners of proteins.

Since introducing protein microarrays the field of applications has been constantly growing. The very first protein arrays were mainly used for proteomics studies, but with time all application fields from diagnostics to therapeutics were covered. In many cases the application fields are overlapping: for example, protein expression research can be used for diagnostic purposes.

Proteomics studies took the idea of dual-color labelling from mRNA expression procedures in DNA expression arrays. Usually protein samples from e.g. control source and sample of interest are labelled with different fluorophores and applied in the ratio 1:1 to the protein array spotted on the substrate (Fig. 5). Fluorescence, that occurs upon binding of the target to the protein array, is scanned with a dual channel fluorescence scanner and data are analyzed. As a result, the ideal protein chip will provide the quanititative data of the protein expression profile. In order to obtain high specificity binding in-between tested proteins and spotted probes, antibodies were introduced as optimal and highly specific capture proteins. So far antibody capture arrays in proteome research have been successfully implemented in cancer research [65], e.g. leukemia [67] and in protein expression studies investigating the response to radiation [66] or drugs treatment [79] of tumors.

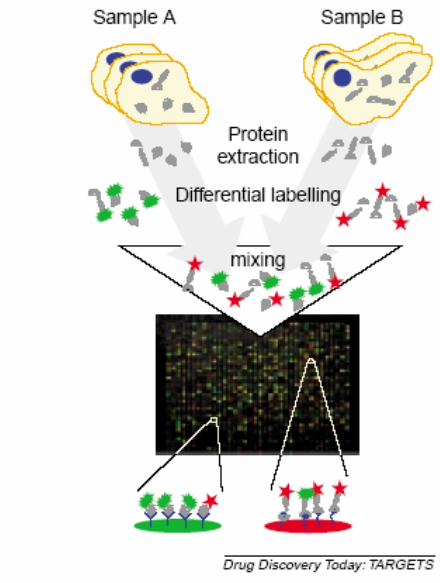

Figure 5 Dual labelling in protein expression microarrays (*like Fig. 3*)

Figure 6 FAST frame for multisample analysis for protein microarrays from www.arraying.com

The diagnostic field of protein microarrays has been growing since their introduction in 2000 and because of the miniaturized formats, they are especially promising in the field of diagnostics. Because of low reagents consumption, ability of leading simultaneous reactions on a single slide (Like in FAST frame shown in Fig. 6) they are providing affordable and fast test at the protein level. For serum based diagnostics antigens are printed onto the slide surface and then reacted with the serum sample, which is then screened for neutralizing antibody response. By contrast, for measuring expressed proteins as an effect of disease antibodies are arrayed onto the chip. Table 2 summarizes the various types of diagnostic arrays. Diagnostic microarrays have been developed for screening of antiviral antibodies[80] as well as for vaccine development [81] and for IgE levels monitoring for prevention and treatment of allergies [82]. Determination of carbohydrate binding pathogens in complex mixtures using *E.Coli* as an example was also reported [83].

Table 2 Use of different types of protein microarrays for various diagnostic applications

	Protein		Tissue	Cell	Carbohydrate	Small molecule
	Ab	Ag				
Cancer	+	+	+	-	-	-
Infectious diseases	+	+	-	-	+	-
Immune response related	+	+	-	[+]	-	-
Neurodegenerative disseases	[+]	+	+	-	-	-
Biodefence	+	+		-	-	-
Technology related	+	+	+	+	+	+
Toxicology	-	-	-	+	-	+

Ab antibody, Ag antigen, From Protein microarrays by Schena

A novel area of diagnostics applications of protein microarrays is food and environmental monitoring, but up to now no chips for high throughput screening have been developed.

Protein microarrays have enormeous potential in the therapeutics field as the use of protein microarrays allows following and understanding the drug interaction with cellular proteins. However, screening for drugs and target proteins is not only reduced to understanding the mechanism of drug activity, but also takes in account how drugs bind to other proteins and what is their cross reactivity and toxicity. For this field of research microarrays seem to be the best choice as they allow multisensing within a single experiment. The reported work on protein therapeutics shows that protein microarrays are advantageous over ELISA as they provide simultaneous information on the clearance rate of the protein and it's in vivo processing. Moreover, using protein microarrays allows the detection of proteins that cannot be detected with ELISA systems. For therapeutic reasons it is also important to follow the immunization patterns [85]

3. Chip manufacture

As protein microarrays are similar to DNA microarrays and are using the same equipment for spotting or data analysis the chip manufacture steps are similar. The differences are mostly in probes preparation, sample handling and labelling as well as in the choice of proper substrates, binding buffers, and appropriate time and temperature for biochemical reaction. A microarray experiment can be divided into 5 basic steps as schematically described in Fig. 7:

- Biological question
- Sample preparation
- Biochemical reaction
- Detection
- Data analysis

First step in the microarray experimental cycle is to formulate the biological question, the aim of the experiment that will allow further experimental planning. The second step that comes into microarrays experiment cycle takes in account the probe and sample preparation as well as possible sample modification. The choice of spotted proteins strongly depends on the assay type (described in 2.1) and sample. The possible types of proteins widely used in protein microarrays are described in the *Probe preparation* section. It needs to be pointed out that there is no generally applicable design of protein arrays. Each protein array needs to be adapted to the specific application, the assay format and equipment available, e.g. quantification of protein is dependent on the specificity and uniform binding affinity of the probes (e.g. monoclonal antibodies). The most important difference in protein and DNA arrays is the complexity of the protein´s structure and its sensibility to changes in the environmental conditions. Thus, difficulty of working with protein microarrays is mostly concerned with preventing protein from denaturation during

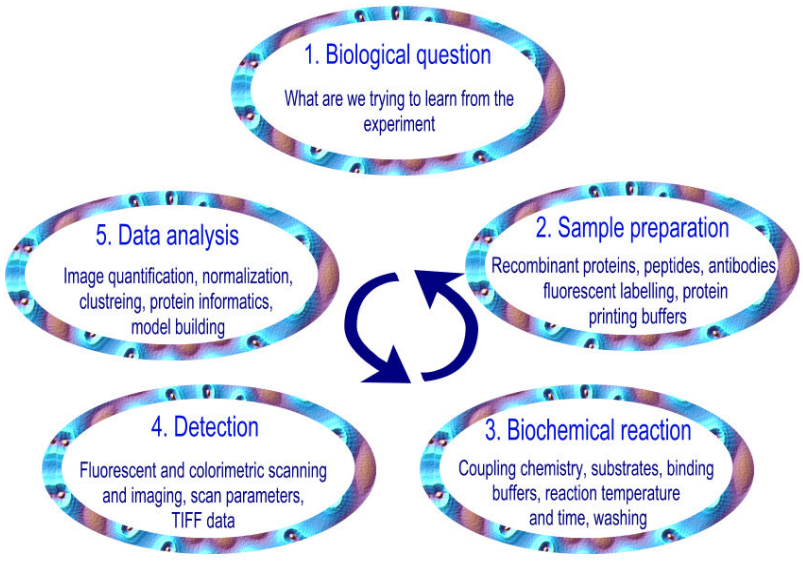

Figure 7 Microarray experimental cycle. From Protein Microarrays by M. Schena

manufacture, and during the microarray experiment. The best-known denaturizing agents that can disrupt protein´s structure are high temperature, pH changes, organic solvents (like formamide) as well as inorganic salts, high pressure and ultrasounds. Additionally, proteins have the tendency to adsorb nonspecifically to solid substrates causing background problems and therefore low assay sensitivity due to low signal-to-noise ratios. For this reason experimental design needs to take into account not only the right choice of probes like antibodies or peptides, but also protein printing buffers that allow protein binding without activity loss. The third step in a microarray experiment is the biochemical reaction occurring between the sample and the spotted probes. Here the most important issues to consider are coupling chemistry, optimization of reaction time and temperature and washing conditions having in mind the high sensitivity of proteins to changes in environmental conditions. The detection step allows implementing scanning and reading devices based on fluorescence, chemiluminescence, or radioactivity and takes in consideration different imaging modes and scan parameters. The last step of the microarray cycle is data analysis and image quantification, which is usually the most time consuming part involving normalization procedures, statistics, transformations and filtering of outliers. The five key components in the manufacture of high quality microarrays are:

- motion control system
- spotting pins
- probe preparation
- substrate support
- printing environment.

If any of these five criteria is not optimized, the quality of the resulting microarray will be compromised. All mentioned components are described in the next chapters.

3.1 Probe preparation

Before starting the microarray experiment several steps ranging from probe preparation to slide processing need to be implemented. Taking into account the complexity of proteins as well as their sensitivity to changes in environmental conditions special precautions are required for probe preparation. Depending on the spotted protein as well as on the assay format there are several possibilities to build up a protein microarray (see also Table 3 [39]). Widely employed capture agents are as following:

- Antibodies
- Proteins
- Peptides
- Receptors
- Aptamers
- Scaffolds
- Haptens

The most widely used probes are antibodies (AB). They are made from cells derived from the B-lymphocyte (B-cell). Each of these cells makes only one specific antibody. The antibody itself consists of four chains of amino acids chemically bonded to each other. They are "Y" shaped having two identical smaller *light chains* and two identical larger *heavy chains* forming each side of the "Y" (Fig. 8). The antigen binding sites on the top of the antibody are called Fab fragments, the constant antibody region is called Fc fragment, and this part of antibody attaches to the phagocytes.

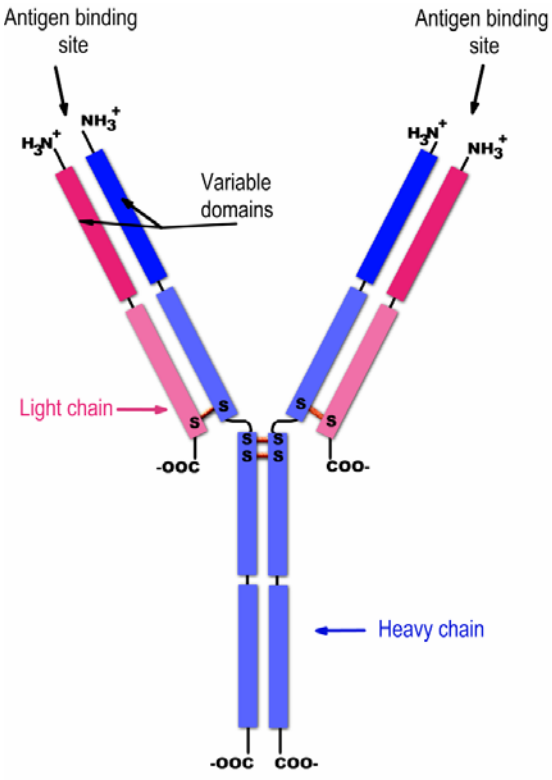

Figure 8 Scheme of the antibody

Antibodies are used for antigen detection and serum based diagnostics as well as in protein expression studies. There are two types of antibodies:

- Monoclonal (mAB) antibodies
- Polyclonal (pAB) antibodies

Monoclonal antibodies are produced by cell lines or clones obtained from animals that have been immunized with the target substance. These antibodies are found to have excellent stability and binding specificity, but the cost of production is high. Another possibility is to use polyclonal antibodies, which are less specific than monoclonal ones, but represent excellent stability and binding affinity. Production requires a high amount of antigen during the production process and can exhibit cross-reactivity as coming from animal immunization. In the microarray field, not only whole antibodies are used, but also their fragments (Fab or Fc fragments).

Furthermore, epitopes are used as probes in microarrays because they play important roles being the specific targets of immune responses. When using peptides as specific antibody epitopes, quantitative

measurements of antibody/epitope binding are possible. This approach can be used not only for immunological studies, but also for screening of biomarkers or in the vaccine development.

Table 3 Protein microarrays applications depending on the probe type and interaction

Probe type	Interaction	Application
Antibodies	antibody–antigen, antibody–RNA, antibody–DNA, antibody–cell surface, antibody–organelle	Epitope mapping, evolution, gene expression, genotyping, post-translational analysis, proteomics, structure–function
Cell extracts	general biochemical, proteins	General biochemical, reverse- phase protein microarrays
Enzymes	enzyme–substrate, enzyme–effector, enzyme–inhibitor	Kinetics, substrate specificity, inhibitor analysis
Peptides	protein–protein, protein–RNA, antigen–antibody	Epitope mapping, evolution, gene expression, genotyping, post-translational analysis, proteomics, structure–function
Proteins	protein–DNA, protein–small molecule, protein–RNA, protein–protein, protein–receptor	Drug discovery, epitope mapping, evolution, gene expression, genotyping, post- translational analysis, proteomics, structure–function, two hybrid analysis
Small molecules	small-molecule–protein	Binding kinetics, drug discovery
Whole cells	receptor–hormone, receptor–antibody, sugar–antibody	Cell typing, secretion studies, clonal analysis
Carbohydrates	sugar–protein, sugar–antibody, sugar–receptor	Docking, signaling

Synthetic peptides consisting of 10-80 amino acids are easily obtained from a solid phase chemical synthesis with high purity and ability to be produced in huge amounts. This type of probes is widely applied in focused microarrays e.g. epitope analysis and in quantitative measurements of protein binding or enzymatic activities. The use of peptides thanks to their small size allows obtaining high density chips (up to thousands of peptides/chip).

Another important group of probes are recombinant proteins that are well known to provide one of the easiest and most appropriate ways to study protein functions diversity. They are produced in vivo as result of expression in recombinant host cells. Recombinant proteins are mostly employed in proteomic studies on protein-protein binding, protein-drug binding and specificity of enzymes.

Alternatively, aptamers are used in protein microarrays [97]. Aptamers are single stranded DNA or RNA molecules that can form tertiary structures capable of recognizing and binding non-nucleic acids structures like proteins with high specificity [86]. Having high-affinity to protein ligands, aptamers have the advantage of high stability and ease in modification. Aptamers are also known as the most

effective tools for the detection of biomolecular interactions and the identification of protein targets that cannot be detected by antibodies.

In a few cases affibodies and ankyrin repeat proteins are used as probes in protein microarrays. Affibodies are robust proteins based on the structure of protein A whereas ankyrin repeat protein are known to be termostable and easy to produce [87].

3.2 Chip substrates & protein immobilization

When looking at the protein properties and the amino acids they are build from there are many reactive groups that can be incorporated in binding to a slide surface. The use of monolayers as well as several glass polymer coatings and gel pads have been reported [22,24,27,100].The microarray chip format allows almost unlimited miniaturization and high throughput analyte detection using small sample volumes. The protein microarray approach offers a large variety of immobilization chemistries: in Table 5 protein coupling to a solid surface is described with respect to the reactive group of the protein, the surface modification and the type of binding. Despite of the numerous possibilities one has to keep in mind that proteins require a quasi-liquid microenvironment that can maintain the proteins structure and activity.

Table 5 Methods of coupling proteins on a microarray slide

Functional group of peptide		Available surface derivatization	Type of binding
natural	- COOH Asp	Amino	Electrostatic Covalent amide after carboxy activation
	- NH_2	Carboxilic acid, active ester, epoxy, aldehyde	Electrostatic
	Lys, Gln, Arg		Covalent amide
	-SH Cys	Malemide	Covalent thioether
	- OH Ser, Thr	Epoxy	Covalent ether
synthetic	His-tag	Ni-NTA complex	Coordination complex
	Biotin	streptavidin	Supramolecular complex

From ``Protein microarray technology`` by D.Kambhampati

3.2.1 Binding chemistry

A key issue in microarray fabrication is the design of the substrate material, which needs to robustly bind proteins to the surface without loss in activity. As proteins are diverse, there is no optimum substrate, and as a consequence several chip surfaces are available for use in protein arrays. The way the proteins are immobilized on the substrates is crucial for the functional properties of the microarray. Because surface chemistry strongly influences molecular interactions and the bioactivity and target binding skills of the bound species, development of reactive surfaces is inevitable.

In principal, all immobilization chemistries are based on three binding mechanisms (see Table 6 and Fig. 9):

- Adsorption
- Covalent binding
- Affinity binding (biorecognition)

Table 6 Attachment strategies for protein microarrays

Surface chemistry	Binding mechanism	Type of assay
Hydrazide-activated polyacrylamide gel	Antibody carbohydrates oxidized with sodium periodate ($NaIO_4$)	
Hydrogel	Adsorption/molecular sieving	Antibody and sera, [53] antibody and antigen [49]
Agarose gel activated with $NaIO_4$	Amino groups	Sandwich assay [54]
Aminosilane	Electrostatic adsorption	Antigen- antibody [55], membrane proteins and lipids with ligands [56]
Poly-L-lysine	Adsorption	Cell and tissue [57], antibody and antigen [58]
Poly(phenylalanine lysine)	Adsorption	Antibody and cytokines in ELISA[59]
Nitrocellulose	Adsorption	Bacterial antibody array [60], Antibody and antigen [61,62]
Aminosilane with oxidised dextran	Schiff base via protein amines	Microarrays based ELISA for IgG and IgE [63]
Cyanosilane	Adsorption via antibody carbohydrates	Cytokine array[64]
Aldehyde silane	Schiff base via primary amines	Antibody and cell line lysates [65], antibody [61]
BSA with a bifunctional crosslinker	Amino groups	Antibody[20], Antibody and cell lysates[66]
Poly-L-lysine with photoreactive crosslinker	Amino groups	Antibody and sera [53]
Aminosilane with bifunctional N-hydroxysuccinimide	Amino groups	Antibody and sera [26]
Epoxy silane	Amine , thiol, and hydroxyl groups	Antibody antigen [57] , peptide assay [67], small protein [68]
Nickel coating	Histidine Tag	Antibody antigen [69]
Dendrimers	Amino groups	Streptavidin and biotin [70,71]
Avidin	Biotinylated antibodies	Peptide based array [72] , antibody and secondary antibody [73]

From Protein microarrays by Schena

Physical adsorption of proteins on surfaces is a simple and effective method of immobilization, especially for large proteins, but mostly random and uncontrolled, whereas covalent binding of proteins results in stronger, "statistically oriented" binding which occurs between the reactive groups of the

proteins and those available on the surface. Affinity binding between protein and surface is site-specific and most effective.

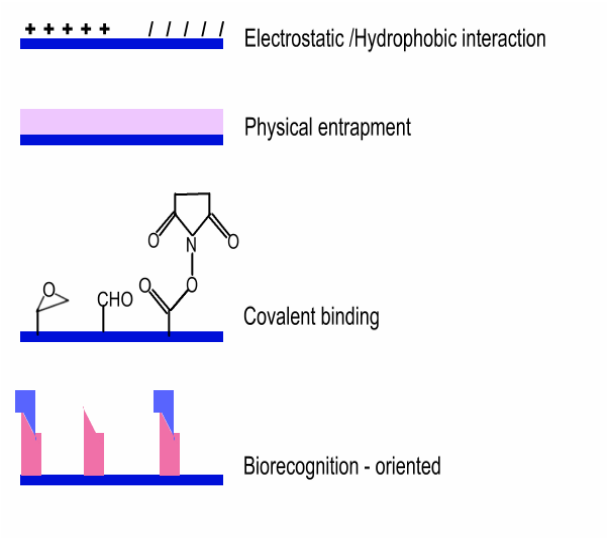

Figure 9 Immobilization mechanisms for proteins (not in scale)

The simplest immobilization strategy based on adsorption is usually unspecific and can result in protein unfolding and inactivation. The most widely used materials are aminosilane [33], poly-L-lysine [34], poly(phenylalaninelysine) [35] and cyanosilane [36]. Adsorption was already used before in ELISA and western blot. Adsorption can be done in two ways, via electrostatic forces when using aminosilane slides for example or via hydrophobic interactions on e.g. nitrocellulose membranes. Membranes are known to have very good loading capacity, they provide a stable environment for proteins, but have the disadvantage of increased nonspecific binding.

The slides available for protein adsorption (e.g poly-l-lysine, aminosilane) attach proteins either via carbohydrates (cyanosilane) or electrostatically (aminosilane).

Additionally, slides can be further modified with cross-linkers that are introducing to the surface active amino, thiol (mercaptosilane [39]) or aldehyde groups for more specialized and stronger, covalent binding of spotted protein. When using the covalent attachment approach, the bond is formed between the functional groups of the protein and the complementary coupling groups of the slide surface. Fig. 10 shows the mechanisms of various covalent attachment chemistries.

Covalent binding can occur via amino, thiol and hydroxyl groups of proteins. Thereby, primary amines from lysines and arginines bind covalently to epoxy- or aldehyde-modified surfaces (eg. aldehyde slilane [37, 38], Fig. 10 a) and e) respectively) forming secondary amines. On surfaces modified with succinimidyl ester (Fig. 10 d) the protein is bound via its primary amines under formation of a stable amide linkage. Unreacted surface succinimide groups can be deactivated by the use of a blocking solution containing e.g. glycine that in addition reduces the background. The thiol group from cysteins can be employed for protein coupling on maleimide or thiol surfaces resulting in a stable thioether bond (see Fig. 10 c)). Covalent binding is the most preferred immobilization technique because of its stability, and very high density of immobilized protein which in most cases is directly translated into highly sensitive detection. However, one has to keep in mind that binding can result in nonspecific

protein orientation and too high density of reactive groups in protein unfolding and denaturation. Moreover, the reactive sites of the protein can be blocked by immobilization procedures which are reason for reduced activity.

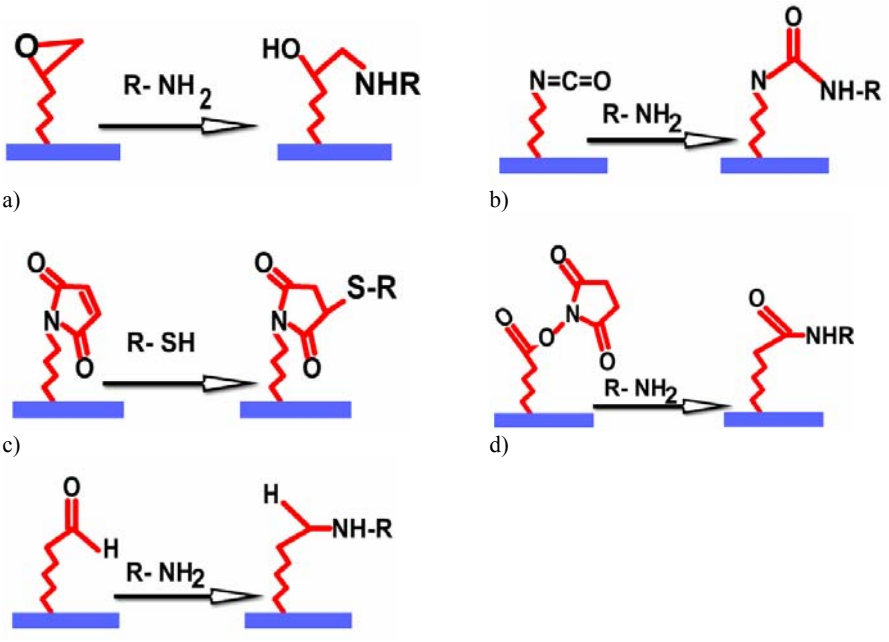

Figure 10 Covalent attachment mechanisms of proteins: a) glicydoxy with amineresт, b) isocyanate with amino rest, c) maleimide with thiol rest, d) NHS with amino rest and e) aldehyde with amino rest

Despite of adsorption and covalent binding of proteins to solid surfaces proteins can be attached via affinity binding. The principle of affinity binding is shown in Fig. 11 using the streptavidin - biotin reaction as an example. In the presented scheme the microarray substrate is modified with streptavidin, which reacts with biotinylated protein. Each of the strepatvidin molecules has four active sites for biotin binding resulting in as much as four protein molecules that can be attached to the surface per streptavidin unit. Other molecules suitable for affinity binding are proteins A, G and L and metals, such as Ni^{2+}, which however are described in more details in the *3.2.3 Orientation chapter*.

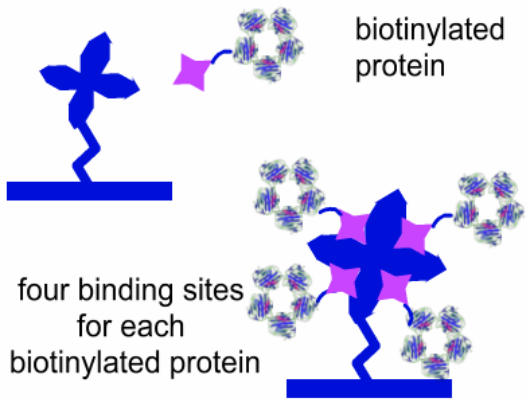

Figure 11 Principle of streptavidin affinity binding

In order to choose the optimum substrate for the immobilization of the protein of interest, one has to consider the accessibility of the immobilized protein for the respective target. This strongly depends on the size of the spotted protein. When spotting small sized probes all strategies are suitable for good

target accessibility, whereas for larger probes going up to cells sizes the best of results are obtained when using adsorption and covalent linking techniques (Table 7).

Table 7 Immobilization strategies for different types of protein assays

Assay type	Physical adsorption	Covalent immobilization onto flat surface	Covalent immobilization onto gel coating	Cross-linking
Small ligands	+	+	+	+
Proteins	+	+	+/-	-
Large organelles and cells	+	+	-	-

From Protein microarrays by Schena

When speaking of protein immobilization in microarrays one important issue is the surface concentration of immobilized protein. A key role for the optimum surface density needed for successful signal detection plays the detection technique: when using confocal microscopy the sensitivity of the measurement is high and thus less protein per surface area is needed for sensitive detection of analyte, whereas less sensitive and less expensive techniques require higher amount of immobilized protein to reach sufficient assay sensitivity [37, 74]. However, the amount of bound molecule strongly depends on the immobilization method and the used surface.

Table 8 Properties of immobilization techniques in protein microarrays

	Physical Adsorption	Entrapment into hydrogel	Covalent immobilization onto flat surface	Covalent immobilization onto gel coating	Cross-linking
Access to large ligands	Yes	No	Yes	Difficult	No
Density of immobilized protein	Low	High	Low	High	Very high
Stability of immobilization	Relatively low	Relatively low	High	High	Very high
Preservation of functional activity	Poor	Very high	Fair	fair	Moderate

From Protein microarrays by Schena

Table 8 summarizes immobilization density, access of ligands and stability and preservation of protein depending on the used substrate. As expected, entrapment in hydrogel results in very high density of immobilized protein as well as the best of all preservation of protein functional activity.

3.2.2 Dimensionality

1-dimensional coatings (1D)

1D coatings are usually made on derivate glass substrates that are modified with poly-l-lysine or aldehyde- and amino-silanes. Poly-l-lysine slides were widely implemented in antibody-antigen microarrays, e.g. in multiplex arrays of 115 to 196 proteins for detection of biomarkers specific for

prostate cancer [25] and rheumatoid arthritis [58]. Silanized glass typical for DNA arrays was applied e.g. for simultaneous analysis of cytokines in human blood [75].

A prominent type of 1-dimensional surfaces are self assembled monolayers (SAMs) that consist of a single layer of molecules on a substrate. They can be prepared with the use of different molecules and different substrates depending on the application. A common example is an alkane thiol on gold. SAMs are known to enhance the biocompatibility with proteins and thus binding capacity. As proteins contain a wide range of functional groups, all of them can be used for chemical immobilization on a slide surface. Most commonly alkylsiloxane monolayers, fatty acids on oxidic materials, alkanethiol monolayers and surfactants are employed. The most common substrates are gold, glass, quartz and other metal surfaces. Alkanethiols that chemisorb on gold surfaces through the thiol headgroup provide a surface whose chemical and physical properties are precisely controlled by varying the terminal chemical functionality of the alkanethiol molecule. Desired molecular recognition properties can be tuned by proper selection of the alkanethiol terminal functional group. The combined work of Lee [25] with the use of monolayers proved that it is possible to monitor antibody–antigen adhesion as well as cell adhesion. In order to control the properties of the chip surface and especially, the density of reactive groups SAMs of mixed alkanethiols are employed. SAMs on gold play an important role in surface plasmon resonance (SPR) studies, as the thickness of the immobilized protein layer is critical and compromise the detection sensitivity.

2-dimensional coatings (2D)

The application of 2D coatings derives from the medical use of poly-ethylenglycol (PEG). Since years PEG is implemented in medicine in implants and drug delivery systems. It's a neutral hydrophilic polymer that prevents surface fouling by proteins and unspecific protein binding. As PEG can be a derivative with different reactive groups, several strategies for protein binding can be followed: Using PEG as a spacer between surface and protein was very successfully used to prevent steric hindrance [40]. Additionally, PEG side chains can be copolymerized with polymer backbones under formation of high density structures. Surfaces based on high molecular weight PEG molecules are sometimes problematic, because of their very poor grafting efficiency.

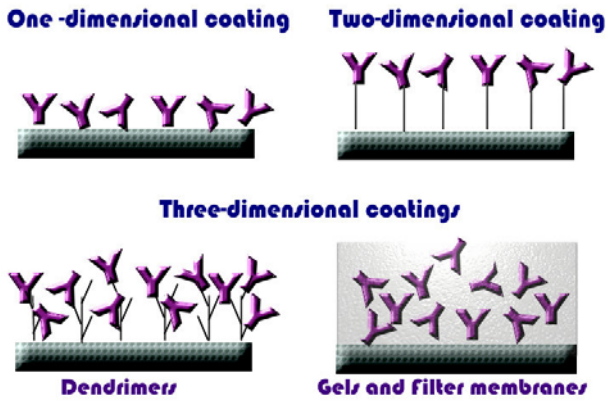

Figure 12 Representation of various forms of microarray slide coatings

3-dimensional coatings (3D)

The main advantages of 3D coatings are:

- Increased loading capacity
- Reduced protein denaturation because of homogeneous aqueous environment
- Low nonspecific binding

The oldest in history support media for protein microarrays are nylon and nitrocellulose membranes as they provide high binding capacity [21-23]. For this reason, such membranes are now attached to glass and used in the microarray format. The commercial example is the FAST slide from Schleicher & Schuell based on 15 µm thick nitrocellulose tightly attached to a glass slide (25 x 75 mm).

Nowadays 3D surfaces, such as hydrogels and dendrimers (branched polymers) are becoming more popular, even though they are rather difficult to prepare and commercial slides tend to be expensive. Until now only a limited number of 3D surfaces is available on the market: the most famous ones are the Hydrogel Slide based on polyacrylamide from Perkin Elmer developed by Mirzabekov [44] and Nexterion Slide H from Schott.

Hydrogel slides are usually quite thick reaching up to 30 µm, which may cause diffusion problems due to limited mass transport [45] Apart from improved protein stability due to a homogeneous quasi liquid microenvironment and high loading capacity 3D surfaces offer the advantage of better separation of protein spots as they are quite limited in movement because of soaking into the polymer.

Recently reported 3D supports are functional micro- and nanoparticles which combine the advantages of both planar surfaces and suspension arrays resulting in greater signal-to-noise ratios.

3.2.3 Orientation

Orientation of protein on the slide surface is seen as one of the key features in microarrays. When there is no forced protein orientation proteins bind randomly to the slide surface. This can cause loss of activity of spotted protein and reduced signals, as the active site of the protein is no longer accessible for the target. Oriented protein attachment provides better accessibility of native sites for the analyte, less variation in protein activity and increased protein stability. When working with antibodies that are homogeneous, it is possible to provide oriented attachment via thiol groups in the hinge region or between the light and heavy chain [46] by their carbohydrate residue or by specific epitopes and chemical tags. Most probably protein denaturation can be avoided, when blocking the antigen-binding site. In [94] ten times increased analyte binding capacity and higher stability of both full-size antibodies and Fab-fragments were reported when using random, non-oriented immobilization. The oriented coupling usually leads to a lower surface coverage [95] and this is most likely the reason why there are usually no positive effects on the assay performance [96].

Oriented immobilization can be provided by using:

- Proteins A, G and L
- Streptavidin
- Carbohydrates
- NTA
- Nucleic acids

Proteins A, G and L are used for antibody immobilization because of their antibody binding specificity [88]. Protein A (derived from *Staphylococcus aureus*) binds to the Fc region of the antibody of mammals. Protein A, which is nowadays produced via genetic engineering, has high surface immobilization capacity as well as high ability of immunoglobulin orientation [89]. Protein G that originates from *Streptococcus* shows a broader application range than protein A, as it binds to more classes of antibodies. Binding also occurs in the Fab fragments region. Another type of proteins used for oriented binding of antibodies is protein L which originates from *Peptostreptococcus magnus*. It binds Igs through interaction with the light chains. Since no part of the heavy chain is involved in the binding, Protein L binds a wider range of Ig classes than Protein A or G. Protein L binds to representatives of all classes of Ig, including IgG, IgM, IgA, IgE and IgD. Described proteins can bind at least two antibody molecules per protein [90, 91].

Furthermore, the high affinity of biotin for streptavidin can be applied for site-specific immobilization of proteins. Usually biotinylated molecules, such as biotinylated antibodies are bound to slide surfaces

modified with streptavidin [47]. Biotin modification of antibodies is usually done via primary amines, resulting in 3 to 6 biotins per antibody. Biotinylation can cause loss of protein activity, when the primary amines are located in the functional regions of the protein. In order to avoid this problem, site specific biotin attachment is done by genetic engineering.

Moreover, oriented protein immobilization can be reached by the use of carbohydrates. The carbohydrate chains that are placed in the heavy chain part of the antibody can be oxidised to reactive aldehydes by periodic acid or by enzymes and coupled covalently to the chip surface containing amino groups [93].

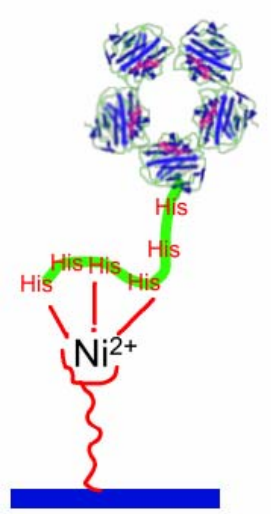

Figure 13 Principle of His-Tagged protein binding

The use of recombinant tags (most common is poly-histidine: His-Tagged) was also reported in literature [69]. The idea originates from immobilized metal ion affinity chromatography (IMAC) where metals like Ni, Zn and Co were attached to the columns and proteins with affinity to the metal ions were bound. Mostly for microarray application nitrilotriacetic acid (NTA) coupled to Ni is used for oriented immobilization of His-tagged proteins. One of the major advantages of NTA-Ni^{2+} immobilization is that it does not effect the structure and functionality of the protein and allows to keep the proteins activity up to 80% [95]. In order to immobilize Ni^{2+}on the slide surface chelating material (mostly used is NTA or iminodiacetic acid (IDA)) needs to be incorporated in a lipid layer or immobilized on gold-coated glass slides. The resulting surface layer is then ready for His-tagged protein immobilization (Figure 13).

Alternatively, DNA coupling can be used to attach proteins in an oriented way (nucleic acids tagged proteins) [94]. The idea behind this approach is that the protein modified with a nucleic acid (mRNA, cDNA, oligonucleotide) is attached to a slide previously spotted with nucleic acid complementary to the protein linker (see Figure 14). The complemetary nucleic acid strand is then modified with streptavidin in order to bind with the biotinylated protein. This immobilization process is applied for binding of delicate proteins with low non-specific binding and allows oriented immobilization without loss in protein activity, similar to His-tagged proteins.

Figure 14 DNA oriented protein immobilization

Moreover, proteins can be tagged on their amino or carboxyl termini to enable site-specific attachment.

3.2.4 Chip formats

The most common used microarray format is the glass slide, but nevertheless also nanowell approaches have been introduced. The reason for searching for new chip formats is the proteins sensitivity for environment conditions. In order to keep proteins in an active state during the printing process printing needs to be done in a moisturized environment. To meet this requirement the main focus in research & development of new surfaces are assay formats that combine the well format providing the moisturized environment with the format of easy to handle planar glass slides.

The majority of microarray studies was done on glass slides implemented from DNA microarray technology in the field of protein chips. Glass offers a number of practical advantages, such as mechanical stability and low autofluorescence. One of the main advantages of using glass slides as support for protein microarrays is their compatibility with the standard microarray and detection equipment. The only disadvantage of glass slides is the high evaporation rate of the spotted solution and the risk of cross contamination as a result of carry over [3].

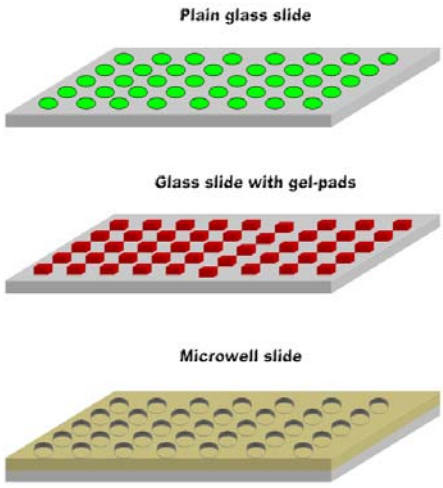

Figure 15 Types of protein microarray formats [31]

The idea of immobilizing proteins in tiny gel pockets that are attached on the glass slides was first realized by Mirzabekov et al. [50,51]. A variety of assays, immuno- as well as enzymatic assays were carried out using this slide format. Currently, gel-pads slides are commercially available as HydroGel® slides from Perkin Elmer, representing glass slides providing polyacrylamide pads of different size depending on the users needs.

Despite of planar glass slides and gel pads microwell as well as nanowell microarrays are employed. They are compatible with the standard microarray equipment. However, adjustments with respect to the printing and scanning devices are usually needed. This method can be an attractive alternative for solution based assays and multi-component reactions. The advantage of these chips is the highly reduced evaporation, no cross contamination and low cost of production. As the basis of microwell arrays silanes are usually used, e.g. polydimethylsiloxane (PDMS). Microwell arrays allow small volumes of different analytes to be densely packed onto a single chip, with the advantage of high segregation during processing. The disadvantage of this technique is the need for specialized equipment allowing the nano- or microwells loading with protein solutions.

3.3 Printing

One of the crucial points of chip fabrication is printing the array on the slide surface. The first in history high throughput spotting experiments were performed by Silzel in late 1990s, when printing antibodies on a polystyrene film [52] using a standard desktop printer. Protein arraying is nowadays possible with fully automatized roboters (spotters), which give reliable and reproducible printing results. In order to obtain strong signals within the spot, probe concentration as well as print buffer needs to be optimized. According to the theory of Ekins [18] smaller spots provide higher density of immobilized protein within the spot leading to greater signals. On the other hand the smaller spot size can negatively influence the signal intensity because of poor spot quality. Thus, in practice the concentration of spotted capture protein needs to be investigated for each newly set up experiment. The optimization of the spotting conditions covers the optimization of print buffer composition as well as adjustments in the arraying environment, e.g. humidity. The consideration from which buffer the proteins are going to be spotted depends on the coupling reaction and the properties of the chip surface. When using hydrophobic substrates the printed spots are by far smaller than on hydrophilic surfaces. In order to manipulate the spot size surfactants, such as Tween-20 or SDS (sodium dodecyl sulphate) are added, which not only lead to an increase in spot diameter due to improved surface wettability, but also enhanced spot homogeneity which has impact on data reliability. Furthermore, reagents, such as betaine or trehalose are added to stabilize the proteins in the printing solution, whereas additives, such as DMSO are employed to slow down evaporation of the probe solution during the printing process.

There are two spotting principles: contact and non-contact spotting.

Contact spotting is best for spotting a high number of probes onto many surfaces, whereas non-contact spotting allows spotting of only a low number of probes, but is especially suitable for soft surface materials.

The non-contact type of spotters are either solenoid or piezoelectric. The solenoid type is best for low probe numbers, but high numbers of spots. The tips for solenoid type spotters are built from high quality ceramics, providing different sizes for different spot diameters and volumes of spotted probe. The piezoelectric spotter uses electric current for the ´´firing´´ glass capillaries, which cause the probe droplet falling onto the slide surface. This type of spotting is typically slower than the contact spotting as the devices are limited to 4 - 8 pins.

Because of problems with non-contact spotting the use of contact spotters is expanding. Contact spotters allow use of multiple print heads (Fig. 16 b) and are able to print up to 48 spots within one touch of the slide surface. Two types of contact spotting pins are available: solid pin tips or split pins. Split pins can hold a larger probe volume and dispense it on the slide, whenever the pin touches the surface. The advantage of using split pins is avoiding redipping for a probe, which speeds up the printing process. Regardless the advantages of split pins in most microarray laboratories solid pins are used, mostly because of their lower price, higher robustness and lower risk of pin blocking.

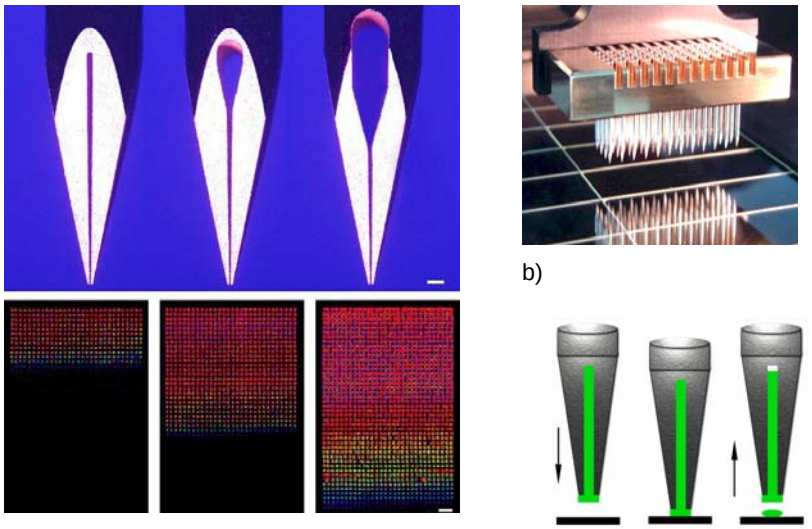

Figure 16 Printing devices a) pins of a different load of probe, b) print head with 48 pins and c) printing principle http://arrayit.com/

Moreover, solid pins are not as sensitive to buffer salinity and viscosity as glass capillaries of noncontact spotters. The major advantages of this type of spotters are the small probe volume (available pin loads are 0.25 µl, 0.6 µl and 1.25 µl), and the small spot diameters (75 to 300 µm) that allow spotting thousands of probes on the slide for multiple screening.

When using a contact spotter the maximal number of probes that can be spotted within one dip of the smallest capacity pin is 200, when taking in account small molecules like dyes, and about 100, when using protein probes. In order to eliminate carryover of different probes the pins are washed and dried between probes, which generally occupies most of the time in the spotting process.

4. Surface characterization

4.1 Profilometry

One of the non-intrusive high-resolution surface diagnosis tools is optical profilometry. Scanning white light interferometers capture optical intensity data at series of positions along the vertical axis, determining surface location by using the shape of a white light interferogram. The technique involves projecting computer-generated patterns of light and dark fringes onto the sample surface using a spatial light modulator. The fringe frequency and phase are varied over time in an optimized sequence of patterns that are generated at video frame rates. Light scattered back from the sample surface is imaged by digital video cameras that record distortions in the fringe patterns due to the 3-D surface profile. Profilometry is used for surface topography studies as well as for determination of layer thickness.

4.2 AFM

Atomic force microscopy (AFM) is one kind of scanning probe microscopies (SPM) that measures local properties, such as height, friction, or magnetism.
To obtain an image, the atomic force microscope scans over a small area of the sample, measuring the local property simultaneously.

The atomic force microscope (AFM) was invented in 1986 by Binnig, Quate and Gerber. The principle of AFM is based on the sharp probe moving over the surface of a sample in a raster scan. In the case of AFM, the probe is a tip on the end of a cantilever which bends in response to the force between the tip and the sample.

The principle of AFM is shown in the diagram (Fig. 17). When the cantilever bends, the light from the laser is reflected onto the split photo-diode. By measuring the difference in signal, changes in the bending of the cantilever can be measured and a topographic picture of the sample is generated. The movement of the tip or sample is performed by an extremely precise positioning device made from a piezo-electric tube scanner. The scanner is capable of moving in a sub-angström resolution in all directions.

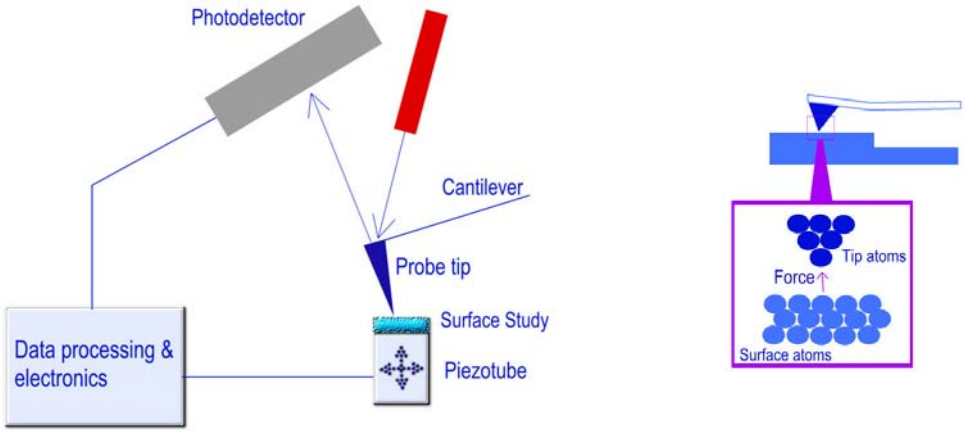

Figure 17 AFM principle and interaction in-between cantilever tip and surface atoms

When using AFM in contact mode, the tip gets in soft contact with the sample. In non-contact mode the cantilever is vibrated near the surface of the sample. The spacing between the tip and the sample is of the order of tens of nanometres. Non-contact AFM provides a means for measuring sample topography with little or no contact between the tip and the sample. This is the reason why non-contact AFM is widely used for studying soft or elastic samples like bio-molecules [99].

AFM measuring techniques can be divided in terms of dimensionality:

- 1D Force distance AFM is measuring forces above the surface
- 2D Imaging AFM gives the answers to surface topography or force distribution
- 3D combination of 1D and 2D AFM modes describes force distance topography

AFM provides the ability to view and understand events as they occur at the molecular level. This will increase our understanding of how systems work and lead to new discoveries in many fields including life science, materials science, electrochemistry, biophysics and nanotechnology.

4.3 Contact angle

Contact angle (CA) measurement is a very simple method for analyzing parameters concerning surface energy and tension by measuring the ability of a liquid to spread on a surface (see Fig. 18). When a drop is deposited on a planar solid surface, the angle between the outline tangent of the drop at the contact location and the solid surface is called contact angle.

The contact angle measurements give 3 informations:

- The affinity of a liquid to a solid surface: if water is used to measure the contact angle one can deduce the hydrophobic (great angle) or hydrophilic (small angle) character of the surface.
- If several reference liquids are used, the surface energy of the solid can be calculated, discriminating between polar and dispersive components.
- The measure of the hysteresis between advancing angle and recessing angle give information on non homogeneity of the surface (rugosity, contamination)

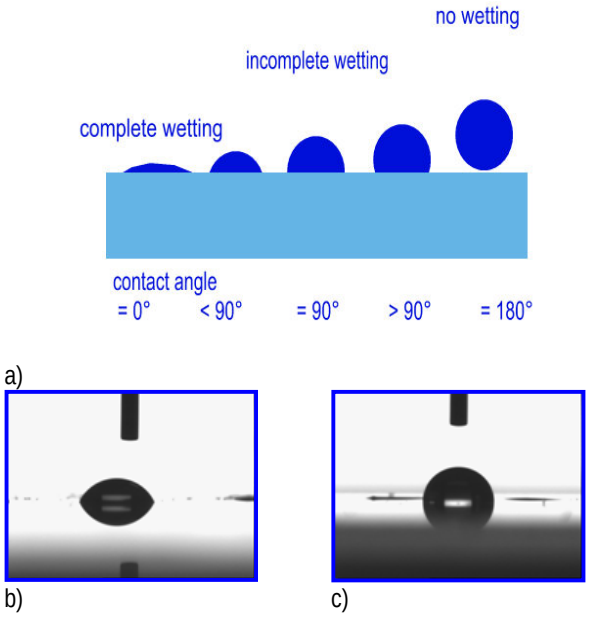

Figure 18 Contact angle dependence on the surface wettablity: a) the principle, and snap shots of real measurements for b) hydrophilic and c) hydrophobic surface

This technique is extremely sensitive allowing to even detect properties on monolayers, furthermore detect the presence of films, coatings, or contaminants with a surface energy different from that of the underlying substrate. Because of its simplicity, it's widely used for material surface analysis related to wetting, adhesion, and adsorption.

4.4 ζ-potential

ζ-potential is a physical property which is exhibited by any particle in suspension. ζ -potential is the electrical potential that exists at the "shear plane" of a particle, which is some small distance from its surface. The development of a net charge at the particle surface affects the distribution of ions in the surrounding interfacial region, resulting in an increased concentration of counter ions (ions of opposite charge to that of the particle) close to the surface (Fig. 19). Thus an electrical double layer is formed

around each particle. The liquid layer surrounding the particle can be divided in two regions: an inner region, called the Stern layer, where the ions are strongly bound and an outer, diffuse, region where they are less firmly attached.

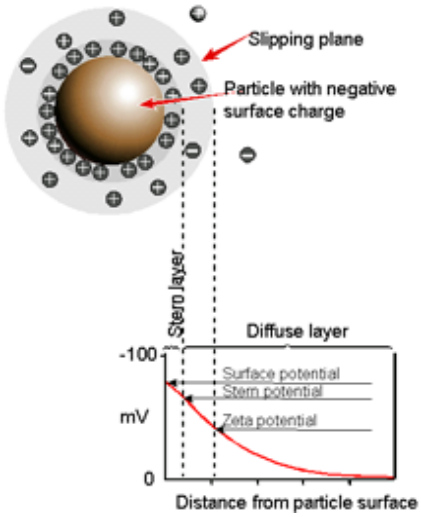

Figure 19 Principle of ζ-potential measurement

Within the diffuse layer there is a notional boundary inside which the ions and particles form a stable entity. When a particle moves (e.g. due to gravity), ions within the boundary move with it, but any ions beyond the boundary do not travel with the particle. This boundary is called the surface of hydrodynamic shear or slipping plane. The potential that exists at this boundary is known as the ζ-potential. The most important factor that affects ζ-potential is pH. A ζ-potential value on its own without a quoted pH is a virtually meaningless number.

5. On-chip immunoassays

When performing an on-chip immunoassay several parameters need to be taken into consideration. The most important ones are:

- spotted probe concentration
- concentration of the labelled target
- assay time
- crossreactivity
- composition of the spotting buffer

First step is the optimization of the spotted protein concentration. Depending on the chip surface and the type of the assay the optimal protein concentration can differ in folds of magnitude. As shown in Fig. 20, when the concentration of spotted protein reaches a certain level (in Fig. 20 the level of 5 mg/ml anti-albumin antibody), saturation occurs and no further signal enhancement is achieved. When using e.g. anti-IgG antibody good results are obtained with 0.5 mg/ml. The optimal concentration of spotted protein always needs to be determined empirically having in mind that the number of capture molecules needs to be low in competitive, but high in sandwich immunoassays.

Furthermore, the probe concentration has great impact on the spot morphology: too high protein concentrations result in overloaded spots that produce smears and comets and compromise the quality of data.

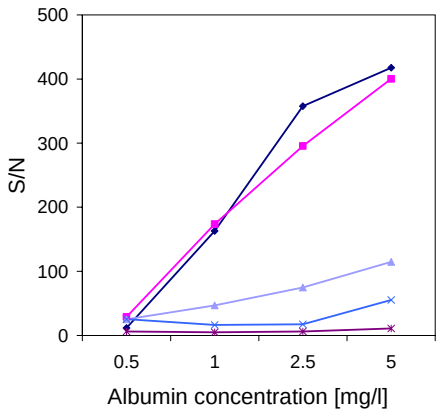

Figure 20 Calibration curves for albumin. S/N dependent on the capture antibody concentration (mg/ml): ◆10, ■5, ▲2.5, ✳1.25 and ⎯ 63 mg/ml

Secondly, the concentration of labelled target needs to be optimized because too much target may cause strong background, and moreover, especially in competitive assays increase the limit of detection.

Furthermore, assay time needs to be optimized: If working with competitive as well as with ELISA type of assay the incubation times have impact on the assay sensitivity in the way that too long incubation times cause increased background noise and too short time may not produce sufficiently high assay signals to detect very low analyte concentrations.

One of the main requirements for immunoassays is high specificity and thus low crossreactivity. However, especially polyclonal antibodies tend to crossreact. The easiest way to check the antibodies for cross reactivity is to perform an assay with antibodies of the same origin like IgGs, but obtained in different hosts, or using antibodies against other proteins. Fig. 21 shows the fluorescence image of the cross reactivity check for the reaction of anti-rabbit IgG with rabbit IgG, goat IgG, human IgG and anti-CRP. As shown in the figure bright signals were obtained for rabbit IgG, whereas almost no signal was obtained for the other tested proteins.

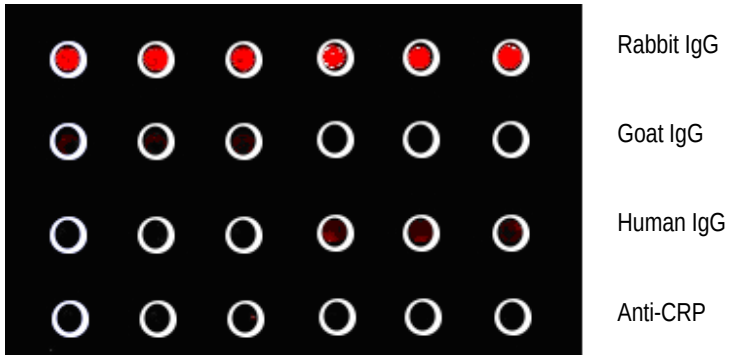

Figure 21 Crossreactivity check for the IgG assay.

Another important parameter to optimize is the print buffer (see Fig. 22), as it plays a crucial role in spot morphology, protein´s activity and assay sensitivity. Thereby, optimization takes in account the different buffers of varying compositions, concentrations and pHs as well as additives. The printing buffer should be as simple as possible unless there are special demands for complicity. The spotting buffer needs to be optimized not only with regard to the spotted protein, but also with regard to the surface. In general, hydrophobic surfaces give smaller spots whereas hydrophilic result in larger ones. Usually, small amounts of detergents (on the level of 0.01% and lower) are added to the buffer solution to improve spot morphology and reduce non-specific interactions. Too high amounts of detergents may result in spot spreading. Additives like betain or trehalose are often added as stabilizing reagent to prevent the spotted protein from drying and thus denaturation on the surface.

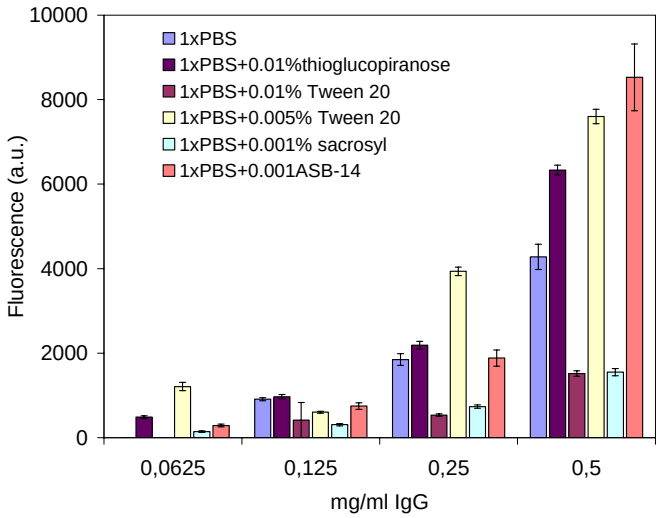

Figure 22 Influence of the spotting buffer on the signal height using an IgG immunoassay

6. Detection

Many detection techniques have been implemented in microarray technology: optical techniques, radiolabelling, and electrochemical as well as piezoelectric methods. While each methodology has advantages and disadvantages, the only technique that has been commercially successful is fluorescence. The detection techniques for microarrays can be divided into:

- Label based detection
- Label free detection

Label based techniques can be further divided into fluorescence, chemiluminescence and radioactivity detection. Fluorescence detection is usually the preferred one as it is simple, safe, sensitive and allows images with high resolution. Usually, Cy3 (λ_{ex}= 532 , λ_{em}= 570) and Cy5 fluorophores (λ_{ex}= 635 , λ_{em}=670) previously introduced in DNA microarrays are employed in protein chip technology. Chemiluminescence is used for the detection of proteins recognized by secondary antibodies labeled with HRP. The oxidation of a substrate like luminol causes light emission that can be monitored[101]. Radiolabelling is performed with radioisotopes incorporated in the protein detecting the signal by audioradiography. Though radiolabelling is one of the most sensitive detection methods, it´s no longer in use because of safety issues and waste problems.

As mentioned at the beginning read-out of microarrays is usually done with the use of fluorescence scanners. The scanners work like a fluorescence microscope specialized for acquiring microarray fluorescence images on the standard microscopic slide format. Most of the commercially available scanners are equipped with two lasers for detection of probes and targets labelled with Cy3 and Cy5. As the market of microarrays is growing very fast, the new generation of scanners has already slots for four lasers of different wavelengths that can be adapted with filters to many different fluorescence modes. Parameters that are crucial for a high-quality performance of a microarray scanner are resolution, sensitivity, dynamic range and detectivity. Scanner performance, comparison and further development has been reviewed in [102].

The scanners available on the market are now fulfilling the demands of the users: For qualitative analysis (detection of the presence and absence of spots) less complicated scanners are sufficient, whereas for quantitative analyte detection instruments with higher resolution are required in order to be able to differentiate in-between spot signals as well as detect very low signals. Nowadays confocal scanners are used because of their ability to scan large areas and detect single molecules of fluorescent dyes on a surface with 0.5 µm to 10 µm resolution. The use of a confocal scanner has the advantage of reducing out of focus light, since the fluorescence is detected only in a single plane ignoring

fluorescence that comes from neighboring spots (Fig. 23). However, the correct focus plane is critical and if the area of interest is not plane and even, loss of light occurs, as the sample gets out of focus during scanning. The nonconfocal scanner provides larger focus depth, and thus does not suffer from this problem. With nonconfocal scanners all light reflected from the surface is captured resulting in high signal-to-noise ratios and good signal quality.

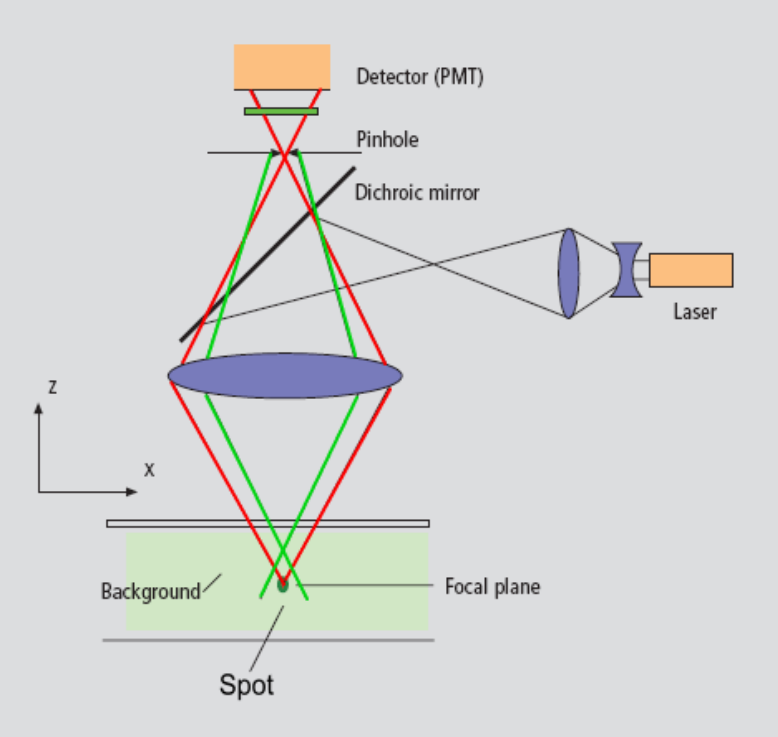

Figure 23 Principle of confocal scanning

Label free detection techniques have been developed as labeling with fluorophores can increase the assay cost and moreover the labeling can affect the biological activity of proteins. The most used methods are: surface plasmon resonance (SPR), ellipsometry and polarization methods. SPR is a technology that allows screening of protein interactions in real time. Special chips based on a thin gold layer covered with functionalized dextran for protein immobilization are necessary. Disadvantages of label free techniques are the need for sophisticated equipment and the low number of probes that can be investigated.

GLOSSARY:

antibody	A protein (immunoglobulin) molecule, produced by the immune system, that recognizes a particular substance (antigen) and binds to it.
antigen	A molecule that is recognized by antibody (immunoglobulin) molecules. Generally, multiple antibody molecules can recognize a given antigen.
biochip	
cDNA	Synthetic DNA transcribed from a specific RNA through the action of the enzyme reverse transcriptase.
DNA microarray	known as gene or genome chip, DNA chip, or gene array is a collection of microscopic DNA spots attached to a solid surface, such as glass, plastic or silicon chip forming an array for the purpose of expression profiling, monitoring expression levels for thousands of genes simultaneously, or for comparative genomic hybridization
ELISA	The Enzyme-Linked Immuno-Sorbent Assay, or ELISA, is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample.
fluorophore	component of a molecule which causes a molecule to be fluorescent. It is a functional group in a molecule which will absorb energy of a specific wavelength and re-emit energy at a different (but equally specific) wavelength.
genetic markers	Alleles used as experimental probes to keep track of an individual, a tissue, a cell, a nucleus, a chromosome, or a gene
G-Protein	A member of a family of proteins that contribute to signal transduction through protein–protein interactions that occur when the G-protein binds GTP but not when the G-protein binds GDP.
hapten	small molecule which can elicit an immune response only when attached to a large carrier such as a protein; the carrier may be one which also does not elicit an immune response by itself
hybridization	see *hybridize*
hybridize	To anneal nucleic acid strands from different sources
Ig (immunoglobulin)	See *antibody*
IgG	immunoglobulin G
MALDI	Matrix-assisted laser desorption/ionization (MALDI). In proteomics, MALDI is used for the identification of proteins isolated through gel electrophoresis: SDS-PAGE and two-dimensional gel electrophoresis. One method used is peptide mass fingerprinting by MALDI-MS, or with post ionization decay or collision-induced dissociation (further use see mass spectrometry).
marker	See *genetic markers*
messenger RNA	See mRNA.
mRNA.	An RNA molecule transcribed from the DNA of a gene and from which a protein is translated by the action of ribosomes.
northern blot	Transfer of electrophoretically separated RNA molecules from a gel onto an absorbent sheet, which is then immersed in a labeled probe that will bind to the RNA of interest.
Oligonucleotide	A short segment of synthetic DNA
PCR	see *polymerase chain reaction*
Polymerase Chain Reaction	is a biochemistry and molecular biology technique for enzymatically replicating DNA without using a living organism
Protein A	A 40-60 kD surface protein originally found in the cell wall of the *Staphylococcus aureus* that has its ability of binding to immunoglobulins. of mammalian species,

	mostly IgG's. It binds with the Fc region of immunoglobulin through interaction with the heavy chain.
Protein chromatography	Technique used to purify and further identify the proteins
Protein electrophoresis	Method of analysing a mixture of proteins by means of gel electrophoresis, mainly in blood serum
Protein G	Streptococcal is a cell surface-associated protein from streptococcus that binds to IgG with high affinity. It has three highly homologous IgG-binding domains.
Protein L	IgG binding protein from *Peptostreptococcus magnus that* binds antibodies through the light chains. Protein L binds to representatives of all classes of Ig, including IgG, IgM, IgA, IgE and IgD. ScFv and Fab fragments also bind to Protein L.

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Chapter 1

Effect of surface parameters on the performance of protein-arrayed hydrogel chips: a comprehensive study

***Submitted to Langmuir**:* Katarzyna Derwinska, Levi Gheber, Ursula Sauer, Leopold Schorn, Claudia Preininger. Effect of surface parameters on the performance of protein-arrayed hydrogel chips: a comprehensive study

Abstract

In this paper, the assay performance of three-dimensional polyurethane (PU) hydrogel surfaces, used either plain or modified with crosslinkers and additives in a direct immunoassay for IgG, is correlated with chip surface parameters, such as water content and expansion, mechanical stability, hydrophilicity, thickness and surface topography. The commercial chip surfaces ARChip Epoxy, Nexterion slide H and HydroGel are used as a reference. A strong correlation between assay sensitivity and physical surface parameters was only found for various hydrogels of the same chemical composition, in which cases assay sensitivity increases with decreasing hydrogel concentration as well as decreasing roughness, water content and expansion. However, as is the case with all hydrogels tested, more hydrophobic layers with low water content are more highly reproducible from one measurement to another.

1. Introduction

The rapid development as well as the broad range of applications of protein chip technology [1-9], including protein expression profiling, serum-based diagnostics, antigenicity and protein functionality, discovery of biomarkers, drug target binding, and epitope mapping, make it imperative to control chip quality, optimize chip surfaces with regard to selectivity, sensitivity and capacity, and to further develop and optimize new surfaces.

However, developing protein chip surfaces is a challenging task, particularly if one takes into account the enormous chemical and structural complexity and heterogeneity of proteins, factors that dictate and complicate their interactions with solid surfaces. Since proteins often loose their biological activity due to dehydration, denaturation or oxidation when bound to a solid substrate, the properties of immobilized proteins are unpredictable. This makes it extremely difficult to define general protein immobilization strategies. In addition, proteins have a strong tendency to adsorb non-specifically to solid substrates, which causes unwanted background noise. Taken together, all of these factors may compromise the performance of protein chips and limit specificity and sensitivity of diagnostic devices, particularly of those that handle real-world biological fluids.

The surfaces available for protein microarrays can be classified according to binding origin and structure. Surfaces such as aminosilane, poly-L-lysine, polystyrene and nitrocellulose bind proteins via electrostatic adsorption and hydrophobic adhesion, whereas epoxy or aldehyde slides bind proteins covalently via their lysin, arginine, serine or cystein residues. Affinity binding is achieved through biotin-streptavidin attachment [10], Ni^{2+} or Cu^{2+} chelates [11, 12], calixarene [13] or salicylhydroxamic acid (SHA) complexation [14]. In terms of dimensionality, a distinction can be made between two-dimensional (2D) planar surfaces, such as epoxy [15] and aldehyde glass slides [16], and three-dimensional platforms (3D) widely represented by hydrogels such as agarose [17] and polyacrylamide [18-21], modified dextrans [22], polyethylene glycol (PEG) platforms [23] and dendrimeric structures [24]. 2D slides usually use the covalent or electrostatic approach of binding directly to the surface, while 3D slides utilize physical adsorption within the gel structure. 3D gel surfaces are considered to be the most suitable for protein microarrays because of their high binding capacities and because they create a homogeneous aqueous environment that prevents protein denaturation. Compared to planar surfaces, hydrogels display less non-specific adsorption.

P. Angenendt et al. [25] compared 11 gel-coated and non-gel-coated glass or plastic surfaces. It was revealed that hydrogel slides, while producing up to four times as much inter-field variation

in antibody arrays, outperform non-gel-coated slides with regard to detection limit. The authors' results indicate that each antibody to be used in an antibody array needs to be tested individually and the chip surface selected accordingly. E.W. Olle et al. [26] compared acrylamide, nitrocellulose, aldehyde- and epoxy-silane slides using anti-goat IgG and found that the tested epoxy surface ES resulted in the greatest degree of binding along with minimal background (the optimum substrate was defined as the one binding the greatest amount of antibody while at the same time resulting in a low background). C. Steinhauer et al. [27] evaluated porous silicon-based substrates developed in-house along with five commercially available glass substrates with respect to biocompatibility and probe binding capacity as judged by spot morphology, signal intensities, signal-to-noise ratios (S/N), dynamic range, sensitivity and reproducibility. The silicon substrates and the HydroGel slides were superior in dynamic range, while planar SpotOn slides, 3D silicon substrates and HydroGel slides displayed the highest signal-to-noise ratios.

In summary, the studies on various substrates reported in [1, 25, 27] demonstrate that no optimum substrate exists that can be generally applied in protein microarrays. The choice of substrate, influenced by the kind of assay as well as probe characteristics (size, charge, structure), has to be determined for each protein individually. Furthermore, the criteria to be fulfilled for optimum substrates are not generally applicable, but set by the users for a specific application.

In the following, we attempt to define hydrogel requirements for optimum protein immobilization using three-dimensional hydrogel surfaces based on polyurethane, used either plain or modified with crosslinkers and additives in a direct immunoassay for IgG. Hydrogel surfaces were evaluated with respect to immobilization capacity, signal-to-noise ratio and data reliability and compared with ARChip Epoxy, HydroGel and Nexterion Slide H. Since hydrogel surfaces mimic the properties of bulk solutions, the effect of hydrogel water content and swellability on immobilization capacity and S/N value was also investigated. In addition the effect of roughness and layer thickness on assay performance was examined.

2. Materials and methods

Materials

Hydrophilic polyether polyurethane hydrogels PU1, PU2, PU3 and PU4 were purchased from Cardiotech International (water content and expansion: PU3>PU2>PU4>PU1). Thermoplastic aromatic polyurethane PU6 was obtained from Noveon. Cationic methacrylate Meth2 was provided courtesy of Degussa Röhm Pharma Polymers. Monochlortriazinyl-ß-cyclodextrin sodium salt (MCT) was acquired from Wacker. The crosslinkers hexamethylenediamine (HDA), poly(allylamine hydrochloride) (PAH), polyethylenimine (PEI), poly(vinylsulfate), and dextransulfate were analytical grade (Aldrich). Chitosan (food grade) from Dalwoo was used. Cystenaminhydrochloride (CAHCl), mercaptoethanesulfonic acid (MESA) and polystyrenesulfonic (PSSA) acid were obtained from Fluka. Dodecyl sulfate sodium salt (SDS) was purchased from Merck and phosphate buffered saline (PBS) from Gibco. Dy633-labelled anti-rabbit IgG (DyAB) was purchased from Dyomics. Rabbit IgG (technical grade) was from Sigma. All other reagents were analytical grade.

Chip fabrication

Silane Prep™ slides (25 x 75mm) (Sigma, S4651) were dip-coated, using the dip coater from KSV Instr. Ltd, from plain hydrogel solutions of 2% PU1 to 2% PU4 in EtOH/H2O (95/5) and 2% PU6 in cyclopentanon, and respective hydrogel/additive cocktails (polymer concentrations were optimized) (surfaces 20, 21, and 8 were prepared in EtOH/H2O (71/29)). Composition, thickness and roughness of the best-of surfaces are indicated in Table 1. HydroGel® slides from Perkin Elmer, and Nexterion® Slide H from Schott were employed as a reference.

Surface modification

Surfaces of 2% PU2, 2% PU3 and 2% PU4 were modified according to the following procedures:

a) MCT: Slides were immersed in a solution of 10% MCT (adjusted to pH 10 with sodium carbonate) for 1 h. Then slides were dried at room temperature [28].

b) PAH: Slides were incubated in a solution of 2 mg/ml PAH (pH 8) for 6 h [29].

c) PEI: Like b).

d) Chitosan: Slides were incubated in an aqueous solution of 1% chitosan for 24 h.

e) HIO_4: The slides were immersed in 1% aqueous HIO_4 for 1 h and then washed twice with MiliQ water.

All treatments were performed on a belly dancer (speed 5).After treatment the slides were washed twice in MiliQ water and dried with compressed air.

Microarray printing

Three replicates of 0.005-1 mg/ml rabbit IgG in 1x PBS (pH 7.2) were arrayed onto the chip surfaces using the OmniGrid contact spotter from GeneMachines (pin SMP3). The spot-to-spot distance was 400 µm, spot volume was 0.6 nl.

Postarraying & blocking

After arraying the slides were incubated in a humid chamber at 4 °C overnight to complete probe immobilization. Surface blocking was performed in 1x PBS (pH 7.2)/0.1% Tween-20 to rinse off unbound protein and deactivate reactive surface groups. Finally, the slides were washed twice in 1x PBS (pH 7.2), blow-dried with compressed air or spin-dried using a centrifuge (900 rpm for 3 min).

Direct immunoassay

Protein slides were processed with 4 ng/µl Dy633-labelled anti-Rabbit IgG (λ_{ex}=635 nm, λ_{em}=670 nm) in 1x PBS (pH 7.2)/0.1% Tween-20 at 4 °C for three hours, then washed twice in 1x PBS (pH 7.2) and blow-dried with compressed air or spin-dried using a centrifuge (900 rpm for 3 min).

Fluorescence detection

Slides were stored in the dark and scanned on the same day the immunoassay was performed. Fluorescence measurements were taken using the Genepix^{TM} 4000B non-confocal scanner from Axon Instruments. For data analysis the Genepix software was used.

Determination of immobilization capacity

Immobilization capacity in fmoles/mm² was calculated by taking the median fluorescence minus the local background of 27 replicate spots of dye-labelled anti-IgG before and after blocking (30 min) multiplied by spotted protein concentration and divided by molecular mass of labelled protein and square radius of the spot. The calculation was done according to the formula. The factor $1.9 \cdot 10^6$ is calculated from the volume of the protein solution per spot (0.6 nl/spot) and the Π.

$$I = 1.9 \cdot 10^6 \frac{F_A}{F_B M_{LP} R^2} C_{LP}$$

Where:

I - Immobilization capacity

F_B – Fluorescence before blocking

F_A – Fluorescence after blocking

M_{LP} – Molecular mass of labelled protein

R – Spot radius

CLP- concentration of spotted protein

Surface characterization

Layer thickness was measured over 2.5 x 2.5 mm^2 surface areas using the Wyko NT1100 optical profiling system (Veeco) and Vision32 Veeco software. The values in Table 1 are mean values of two measurements.

AFM studies were performed with a NSOM/AFM 100 from Nanonics Ltd., Israel. Samples were scanned in contact mode with Mikromasch probes having spring coefficients of 0.03 N/m. The AFM system is mounted on the stage of an optical microscope, allowing examination of the sample at high magnification and choosing the area of interest for AFM imaging.

Images were analyzed using SPIP - a commercial software package from Image Metrology A/S calculating the average roughness, S_a, defined as

$$S_a = \frac{1}{MN} \sum_{k=0}^{M-1} \sum_{l=0}^{N-1} \left| z(x_k, y_l) - \mu \right|$$

Where M, N is the number of pixels in the x and y directions, respectively, z is the height of the point at (x, y) and μ is the mean height of the area:

$$\mu = \frac{1}{MN} \sum_{k=0}^{M-1} \sum_{l=0}^{N-1} z(x_k, y_l)$$

S_a is the average of the (absolute value of) variations from the average height.

Surfaces were also analyzed for their texture. The Fourier spectrum of the images was calculated along equidistantly separated, concentric, semicircles with a centre on the horizontal lower edge of the image. The radius of the semicircle with the highest Fourier amplitudes was used to calculate the dominating radial wavelength (Srw). Srw is a measure of the size of the typical corrugation of the surface.

3. Results and discussion

Assay performance of plain hydrogels

PU1, PU2, PU3, PU4 and PU6 were tested as an immobilization matrix for proteins in a direct immunoassay using 0.005 to 1 mg/ml IgG. Evaluation parameters were immobilization capacity, signal-to-noise ratio (S/N) and dynamic range of the calibration curves. The highest immobilization capacity was reached for 2% PU2 and 2% PU4, at more than 400 fmoles/mm^2, whereas for hydrogels PU3 and PU6 the immobilization capacity was about 200 fmoles/mm². PU1 resulted in values below 100 fmoles/mm². The loss of labelled antibody after 3 h incubation, which is the typical assay time, is less than 20% for PU2 and PU4; yet in the case of PU6 the loss was about 60%, and an even greater loss was observed for PU3, reaching as much as 80%.

Fig. 1 shows the calibration curves for 0.005 to 1 mg/ml IgG on the plain PU surfaces tested in comparison with ARChip Epoxy, Nexterion slide H and HydroGel. To facilitate an overview in Fig. 1, no error bars are included, and the mean coefficient of variation (CV) is reported separately in Fig. 5. Among the tested PUs the highest sensitivity, defined as the greatest signal change per concentration, was achieved for PU4. The signal-to-noise ratio (S/N) was comparable to that of ARChip Epoxy and Slide H (except for conc <0.5 mg/ml IgG). The weakest signals and poorest reproducibility (89% CV) by far were obtained for PU1, thus excluding it from further measurements. PU3 in contrast, resulting in signals on the same level as PU1, showed excellent spot morphology, a high number of spots (98%) available for data analysis and good data reproducibility, and thus was further used in modification and surface studies.

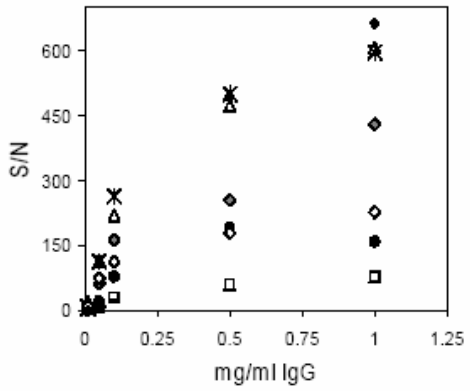

Figure 1. Calibration curves for 0.005-1 mg/ml IgG arrayed onto surfaces of plain hydrogels – PU1, ◆PU2, □ PU3, △PU4, ●PU6, ◇HydroGel, ◆Slide H, and ✳ ARChip Epoxy.

The dynamic range was 0.05 to 1 mg/ml IgG for PU2, PU4, ARCHip Epoxy and Slide H whereas for PU1, PU3, PU6 and HydroGel the range was narrower with 0.1 to 0.5 mg/ml IgG.

Assay performance of hydrogels with additives and crosslinkers

In order to increase the strength of antibody binding and improve assay performance, cationic and anionic reagents as well as functional crosslinkers were employed for surface modification. Plain hydrogels were modified as reported in the materials and methods section, but only those modifications leading to improved S/N values, increased immobilization capacity or enhanced reproducibility in measurement were studied in detail and summarized in Table 1.

No.	PU	additive	modification	Sa (nm)	Spot diameter (µm)	Thickness dry state (nm)	Thickness after 3 h incubation (nm)	Swellability %	factor of signal enhancement
1	PU2	-	-	0.63	77	256.5	211.0	-17.7	-
2	PU2	1% PSSA	-	0.47	110	269.0	143.0	-46.8	0.9
3	PU2	1% CAHCl	-	1.00	127	282.5	226.0	-20.0	1.0
20	PU2	1% dextransulfate	-	6.00	117	280.5	159.5	-43.1	0.4
21	PU2	1% chitosan	-	14.20	92	662.0	638.5	-3.5	0.2
25	PU2	-	HJO4	23.30	85	244.0	227.0	-7.0	1.2
30	PU2	-	MESA	0.78	77	201.0	207.5	+3.2	0.9
32	PU2	-	MCT	1.30	117	243.0	153.0	-37.0	1.1
6	PU3	-	-	2.32	109	121.5	56.5	-53.5	-
7	PU3	4% Meth2	-	87.80	97	322.5	465.0	+44.2	3.2
8	PU3	2% chitosan	-	2.00	113	609.0	273.5	-55.1	6.4
26	PU3	-	HJO4	2.28	67	111.5	93.5	-16.1	8.1
27	PU3	-	HJO4+ PEI	0.86	83	140.0	124.5	-11.1	2.4
28	PU3	-	HJO4+PAH	1.04	117	53.5	61.0	+14.0	3.8
34	PU3	-	MCT	1.38	85	75.0	38.0	-49.3	1.7
5	PU4	-	-	1.18	66	314.5	241.0	-23.4	-
16	PU6	-	-	2.37	97	253.5	257.5	+1.6	-
35	HydroGel			0.88	85	2268.0	2025.3	-10.7	-
36	Nexterion Slide H			0.55	71	17.4	10.9	-37.8	-
37	ARChip Epoxy			5.86	106	22.0	29.0	+31.8	-

Table 1. Composition and characteristics of tested PU surfaces, HydroGel, Slide H, and ARChip Epoxy.

The integration of the surface modification in chip fabrication by means of tailored additives (surfaces 2, 3, 20, 21, 7 and 8) offers the advantage of not requiring any additional process step for increasing surface activity and selectivity. PSSA, dextran sulfate and vinyl sulfate were employed to create an anionic surface, whereas chitosan, CAHCl and Meth2 were used to produce amino-functional cationic surfaces. MESA and MCT were used as crosslinkers in order to stabilize the hydrogel layer and enhance antibody binding. In addition, the hydrogels were activated using HJO_4 to produce aldehyde groups for covalent binding of the antibody or further

modification with crosslinkers, such as PEI and PAH. The factor of signal enhancement referred to as S/N of the modified hydrogel surface divided by S/N of the respective non-modified hydrogel surface (set to 1) was taken as measure for the efficiency of surface modification. From the factor of signal enhancement represented by the triangles in Fig. 2 it can be seen that neither kind of modification of PU2 led to signal enhancement and increased assay sensitivity. This applies to the PU4 surfaces as well. In contrast, all tested PU3 modifications resulted in improved assay performance: addition of MCT, Meth2 and chitosan produced 1.7, 3.2 and 6.4 times greater signal-to-noise ratios, whereas oxidation of the surface with HJO_4 - hereby increasing the amount of bound antibody by 1.8 times - resulted in 8.1 times enhanced signals dropping to 2.4 to 3.8 times on further modification with PEI, and PAH. Obviously, the electrostatic interaction and adhesion forces at pH 8 are stronger between the anionic PU3 surface and the polyamine with the hydrophobic backbone PAH than between PU3 surface and hydrophilic polyamine PEI. This is in good agreement with the conclusions of Jiang et al. [29], who reported that PAH films preferentially deposit on polyether surfaces.

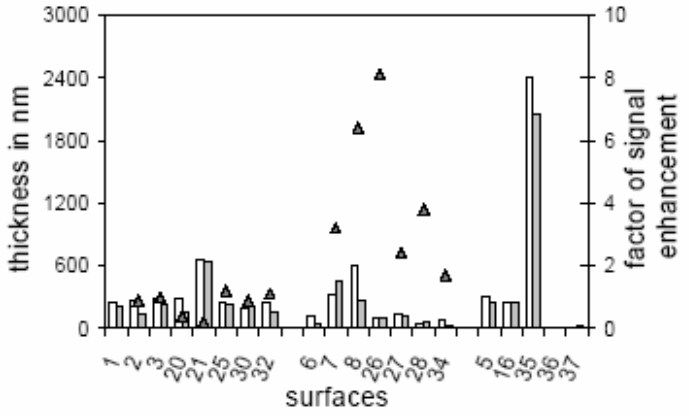

Figure 2. Correlation between layer thickness before (□) and after 3 h incubation (■) and factor of signal enhancement (▲) upon surface modification.

A reason for the more effective modification of PU3 might be the higher molecular weight, and thus increased number of surface hydroxy groups and the more than two times greater swellability of PU3 which allows an improved reagent penetration and accessibility.

In addition, oxidation of PU2 and PU3 produced more stable hydrogel layers, reducing the loss in material during incubation. Crosslinking of PU2 with MCT led to increased loss of hydrogel as compared with plain hydrogel, whereas crosslinking PU3 with MCT had no impact on the mechanical stability of the layer. In both cases there was almost no improvement in assay

performance (enhancement factor 1.1 and 1.7). Modifying PU2 by the addition of PSSA and dextransulfate resulted in more than twice as much diffusion of the hydrogel out in solution yet resulting in fluorescence signals of a strength similar to plain PU2. PU2 modified by the addition of amino-functional CAHCl showed the same mechanical stability and signal strength as plain PU2. Obviously, the amino groups of the additive were available neither for hydrogel crosslinking nor for antibody binding. This might be due to the high volume of EtOH in the cocktail (EtOH/H2O (95/5)), which slows down dissolving of CAHCL and the coupling reaction. In contrast, addition of chitosan (EtOH/H2O (71/29)) led to the formation of a layer of improved mechanical stability which was three times thicker than with plain PU2. However, fluorescence signals were four times weaker than for plain hydrogel, most probably because the chitosan amino groups were used up in crosslinking the hydroxyl-groups of PU2.

Crosslinking PU2 with mercaptoethanesulfonic acid (surface no. 30) produced an anionic hydrogel surface of about the same thickness as with pure PU2 but with improved mechanical stability (slight swelling) and with a 30 % increase in fluorescence signals, but no improvement in S/N as background also increased.

Modification of PU3 with cationic Meth2 and chitosan produced layers that were 2.7 and 5 times thicker respectively. However, the addition of chitosan did not improve mechanical stability – the loss in material during incubation is about 55 % for both plain and modified hydrogel, whereas the addition of Meth2 clearly improved adhesion to glass and increased the stability in the aqueous solution, resulting in extremely high swellability (+44 %) and roughness (Sa 87.8 nm), five times as much immobilization capacity and about a three times higher S/N.

Mechanical stability of the PU surfaces

The layer thickness of the hydrogel surfaces was measured before and after incubation in the reaction buffer using an optical profilometer. The incubation time in 1x PBS (pH 7.2)/0.1% Tween-20 was set to 3 h, as this was the typical incubation time for the direct on-chip immunoassay. The change in layer thickness upon incubation resulted either in shrinkage or loss of surface material (indicated by a negative value) or surface swelling (positive value). Loss and shrinkage of surface material were referred to as low mechanical stability of the surface layer. As is obvious from Fig. 2 most surfaces show a decrease in thickness over incubation time. Nine out of 20 chip surfaces diffused out in solution by up to 20% (no. 1, 3, 21, 25, 26, 27 and HydroGel) and six by 20 to 50% (no. 2, 5, 20, 32, 34, slide H). Surfaces 16, 21, 25 and 30 can be considered stable during incubation, since material loss for no. 21 and 25 (-3.5%, -7%) and swelling for no.

16 and 30 (+1.6% and +3.2%) was extremely low. In fact, surface no. 16 was prepared from a thermophilic polyurethane, which is supposed to show no expansion. Clear swelling, indicated by a strong increase in layer thickness after incubation, was measured for surfaces 7, 28 and ARChip Epoxy (+44%, +14% and +31%).

The swellability of PU2 was -17.7%, about the same swellability level as PU2 surfaces modified by the addition of CAHCl. Except for PU2 surfaces with the anionic additives PSSA and dextransulfate and PU2 surfaces crosslinked with MCT, which resulted in a material loss of about 40%, mechanically stable layers were obtained either through oxidation of the surface with HJO4, formation of an anionic surface by the use of MESA or by the addition of chitosan. Clearly, the latter modifiers all had crosslinking and binding ability, whereas PSSA and dextransulfate were only loosely incorporated in PU2 rather than bound. Though the use of additives led to slightly thicker surfaces after fabrication, the final thickness was about the same or lower than for plain PU2. This implicates that the additives were not well dissolved and incorporated in the hydrogel and therefore were subsequently washed out during incubation, destroying the layer. The mean layer thickness of the PU2 surfaces before and after incubation was 250 to 150 nm, except for PU2/chitosan surfaces; at ~650 nm these were about three times thicker than the plain surface.

In the case of PU3, similar surface thicknesses were produced through the addition of chitosan, which however was washed out of the layer during incubation. Improvement of the mechanical stability of PU3 layers was achieved either through oxidation with HJO4, with and without subsequent crosslinking with PEI or PAH, or through the addition of Meth2. The latter two modifications led to swelling in the magnitude of +14 to +44 %.

The tested commercial hydrogel surfaces, Nexterion Slide H and HydroGel, showed a decrease in thickness by 14.5% and 36.4 %, whereas ARChip Epoxy showed an increase by ~31%.

Addition of chitosan always produced thicker layers by far than the respective plain hydrogels. The thinnest plain hydrogel surface was Slide H (17.4 nm before/10.9 nm after incubation). The order of thickness for plain polyether polyurethanes was as follows: PU4 > PU2 > PU3. The thickest surface layer by far was HydroGel with 2065 nm.

Wettability

The wettability defined as spreading over a horizontal surface was estimated using the spot diameter as calculated by the mean value of eighteen replicate spots (2 slides, 3 arrays) in 1x PBS (pH 7.2) after spotting. As may be clearly seen from Table 1, the most hydrophilic and best wettable PU surface was PU3, showing a spot diameter of 109 µm, whereas PU2 and PU4 were more hydrophobic, displaying spot diameters of 77 and 66 µm respectively. The order of hydrophilicity for the plain surfaces tested was as follows: PU3 > ARChip Epoxy > PU6 > HydroGel > PU2 > Slide H > PU4. The addition of cationic and anionic reagents to PU2 created more hydrophilic surfaces, as indicated by a spot increase of 15 to 40 µm. Oxidation with HJO4 resulted in an increase in diameter of 8 µm, the use of crosslinker MESA did not affect surface hydrophilicity, whereas the crosslinker MCT produced an extremely hydrophilic surface, with spots of 117 µm (increase of 40 µm). Surfaces consisting of PU3 additives and crosslinkers led to more hydrophobic surfaces. In contrast to PU2, oxidation of PU3 with HJO4 led to a more hydrophobic surface by far, as expressed by a decrease in diameter of 42 µm.

Surface topography and homogeneity

The surface topography of the PU surfaces and the commercial surfaces HydroGel, Slide H and ARChip Epoxy as listed in Table 1 was investigated using AFM. Comparing the plain PU surfaces 1, 5 and 6 which have a similar chemical composition (however, no. 6 has a larger molecular weight) the roughness increases from 1 to 5 to 6 (0.63 → 1.18 → 2.32 nm), showing a texture with a decreasing feature size (dominating radial wavelength – Srw) from 5.6 to 2.7 to 0.29 µm. PU6 which is of different chemical composition shows a roughness (Sa) of 2.37 nm which is comparable with the roughness of PU3. When comparing these roughness data with the assay performance (see Fig. 1) it can be cautiously concluded that greater signals are produced on smoother surfaces of similar chemical composition and binding mechanism. However, extending this comparison to surfaces of different chemical composition and binding chemistry, such as modified PU surfaces, HydroGel (0.874), Slide H (0.55) and ARChip Epoxy (5.86) no correlation can be found; especially when pointing out ARChip Epoxy which shows the highest roughness by far, but nevertheless an excellent chip performance. The increased roughness of ARChip Epoxy was due to the high roughness of the plain glass slide which in contrast to the Silane Prep slide (1.7 nm) used as substrate for the PU surfaces was 5.81 nm.

Modification of PU2 led to surfaces of increased roughness, the effect however is ambigous: the modified PU2 surfaces 20 and 21 with respective Sa values of 6 nm and 14.2 nm showed a signal

decrease by 60 and 80% respectively, whereas PU2 surface 25 (Sa 23.3 nm) resulted in a slight signal enhancement. A reason for this might be that due to different modifications completely new surface topographies were created as obvious from Fig. 3.

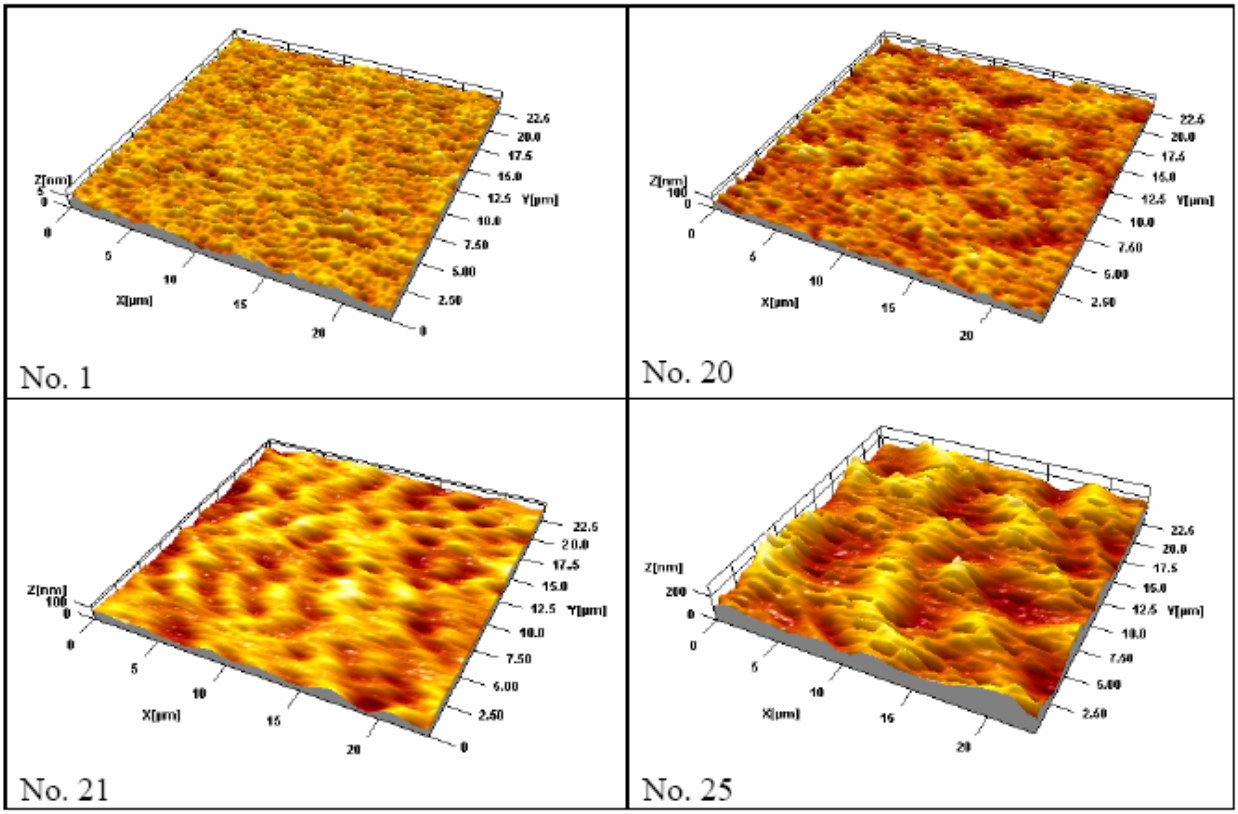

Figure 3. AFM images of PU2 surfaces no. 1, 20, 21, and 25.

By contrast, when comparing surface roughness of PU3 surfaces with the signal enhancement factor a good correlation (except for surface 7) was obtained as demonstrated in Fig. 4. This might be due to the fact that the surface modifications as well as the resulting surfaces are very similar, especially when looking at surfaces 26, 27 and 28. This furthermore supports the idea that a roughness effect becomes visible, only when isolated from other parameters, e.g. if very few surface parameters are changed. In addition, the correlation presented in Fig. 5 agrees well with D. Kuhlmeier et al. [30] who hypothesized that the RMS data reflect the amount of protein bound to the chip surface and therefore are a measure for the immobilization efficiency which in turn is related to the assay sensitivity.

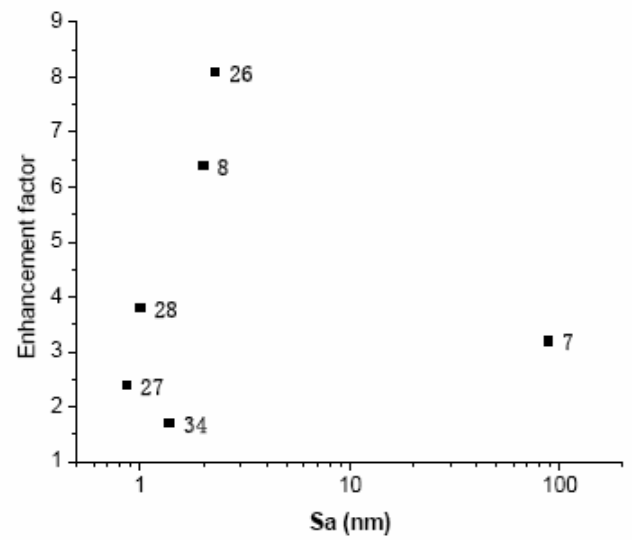

Figure 4. Correlation of surface roughness for unmodified and modified PU3 surfaces 7, 8, 26, 27, 28 and 34 with the signal enhancement factor.

Reproducibility within measurements

The direct on-chip immunoassay was repeated three times using freshly prepared, plain and modified PU slides. Signal-to-noise ratios (S/N) were calculated from the mean fluorescence of 27 spots; three slides per slide type with three subarrays per slide and three replicate spots per subarray. No normalization or special filtering was performed. As a measure for data reproducibility the coefficient of variation (CV) in % was determined for each IgG concentration as mean value out of three CVs (corresponding to the three experiments). In Fig. 5 the CVs for the tested plain chip surfaces are compared. Clearly, the greatest CV at all IgG concentrations was obtained for PU2 and PU3, the PU surfaces with the highest water content and expansion. PU2 as well as the HydroGel slide showed increasing CV with increasing probe concentration. This might be due to increasing spot roughness as a result of increased surface coverage (homogeneous, fully covered spots at any concentration, but thicker spots and increasing amount of IgG within the spot) as reported for microcontactprinted IgG in [31]. On the contrary data reproducibility was improved on ARChip Epoxy and Nexterion Slide H with increasing IgG concentrations: the CV for 0.01 to 1 mg/ml IgG diminished from 46% to 18%. The poor reproducibility at low IgG concentrations is most probably due to incomplete coverage of the spot area with IgG. However, with increasing IgG concentration the spot area is filled up thereby decreasing spot roughness and improving assay reproducibility.

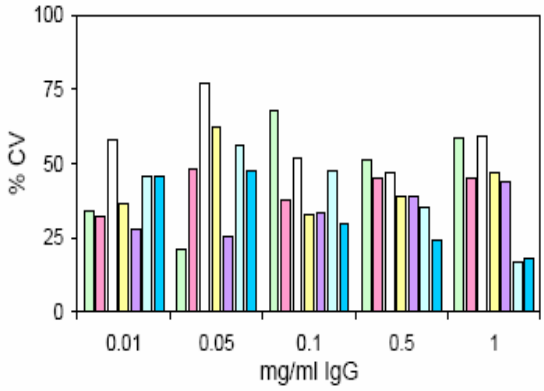

Figure 5. Reproducibility within three experiments expressed as %CV for 0.005 to 1 mg/ml IgG spotted on surfaces made of ■ PU2, □ PU3, ■ PU4, ■ PU6, ■ HydroGel, ■ slide H, and ■ ARChip Epoxy.

Data reproducibility of PU2 surfaces modified with PSSA (no. 2) and CAHCL (no. 3) were improved by 25%, whereas two times higher CVs than for plain PU2 surfaces were obtained for surfaces 21 and 32, especially at low protein concentrations. Data reproducibility of modified PU3 surfaces was similar to that of plain ones, except for surfaces 7, 26 and 27 (CV ~45%). Clearly, one has to keep in mind that variation was calculated from raw data, in order to show actual differences in slide performance, while data filtering and normalization techniques applied for routine applications, would result in much lower CVs.

Correlation of surface characteristics with assay performance

As is obvious from the calibration curves in Fig. 1, the assay sensitivity of hydrophilic polyether polyurethane surfaces increased with decreasing water content according to the following order: PU1, PU3 < < PU2 < PU4. This correlates well with the hydrophilicity of the PU surfaces as estimated by the spot diameter (see wettability). According to the materials data sheet, PU3 shows >100% expansion, whereas PU1 shows no expansion at all. The order of hydrogel water content and expansion is PU3 > > PU2 > PU4 > > PU1, indicating that the hydrogels are most suitable for immobilization at medium water content and expansion, whereas at too high and too low water content and expansion the hydrogel layers are of poor mechanical stability, either due to the gel diffusing out in solution (PU3) or due to poor and brittle adhesion (PU1). Interestingly, PU4 showed enhanced signals, especially at concentrations >0.1 mg/ml, even though water content and expansion was 20% less than for PU2; thus, providing for a less aqueous

environment appears to be not associated with loss of protein's activity. Consequently, water content either plays a less important role than expected or becomes critical only with much less stable proteins than IgG. Though PU6 (like PU1) shows no expansion, twice as strong signals were achieved on PU6 than on PU1, which is most likely a result of the hydrophobic character of the aromatic PU6 and the fact that hydrophobic adhesion of proteins is stronger than ionic adhesion (PU1). With regard to surface modification of polyurethanes, only modification of PU3 led to enhanced assay sensitivity (up to eight times as much). This can be clearly attributed to the improved accessibility of additives and crosslinkers for binding due to considerably greater expansion.

In general layer thickness had no effect on assay performance (see Table 1). However, the layer thickness of the respective hydrogel did determine assay sensitivity, inasmuch as too low a concentration produced incomplete and inhomogeneous coverage of the slide of with hydrogel, whereas too high a concentration resulted in increased BG noise, reducing the S/N and thus assay sensitivity.

In most cases an increase in immobilization capacity evoked stronger fluorescence signals. However, the increase in immobilization capacity is not directly proportional to the increase in signal intensity, which is usually smaller.

When comparing material, surface and assay parameters of the different modified and unmodified tested chip surfaces no correlation within parameters was found despite for the polyurethane surfaces PU1 to PU4: spot diameter and roughness increased with increasing water content and expansion (given by the manufacturer), whereas signal intensity and coating thickness decreased. Data reproducibility deteriorates with increasing water content and expansion. This can be understood in that at high PU expansion more peripheral chains penetrate into the solution giving rise to a more diffuse interface, and as a consequence rougher surface which leads to less reproducible spots and data. The correlation of assay and surface data for surfaces PU1 to PU4 demonstrates that if surface topographies change only slightly due to similar material characteristics or modification and binding mechanism an effect of surface parameters on assay performance becomes visible, as also shown in Fig. 4.

By contrast, when different hydrogels of different chemistry and modifications were compared, no correlation between surface properties and assay performance was found, except for the assay reproducibility, showing that more hydrophobic layers with low water content are more highly reproducible between different measurements.

Authors that report the critical role of surface hydrophilicity for protein immobilization and stabilization we disagree with: According to our experience both fluorescence signals and data reproducibility are increased with less hydrophilic surfaces. Furthermore, in contrast to [25] no difference in assay sensitivity or detection limit was found for hydrogel and non-hydrogel surfaces.

Conclusions

Correlating the on-chip assay performance (immobilization capacity, signal-to-noise ratio, data reproducibility) with the chip surface properties (surface modification, thickness, roughness, water content and expansion) using different hydrogels of the same chemistry, such as PU1 to PU4, reveals that spot diameter and roughness increase with increasing water content and expansion, whereas signal intensity, data reproducibility and coating thickness decrease. No correlation was found, when using completely different surface chemistries following different binding mechanisms and providing different surface topographies. Furthermore, in contrast to previous reports [25] the assay sensitivity was similar on hydrogel and non-hydrogel surfaces. However, data reproducibility was improved on more lipophilic chip surfaces.

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Chapter 2

A comparative analysis of polyurethane hydrogel for immobilization of IgG on chips

***Submitted to Anal. Chim. Acta**:* Katarzyna Derwinska, Claudia Preininger. A comparative study of polyurethane hydrogel for immobilization of IgG ob chips.

Abstract

Hydrogels are considered an optimum material for protein chip surfaces, since they provide a quasi-liquid environment which allows protein activity to be maintained and shows good spot morphology as well as excellent immobilization capacity. In the following we present a polyurethane (PU) chip that electrostatically binds IgG. The PU surface is optimized with regard to layer thickness, hydrogel and immobilized antibody concentration, pH and ionic strength of the print buffer as well as to blocking solution. Evaluation is done in a direct IgG immunoassay using the Nexterion slide H. It is shown that higher IgG concentrations are necessary on slide H than on the PU chip in order to reach the same assay sensitivity.

1. Introduction

Numerous strategies have been developed for immobilizing proteins on solid substrates for microarray application [1-3]. These range from adsorption on hydrophobic materials, such as polystyrene, and covalent binding on functional polymers [4] to DNA-mediated, oriented immobilization [5] on activated glass or plastic supports. For the development process, high immobilization capacity as well as prevention of protein denaturation are important issues. Furthermore, the surface needs to be robust, stable and reproducible. The most widely used substrate for protein microarrays are membranes, as they provide a high binding capacity. With this approach, 1 pg/ml of sample [6] was easily detected. For this reason, filter membranes, such as nitrocellulose or nylon, are now attached to glass and used in the microarray format. Other 3D surfaces are mostly based on hydrogels [7-9] and dendrimers [10] (branched polymers). In addition to polyacrylamide [7, 8] and respective copolymers, agarose [9] has been reported as being easy to prepare as a 3D matrix for proteins using activated aldehyde groups for protein binding.

Hydrogels are defined as colloidal gel polymers in which water is the dispersion medium. This insoluble network of polymer chains has the ability to swell in aqueous solutions, thus providing a semi-liquid environment for attached proteins. As hydrogels are able to react to external conditions, such as changes in pH, ionic strength and temperature, they provide great potential for a variety of fields of application; among these, drug delivery and enzyme sensors are the most prominent.

Yet hydrogels are rather difficult to prepare manually, hence commercially available slides tend to be expensive. Until now only a limited number of 3D hydrogel surfaces have been available on the market. The most well known of these are the hydrogel slides based on polyacrylamide from Perkin Elmer, dating back to the works of Mirzabekow [11], and the Nexterion Slide H from Schott.

Slides coated with hydrogels are usually quite thick, as much as 30 µm in fact. The protein spotted on such a surface evaporates slowly, forming extremely homogeneous spots, and subsequently denaturation is prevented. Moreover, a hydrogel surface allows a better separation of protein spots, since these soak into the polymer and are quite limited in movement. In the following we report on a 3D polyurethane (PU) surface for simple, one-step immobilization of protein for application in an on-chip immunoassay. The PU chip is optimized with regard to layer thickness, hydrogel and immobilized antibody concentration, pH and ionic strength of the print buffer as well as to blocking solution. The immobilization capacity and assay performance of the PU chip is compared with commercial Nexterion H slide for evaluation.

2. Materials and methods

2.1. Materials

Silane Prep™ slides (25 x 75mm) (Sigma, S4651), adhesive slides (Marienfeld, no. 08 100 00) and –NCS modified SAL1-Slides (Asper Biotech) were used as the substrate. Nexterion® Slide H from Schott was employed by way of reference. Hydrophilic polyether polyurethane hydrogel PU was acquired from Cardiotech International. Cystenaminhydrochloride (CAHCl), mercaptoethansulfonic acid (MESA) and polystyrenesulfonic (PSSA) acid were obtained from Fluka. Dodecyl sulfate sodium salt (SDS) was provided by Merck and phosphate buffered saline (PBS) by Gibco. 0.2 M Sörensen buffers of pHs 5.8, 7.2 and 8.6, obtained from Electron Microscopy Sciences (USA), were diluted 1:1 with MiliQ water. Buffer additives aminosulfobetain (ASB-14) and sodium deoxycholate were from Sigma whereas 3-(decyldimethylammonio) propanesulfonate inner salt (SB3-10) and hexadecyltrimethylammonium bromide (CTAB) were from Fluka. Tween-20 was purchased from Fluka and Tween-80 from Sigma. All other reagents were analytical grade.

2.2. Chip fabrication

2% to 10% PU was dissolved in EtOH/H2O (95/5) and dip-coated onto glass slides using the KSVD dip coater by KSV Instruments (velocity: 100 mm/min; retention time: 60 sec; retention time between layers: 2 minutes). 2% PU layers were modified with a solution of either 3.8 mg/ml MESA or 2.6 mg/ml CAHCL in 1x PBS (pH 7.2) for 30 minutes to create an anionic and cationic surface respectively.

2.3. Microarray printing

Three replicates of 0.005-1 mg/ml rabbit IgG (technical grade, Sigma) in various print buffers were arrayed onto the respective hydrogel surfaces using the OmniGrid contact spotter by GeneMachines (pin SMP3). Unless stated otherwise, 1x PBS (pH 7.2) was used as print buffer. The spot-to-spot distance was 400 µm, spot volume was 0.6 nl.

2.4. Postarraying & blocking

After arraying, the slides were incubated in a humid chamber at 4 °C overnight to complete probe immobilization. Surface blocking was performed for 30 minutes using blocking solutions I to IV consisting of: 1x PBS (pH 7.2)/0.1% Tween-20 (I); 1x PBS (pH 7.2)/0.1% Tween-80 (II); 1x PBS (pH 7.2)/3.8 mg/ml MESA (III); and 1x PBS (pH 7.2)/2.6 mg/ml CAHCL (IV). This was

done in order to wash off unbound protein and deactivate reactive surface groups. Finally, the slides were washed twice in 1x PBS (pH 7.2) and then blown dry using compressed air or spun dry in the centrifuge (900 rpm for 3 minutes).

2.5. Direct immunoassay

Protein slides were processed with 4 ng/µl Dy633-labelled anti-Rabbit IgG (λ_{ex}=635 nm, λ_{em}=670 nm) (DyAB) (Dyomics) in 1x PBS (pH 7.2)/0.1% Tween-20 at 4 °C for 3 hours, then washed twice in 1x PBS (pH 7.2) and spun dry in the centrifuge (900 rpm for 3 minutes).

2.6. Fluorescence detection

Slides were stored in the dark and scanned on the same day the immunoassay was performed. Fluorescence measurements were taken using a Genepix^{TM} 4000B non-confocal scanner by Axon Instruments. For data comparison, the PMT (photomultiplier tube) was kept constant within single experiments. All fluorescence (a.u.) data is background-corrected. Additionally, data flagged as bad, according to parameters set in the Genepix software (e.g. spot diameter 40µm - 220 µm; signals >200 a.u. fluorescence), was filtered.

2.7. Profilometry

Layer thicknesses were measured over 2.5 x 2.5 mm^2 surface areas using the Wyko NT1100 optical profiling system (Veeco) and Vision32 Veeco software. The values in Table 1 are mean values for two measurements.

3. Results and discussion

3.1. Effect of substrate, hydrogel concentration and number of layers on immobilization capacity and assay performance

Polyurethane layers of 2%, 4% and 10% PU and one or five layers of 2% PU, on either an amino, isothiocynate or adhesive substrate, were evaluated in a direct IgG immunoassay. The fluorescence (a.u.) thereby achieved for 0.005-1 mg/ml rabbit IgG processed with 4 ng/µl Dy633-labelled anti-Rabbit IgG represents a measure for the immobilized active IgG available for the immunoassay and consequently of the suitability of the hydrogel surface for protein chips. Figures of merit for the PU surfaces are compiled in Table 1, while corresponding calibration curves are shown in Fig. 1.

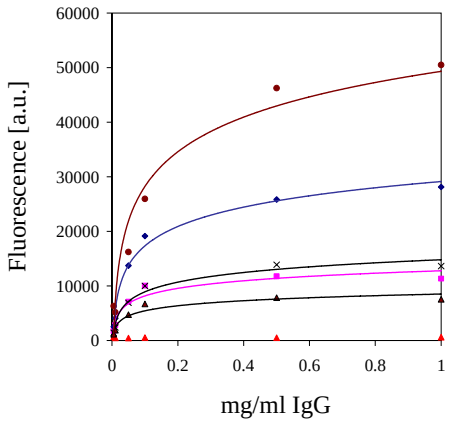

Figure 1 IgG calibration curve on surfaces consisting of: ● 2% PU on amino slides; ♦5 layers of 2% PU on amino slides; ■ 4% PU on amino slides; ▲ 4% PU on -NCS slides; × 4% PU on adhesive slides; and ▲ 10% PU on amino slides.

The data in Table 1 reveals that increasing hydrogel concentration results in decreased layer thickness: layers of 4% PU are three times thicker and 10% PU 7.5 times thicker than those of 2% PU, regardless of which type of substrate was used. Multiple layers of 2% PU resulted in about the same layer thickness as one layer of PU; thickness increased by only 50 µm. As is obvious from Fig. 1, the ability of the surface to immobilize processable IgG, as expressed by the slope of the calibration curve (change in fluorescence signal per concentration unit), decreases with increasing hydrogel concentration: fluorescence values (a.u.) are about 8 times higher for 2% PU than for 4% PU, whereas no significant signal was measured for 10% PU. Multiple layers of PU resulted in a deterioration of assay performance, yet still led to stronger signals than surfaces of 4% or 10% PU. The poor assay performance on 10% PU might be due to an increased density of hydroxy groups on the surface and thus to more closely packed immobilized

antibodies, which are thereby sterically hindered from reacting with the target. This problem is usually solved by mixing functionalized and non-functionalized polymers in order to control the density of reactive groups and, accordingly, immobilized antibodies [12]. Another reason might be the decreased transmittance of light in layers of high polymer concentration, leading to more light being absorbed by the polymer than by the fluorescent label to be activated. The optimum PU concentration is therefore 2%, and the optimum probe concentration 0.5 mg/ml. With respect to data reproducibility (see coefficients of variation [CV] in Table 1), there was no difference between 2% and 4% PU, yet 10% PU showed a tremendously high CV, while there was almost no effect for amino, adhesive and NCS slides when used as activated substrates to promote better adhesion and binding of the hydrogel, allowing the layer to expand in only one dimension. Clearly, the CVs of PU-covered amino (36%), adhesive (32%) and NCS slides (51%) indicate that the fluorescence signals obtained are comparable.

Table 1 Composition and characteristics of the various PU chips.

support	PU conc in %	No. of layers	Layer thickness in nm		Spot diameter in nm	Fluorescence at 0.5 mg/ml IgG	% CV at 0.5 mg/ml IgG
			Before	After			
aminosilane	2	1	**235**	**202**	159	46244	30
aminosilane	4	1	**721**	**686**	165	11782	36
aminosilane	10	1	**1761**	**2300**	165	532	85
adhesive	4	1	**734**	**742**	152	13904	32
-NCS	4	1	**851**	**768**	158	7854	51
aminosilane	2	5	**291**	**254**	143	25839	41

3.2. Modification of PU

Functionality was introduced by the addition of functional or charged reagents or by employing bifunctional crosslinkers. However, no improvement in assay performance was achieved (unpublished results) indicating that simple one-step adsorption of antibody on anionic polyurethane is the immobilization method of choice. In order to investigate the effect of surface charge on IgG binding, PU layers were modified with mercaptoethansulfonic acid, to create an even more anionic surface, and cysteaminhydrochloride, to produce a cationic surface. The results show that signals on PU and cysteamin-modified PU were about the same, whereas the signals obtained on surfaces modified using mercaptoethansulfonic acid were enhanced by 30%, suggesting that electrostatic binding of IgG is strongly promoted on very anionic surfaces (see also 3.1.).

3.3. Effect of print buffer on IgG adsorption

The choice of print buffer for a certain chip surface determines the protein binding capacity and stability as well as the spot morphology and in consequence the signal strength and data reproducibility [13-16]. Therefore the printing solution is optimized by use of additives and with regard to optimum reaction pH and ionic strength.

3.3.1 Buffer additives

In addition to widely used printing solutions, such as 3x SSC, 3x SSC/1.5 M betaine and 1x PBS (pH 7.2), commercial buffers as well as 1x PBS (pH 7.2) containing various anionic, zwitterionic and cationic detergents usually used in electrophoresis to promote solubility of proteins encountering for the hydrophobic sites of the proteins and prevent protein aggregation were employed. Furthermore, kosmotropes, such as sodium sulfate, which enhance hydrophobic interactions and promote protein adsorption, were also used.

Interestingly enough, additives often recommended, such as BSA [14], trehalose [15, 16] or glycerol [4], did not significantly enhance fluorescence. On the other hand, the addition of betaine to 3x SSC led to reduced signals and the addition of sulfobetain (ASB14) resulted in about a 50% increase in signals. It is interesting to note that, when comparing the most appropriate additives, which were ASB14 (zwitterionic), SB3-10 (zwitterionic), sodium deoxycholate (anionic), CTAB (cationic) and Tween-20 (non-ionic), there is no correlation between the charge of the additive and the signal-to-noise ratio measured.

3.3.2. Ionic strength

Protein adsorption on hydrophilically neutral surfaces tends to be relatively weak, whereas adsorption of proteins on hydrophobic surfaces is usually very strong and often partially irreversible. Adsorption of proteins on charged surfaces tends to be a strong function of the charge character of the protein, the pH of the medium, and the ionic strength. The influence of ionic strength on microarray signals was investigated using 0.5x, 1x and 2x PBS (pH 7.2) as the printing solution. The ionic strength of the buffers is also reflected in the conductivity of the solution, which is 9.18, 17.42, and 32.30 mS/mm² respectively. As shown in Fig. 2, the fluorescence increased with increasing buffer salt concentration (except for the lowest probe concentration: 0.03 mg/ml IgG). Up to 40% stronger signals were achieved when using 2x PBS instead of 1x PBS. However, the effect of ionic strength at 0.5 and 1 mg/ml IgG remains ambiguous. From the standard deviations in Fig. 2 it is obvious that both signals can be distinguished significantly, yet this is not always the case with 0.5x and 1x PBS. However, the

effect on the assay performance is rather small when compared with the huge difference in ionic strength and conductivity of the tested buffers (2x PBS provides 4x and ~3.5x greater ionic strength and conductivity respectively than 0.5x PBS, while there is no effect at 0.03 mg/ml IgG, about 0.5 times at 0.13 and 0.25 mg/ml and 0.2 times at 0.5 and 1 mg/ml IgG). The effect is obviously more strongly expressed at low antibody concentrations.

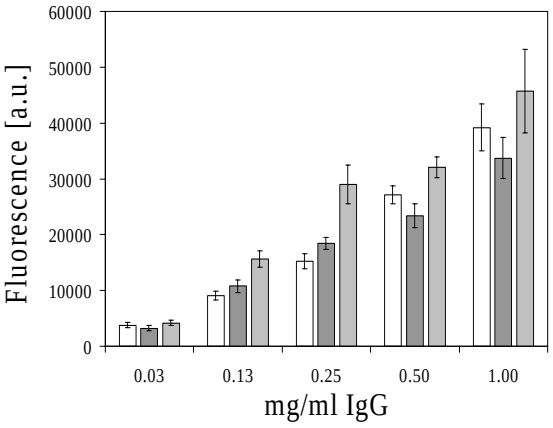

Figure 2 Fluorescence (a.u.) for 0.03 to 1 mg/ml IgG spotted in: ☐0.5x PBS; ■1x PBS; and ■2x PBS (pH 7.2).

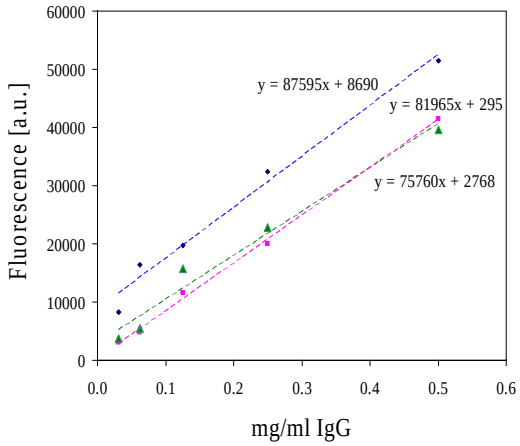

Figure 3 Calibration curves for 0.03 to 0.5 mg/ml IgG in 0.1 M Sörensen sodium phosphate buffers of pHs: ♦ 5.8; ■ 7.2: and ▲ 8.0.

3.3.3. pH of print buffer

The effect of pH on IgG adsorption was investigated when printing 0.03 to 0.5 mg/ml IgG (0.018 to 0.3 ng/spot) in commercial Sörensen sodium phosphate buffers of pH 5.8, 7.2 and 8.0. The strongest signals were measured in Sörensen buffer of pH 5.8, and the signals detected for IgG spotted in buffers of pH 7.2 and pH 8.0 were of about the same strength, but 25 to 60% lower than in buffer of pH 5.8. In conclusion, except for pH 5.8, the effect of the buffer pH on the fluorescence signal is negligible, an observation that has been previously reported [13] and investigated over four pH units (pH 4.5-pH 8.5) by Kusnezow et al. [16]. The stronger signals at pH 5.8 can be attributed to greater immobilization capacity at pHs below the isoelectric point of the antibody, since more antibody is bound to the anionic polyurethane due to stronger electrostatic adhesion of the more positively charged IgG (the isoelectric point of IgG is 6). Clearly, the loading ability of the PU surface is increased at pH 5.8, which produces stronger signals at all tested immobilized IgG concentrations. However, the increase in signal with immobilized antibody concentration follows the same mechanism as at lower pHs, since the linear coefficients are very similar and the calibration curve is merely shifted upward, as may be seen in Fig. 3.

3.4. Choice of blocking solution

Four different blocking solutions were tested: (I) 1x PBS (pH 7.2)/0.1% Tween-20, (II) 1x PBS (pH 7.2)/0.1% Tween-80, (III) 1x PBS (pH 7.2)/mercaptoethansulfonic acid, and (IV) 1x PBS (pH 7.2)/cysteaminhydrochloride. In using different blocking solutions, we aimed at creating surfaces of different hydrophilicity and surface charge. Surfaces that are more hydrophilic are known to decrease non-specific adsorption, whereas charged surfaces were expected to promote either specific or non-specific binding. In order to compare the hydrophilicity of the blocked surfaces, 0.01, 0.05 and 0.1 mg/ml labelled antibody in 1x PBS (pH 7.2) was spotted onto the PU chip. Spot diameter was then taken as a measure for the surface hydrophilicity and calculated as a mean value for 54 spots (spots in triplicate, 6 arrays per slide, 3 slides). The greatest influence on the spot diameter/hydrophilicity was observed when anionic mercaptoethanesulfonic acid and non-ionic Tween-80 were used: the spots increased by 35 µm in comparison to the non-blocked surface. The mean spot diameters obtained for non-blocked PU surfaces and PU surfaces blocked with solutions I to IV were 125 µm, 134µm, 159µm, 158µm and 120 µm. Fig. 4 shows the mean fluorescence (a.u.) for 27 spots (spots in triplicate, 3 arrays per slide, 3 slides) obtained with 0.05 to 1 mg/ml IgG after blocking in solutions I to IV and processing with 4 ng/µl Dy633-labelled anti-Rabbit IgG. The strongest signals by far were achieved when the surface was blocked with Tween-20 and Tween-80. The latter produced signals that were enhanced by 25% compared with Tween-20, whereas solutions III and IV resulted in signals reduced by a factor of 2 to 5. In summary, neither the surface charge – blocking with anionic MESA and cationic CAHCL –nor the hydrophilicity or wettability of the PU surface affected blocking efficiency (compare the diameter of surfaces blocked with MESA and Tween-80).

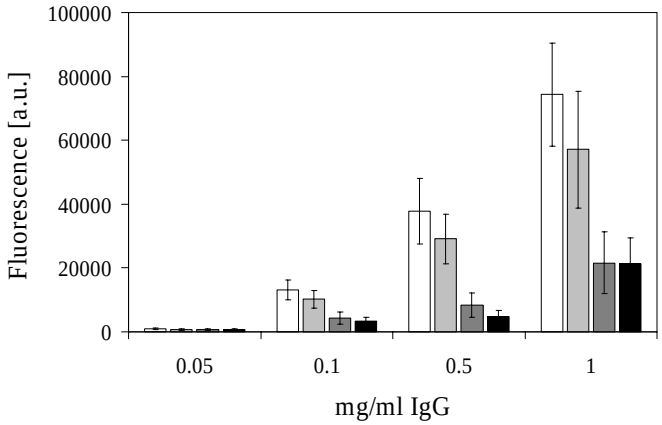

Figure 4 Fluorescence signals for IgG spotted onto the PU chip and blocked with: □ 1x PBS/Tween-80; ■ 1x PBS/Tween-20; ■ mercaptoethansulfonic acid; and ■ cysteamine.

3.5. Comparison with commercial slide H

PU slides were compared with Nexterion® Slide H in a direct IgG immunoassay spotted in 1x PBS (pH 7.2) as shown in Fig. 5 (full lines). The mean CV over the eight data points is 22% for both surfaces. The matching fluorescence images are shown in Fig. 6. In contrast to slide H, which shows a linear loading curve, the PU chip displays a logarithmic dependence on the immobilized probe concentration. It is clear that higher IgG concentrations are required on slide H (1 mg/ml) than on the PU chip (0.5 mg/ml) in order to reach the same assay sensitivity. Since electrostatic adsorption on the PU surface is known to be promoted by print buffers of high ionic strength, 1x PBS (pH 7.2), 2x PBS, 3x SSC and 0.1 M Sörensen buffer (pH 5.8) were examined. Only little difference was observed between PBS and SSC (except for 0.5 and 1 mg/ml IgG), whereas IgG in Sörensen buffer (pH 5.8) produced significantly greater fluorescence, as indicated by the dotted line in Fig. 5. Immobilization of IgG on polyurethane was due to electrostatic adsorption, whereas immobilization on slide H was covalent through amino groups of amino acids side chains on the protein surface. The immobilization on the PU chip occurs in a random manner, whereas binding on slide H is statistically oriented. However, there is no effect on assay performance, as may be seen from Fig. 5.

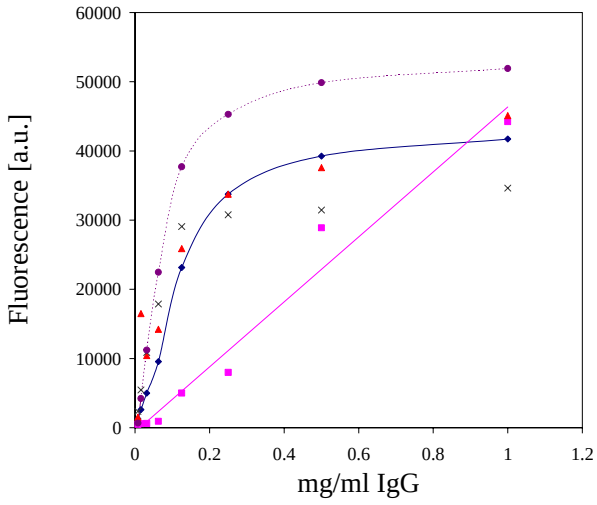

Figure 5 Fluorescence signals obtained for: ♦ 2% PU and ■ Nexterion® Slide H in 1x PBS (full lines); and signals for 2% PU in ▲ 2x PBS; × 3x SSC; ● Sörensen buffer pH 5.8 (dotted lines).

One reason for this might be found in the fact that IgG is a very stable molecule, thus requiring no special conditions for surface immobilization; another factor promoting IgG binding might be the surface charge combined with the gel character of the 3D surface and the additional fact that orientation plays a less important role than often reported in literature, especially in the case of antibodies. In fact, when R. Wacker et al. [5] compared direct spotting, DNA-directed immobilization and streptavidin-biotin attachment, they observed that all three tested formats led

to comparable detection limits, signal intensity as well as assay sensitivity and reproducibility. W. Kusnezow et al. [16] reported that there was no difference in signal-to-noise ratios when the carbohydrate groups of antibodies were activated and the antibodies were immobilized in an oriented manner on the slide. On the contrary, activation and subsequent purification were both time consuming and led to a loss of up to 40% of antibody. In [17], poly-L-lysine slides for electrostatic adsorption as well as aldehyde surfaces for covalent immobilization were judged easy to prepare and robust. The authors reported both very good signal-to-noise ratios and interfield coefficients of variation. Even though according to our profilometry measurements the sensitive layer of the PU chip is more than 100 times thicker than slide H, no binding problems due to slowed diffusion or decreased fluorescence signals as a result of increased background were observed. On the contrary, the PU surface might have the potential of further enhancing assay performance when producing stable hydrogel layers that are thinner than ~200 nm.

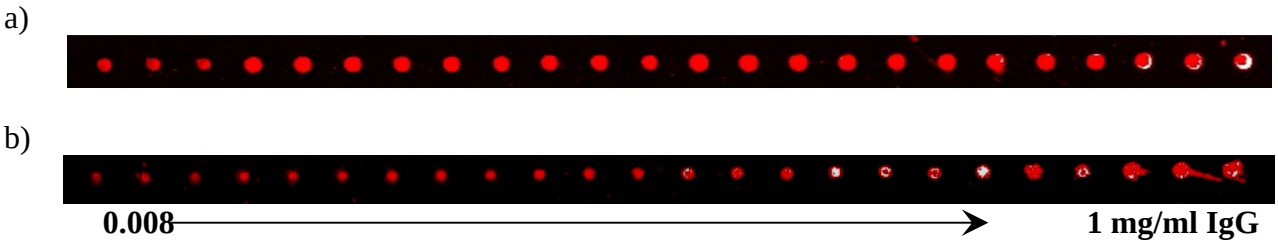

Figure 6 Fluorescence images for: a) the PU chip and b) slide H. Each concentration is in triplicate spots.

3.6. Storage stability

The PU surfaces were stored in the refrigerator or at RT for 2 months without loss in stability or deterioration of assay performance. In contrast to slide H, which is recommended to be stored at -20 °C to prevent hydrolysis of amine-reactive groups, storage of the PU chip at -20 °C led to decreased signal strength and reduced assay reproducibility.

Acknowledgements

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Chapter 3

Comparison of adsorption, covalent random and site-specific IgG immobilization on poly(vinyl alcohol) surfaces

***To be submitted to Anal. Biochem**:* Katarzyna Derwinska, Claudia Preininger. Comparison of adsorption, covalent random and site-specific IgG immobilization on poly(vinyl alcohol) surfaces

Abstract

Plain poly(vinyl alcohol) (PVA) surfaces, PVA surfaces tailored with additives and PVA surfaces crosslinked with functional amino-linkers are evaluated for adsorption, covalent/non-oriented and covalent/site-specific immobilization of IgG. The PVA surfaces were optimized with respect to the type of PVA, PVA concentration and kind of glass substrate. The resulting hydrogel surface of choice consists of 4% PVA (Mw 85000-146000, hydrolysis degree 99+%) coated onto adhesive glass. Comparison of modified and unmodified PVA surfaces revealed three surfaces that showed significantly higher loading capacity than plain PVA: PVA surfaces oxidized with HIO_4, PVA crosslinked with adipic acid dihydrazide and surfaces made of PVA/chitosan. Thereby, fluorescence signals were similar for non-oriented and site-specifically bound IgG.

1. Introduction

The choice of proper surface chemistry in protein chips is critical due to the structural complexity of proteins. Various chip surfaces have been reported [1-3], ranging from silane and gold monolayers to functional polymers and hydrogels. The latter are considered especially suitable for protein immobilization, since they provide a liquid microenviroment that can keep the proteins hydrated and stabilize the structure, which is responsible for the protein´s activity [4]. The hydrogels that have been reportedly used as immobilization matrices on protein chips are: agarose [5], poly(acrylamide) [4, 6-8], polyurethane [9], dextran [10] and polyethyleneglycol (PEG) [11]. Poly(vinyl alcohol) (PVA), by contrast, is used mainly as an encapsulation material for cells, in drug delivery, or as blends in (bio-) sensors. Photosensitive poly(vinyl alcohol-styrylpyridinium) (PVA-SbQ), for example, is cited in [12] as having been used for surface-patterning of a bio-MEMS-based cell chip using recombinant *Escherichia coli*, and according to [13], PVA was part of a polymer blend in a glucose biosensor. In order to produce mechanically stable PVA layers, often polymer blends or co-polymers are developed and employed that combine the mechanical strength of the additive polymer blend or monomer with the biocompatibility and hydrophilicity of PVA. Mechanically stable PVA ormosils, for BOD biosensing in seawater using organically modified silicates (ormosils), were reported in [14]. In addition, improved hydrogel strength has also been obtained by crosslinking PVA with glutaraldehyde [15] or chitosan.

In the following, we describe first additive-functionalized and then crosslinked PVA, which we employed as an immobilization matrix for IgG, activated IgG and site-specifically aminomodified IgG in order to evaluate: 1) the suitability of modified and unmodified PVAs in protein arrays; and 2) the effectiveness of simple, one-step IgG adsorption versus multi-step random and site-specific covalent immobilization.

2. Materials and Methods

Materials

Dodecyl sulfate sodium salt (SDS) was provided by Merck and phosphate buffered saline (PBS) by Gibco. Buffer additives aminosulfobetain (ASB-14) and sodium deoxycholate were from Sigma whereas 3-(decyldimethylammonio) propanesulfonate inner salt (SB3-10), 3-[(3Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), sodium N-dodecanoyl-N-methylglycinate (sarcosyl) and hexadecyltrimethylammonium bromide (CTAB) were from Fluka. Tween-20 was purchased from Fluka. Monochlortriazinyl-ß-cyclodextrin sodium salt (MCT) was acquired from Wacker. All other reagents were analytical grade.

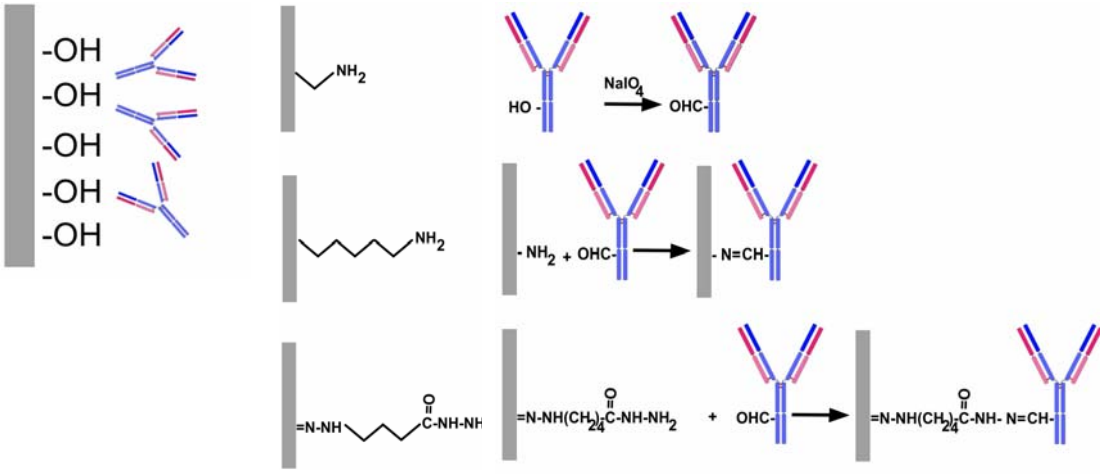
Chip fabrication & surface modification

Aqueous solutions of 1%, 4% and 10% PVA1 (Sigma-36306, Mw 146000-186000, hydrolysis degree 99+%), PVA2 (Sigma-341584, Mw 89000-98000, hydrolysis degree 99+%), PVA3 (Sigma-363103, Mw 146000-186000, hydrolysis degree 87-89+%), and PVA4 (Aldrich-9002895, Mw 85000-146000, hydrolysis degree 99+%), 4% PVA4/2% chitosan (food grade, Dalwoo) and 4% PVA4/2% oligo-chitosan (food grade, Dalwoo) were prepared and dip-coated onto either plain glass (Sigma, 8902), Silane Prep^{TM} (Sigma, S4651) or adhesive Histobond slides (Marienfeld, no. 08 100 00) using the KSVD dip coater by KSV Instruments (velocity: 100 mm/min; retention time: 60 sec; retention time between layers: 2 minutes). For IgG adsorption (schematically shown in Fig. 1A) the PVA slides were used without further treatment, whereas for covalent IgG immobilization adhesive glass slides were coated with 4% PVA4 and modified with bifunctional crosslinkers, such as ethylenediamine (2EI) (Fluka), hexamethylenediamine (HDA), adipic acid dihydrazide (ADH) (Aldrich), chitosan and chitosan-oligo subsequently to HIO_4 oxidation (The slides were immersed in 1% aqueous HIO_4 for 1 h and then washed twice with MiliQ water before incubating the slides in 1% crosslinker solution (pH 8) for 60 min). Schemes of the resulting modified surfaces are presented in Fig. 1B.

Chemical modification of IgG

The carbohydrate groups of IgG were activated using sodium meta-periodate as described in [16]. Briefly, 3 mg/ml IgG in 0.1 M sodium acetate buffer (NaOAc) (pH 5.5) was incubated with sodium meta-periodate (25 mg/ml in NaOAc buffer, pH 5.5). After 1 hour glycerol was added to stop the reaction. The activated antibody was then filled into microfilterfuge tubes (Microcon YM-30, Millipore) and centrifuged to separate the antibody from excess of periodate. Antibody samples were then washed twice with cold NaOAc. The activated IgG (IgG-CHO) was arrayed onto plain 4% PVA4, 4% PVA/2% chitosan and 4% PVA4 surfaces crosslinked with adipic acid dihydrazide

(ADH). The binding principle is shown in Fig. 1C. The concentration of IgG-CHO was determined spectrophotometrically with respect to non-activated IgG using the NanoDrop (ND-1000, protein a280 mode) and the loss of material due to chemical modification was calculated as percentage of the starting material.



A) B) C)

Figure 1 Binding principle schematically shown for (A) antibody adsorption on the PVA surface, (B) covalent binding on PVA surfaces crosslinked with 2EI, HDA and ADH, and (C) site-specific attachment of IgG-CHO on PVA/chitosan and PVA crosslinked with ADH.

Microarray printing

Three replicates of 0.005-1 mg/ml rabbit IgG (technical grade, Sigma) were arrayed onto the PVA surfaces using the OmniGrid contact spotter from GeneMachines (pin SMP3). Unless stated otherwise, 1x PBS (pH 7.2) was used as print buffer. The spot-to-spot distance was 500 µm, spot volume was 0.6 nl.

Postarraying & blocking

After arraying, the slides were incubated in a humid chamber at 4 °C overnight to complete probe immobilization. Surface blocking was performed in 1x PBS (pH 7.2)/0.1% Tween-20 to rinse off unbound protein and deactivate reactive surface groups.

This was done in order to wash off unbound protein and deactivate reactive surface groups. Finally, the slides were washed twice in 1x PBS (pH 7.2) and then blown dry using compressed air or spun dry in the centrifuge (900 rpm for 3 minutes).

Determination of immobilization capacity

Immobilization capacity in fmoles/mm² was calculated by taking the median fluorescence minus the local background of 27 replicate spots of dye-labelled anti-IgG before and after blocking (30 min) multiplied by spotted protein concentration and divided by molecular mass of labelled protein and square radius of the spot. The calculation was done according to the formula. The factor $1.9 \cdot 10^6$ is calculated from the volume of the protein solution per spot (0.6 nl/spot) and the Π.

$$I = 1.9 \cdot 10^6 \frac{F_A}{F_B M_{LP} R^2} C_{LP}$$

Where:

I - Immobilization capacity

F_B – Fluorescence before blocking

F_A – Fluorescence after blocking

M_{LP} – Molecular mass of labelled protein

R – Spot radius

CLP- concentration of spotted protein

Direct immunoassay

Protein slides were processed with 4 ng/µl Dy633-labelled anti-Rabbit IgG (DyAB) (Dyomics) in 1x PBS (pH 7.2)/0.1% Tween-20 at 4 °C for 3 hours, then washed twice in 1x PBS (pH 7.2) and spun dry in the centrifuge (900 rpm for 3 minutes).

Fluorescence detection

Slides were stored in the dark and scanned at λ_{ex}=635 nm and λ_{em}=670 nm on the same day the immunoassay was performed. Fluorescence measurements were taken using a Genepix™ 4000B non-confocal scanner from Axon Instruments. For data comparison, the PMT (photomultiplier tube) was kept constant within single experiments. All fluorescence (a.u.) data are background-corrected. Additionally, data flagged as bad, according to parameters set in the Genepix software (e.g. spot diameter 30 µm - 480 µm; signals >100 a.u. fluorescence), were filtered.

Profilometry

Layer thicknesses were measured over 2.5 x 2.5 mm^2 surface areas using the Wyko NT1100 optical profiling system (Veeco) and Vision32 Veeco software. The values are mean values for two measurements.

3. Results & discussion

3.1. IgG Adsorption

3.1.1. Choice of PVA

4% PVA1, -2, -3 and -4 were tested in a direct on-chip immunoassay using IgG adsorbed on the PVA-surface. The following criteria were taken into account in evaluation: fluorescence signals (a.u.), background (a.u.) and spot morphology. Fig. 1 shows the fluorescence obtained for 0.01 to 0.5 mg/ml IgG (6pg to 300 pg IgG/spot) on PVA surfaces 1 to 4. The fluorescence signals were background-corrected and mean values were calculated for 27 spots. As is evident from Fig. 2, hydrolysis degree plays an important role in the assay performance of PVA. Up to 30% stronger signals were achieved using PVA1 as compared to PVA3, despite the same molecular weight distribution. This is most likely due to the increased number of hydroxy groups available for IgG loading. The influence of molecular weight on fluorescence signals, at a hydrolysis degree of 99+%, is not entirely clear. It can nonetheless be observed that PVA2, with the lowest molecular weight (Mw), and PVA4, containing low and high Mw parts, produce the strongest signals. Signals for the highest Mw PVA tested (PVA1) were 40 to 60% weaker. PVA2, while having a narrower molecular weight distribution than PVA4, shows a similar IgG loading curve and equivalent assay performance. This indicates that the optimum molecular weight for the tested application is between 90.000 and a maximum of 146.000. PVA4 was chosen for further measurements, as the % coefficient of variation (CV) was 11%, i.e. four times lower than for PVA2.

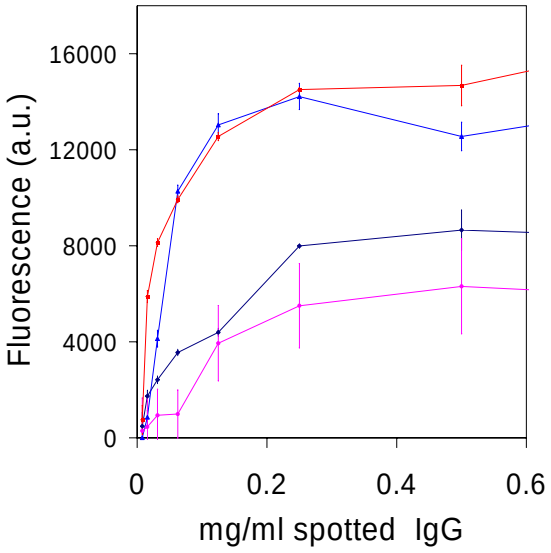


Figure 2 Loading of 0.005 to 0.5 mg/ml IgG on ◆PVA1, ▲PVA2, ●PVA3 and ■PVA4 surfaces. Error bars:SE

3.1.2. Choice of substrate

The choice of optimal substrate is crucial for microarray surface development. The substrate should allow good polymer adherence without peeling off during the microarray experiment and display low autofluorescence at the wavelengths of interest.Three different substrates were coated with 4% PVA4 and evaluated: unmodified glass (Sa = 5.81 nm), aminosilane glass (Sa = 1.73 nm) and adhesive glass (Sa = 12.5 nm). Due to pretreatment, the latter two substrates were expected to foster stronger PVA binding, resulting in a more stable PVA-layer, as hydrogel swelling along the substrate is thereby inhibited. The best results, as shown in Fig. 3, were obtained on the adhesive substrate, whereas the plain glass and the silanized glass resulted in signals that were at least 50% weaker. The maximum IgG loading capacity achieved was 0.5 mg/ml IgG (300 pg/spot), regardless of the substrate used. However, twice as much IgG could be immobilized on PVA-coated adhesive slides than on plain glass and three times as much on aminosilane glass. Although both aminosilane and adhesive glass provide reactive groups that are expected to bind PVA to the substrate more effectively than plain glass, substrate modification evidently had no significant effect with regard to producing stable PVA surfaces. This behaviour contrasts previous studies on poly(urethane) (PU) [D1 paper], which report comparable performance for both aminosilane and adhesive glass. An explanation for this might be found in the electrostatic interaction between PU and aminosilane. This is stronger than between aminosilane and PVA, with PVA displaying only a slight negative surface charge that decreases linearly from -2 mV at pH 5 to -6 mV at pH 9, while PU is more negatively charged, with a ζ potential that decreases linearly from -9 mV at pH 5 to -24 mV at pH 9. In the present case, the improved loading capacity on PVA-coated adhesive substrates is most likely the result of increased substrate roughness (the roughness of adhesive glass is six times greater than that of aminosilane glass). Increased roughness leads to better coverage of the substrate by PVA and to the formation of a rougher PVA layer onto which a higher amount of IgG can be adsorbed, which in turn results in stronger fluorescence signals.

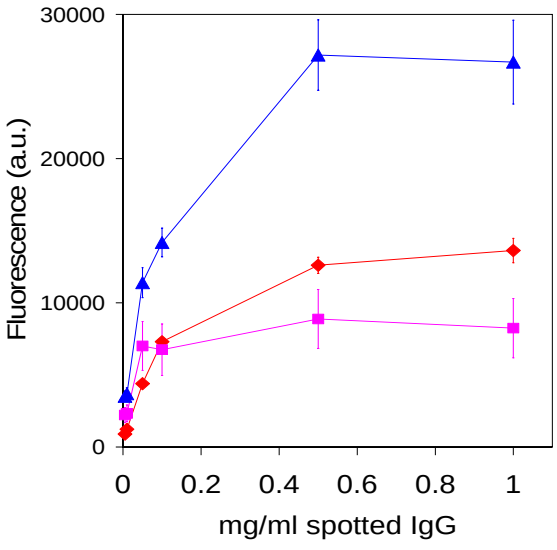


Figure 3 Loading of 0.005 to 1 mg/ml IgG on ◆plain, ■aminosilane and ▲adhesive glass slides covered with 4% PVA4. Error bars:SE

3.1.3. Optimization of PVA concentration

Adhesive slides coated with aqueous solutions of 1%, 4% and 10% PVA4 were evaluated with respect to mechanical stability over incubation time (3 h) and to loading capacity, as determined in an immunoassay and by spotting labelled IgG. As shown in Fig. 4, fluorescence of spotted IgG processed with 4 ng/µl Dy633-labelled anti-Rabbit IgG increases with increasing PVA concentration: When PVA concentration was increased by a factor of 4, signals were 2.5 times stronger, whereas 5 times stronger signals were obtained by increasing PVA concentration by a factor of 10. In conclusion, increasing PVA concentration by a factor of x leads to signal enhancement by roughly 0.5 times x. This is most likely due to better substrate coverage with PVA4 at higher hydrogel concentrations. In fact, the thickness of the hydrogel layer increased significantly with increasing PVA concentration: The layer thickness (dry state) for 1% and 4% PVA as determined by profilometry was 46.5 nm and 407.5 nm respectively. Thus, increasing the hydrogel concentration by a factor of 4, enhances the layer thickness by 9 times, furthermore resulting in improved mechanical stability: when using 1% PVA surfaces twice as much hydrogel dissolves out in solution during incubation (3 h) than with 4% PVA surfaces. As a consequence, hydrogel layer thickness for 1% and 4% PVA is reduced by 67.7% and 38.2% respectively. Apart from the improved mechanical stability of surfaces consisting of high PVA concentration, the immobilization capacity is drastically improved on thicker gels. This is obvious from the loading curve in Fig. 4 as well as from the immobilization capacity calculated for labelled IgG: 1% PVA4 – 16 fmoles/mm², 4% PVA4 - 91 fmoles/mm², and 10% PVA4 126 fmoles/mm². Moreover, the improved IgG loading on 4% and 10% PVA4 surfaces may be a result of increased density of OH-groups on the surface that due to their polarity promote the interactions between local dipoles existing on the interacting molecules [17]. In further experiments 4% PVA4 surfaces were employed because of their lower viscosity and thus easier fabrication compared with 10% PVA.

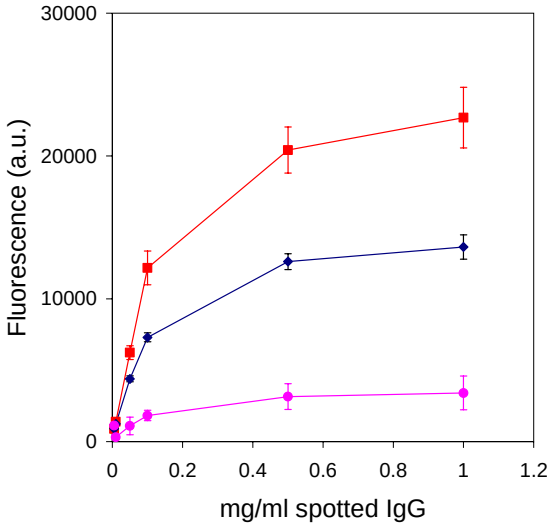


Figure 4 Loading of 0.005 to 1 mg/ml IgG on chip surfaces made of ●1%, ◆4% and ■10% PVA4. Error bars:SE

3.1.4. Effect of print buffer composition on IgG adsorption

Adsorption is a simple, one-step immobilization method. The attachment occurs via electrostatic and/or hydrophobic interaction forces. Thus, the printing solution as it contains additives of various polarity and ionic charge can influence the strength of IgG adsorption. Several additives ranging from 0.01% to 0.001% in 1x PBS (pH 7.2) have been tested: ASB-14, Tween-20, sarcosyl, SDS, MCT, CHAPS, CTAB, thioglucopyranose, SB3-10, sodium deoxycholate, cysteamine chloride, glycerol. In Fig. 5 the triplicate spots of the most suitable print buffers compared with plain PBS are highlighted. The respective fluorescence intensity values and coefficients of variation (%CV) are indicated in the figure caption. PBS containing 0.005% Tween-20, 0.01% thioglucopyranose and 0.01% ASB14 respectively resulted in 1.8, 1.5 and two times stronger fluorescence signals. As also reported in [18] the tuning of the additive concentration is critical, i.e. addition of 0.005% Tween-20 led to signal enhancement by 30%, whereas addition of 0.01% Tween-20 resulted in 1.5 times reduced fluorescence signals as compared to signals obtained in plain 1x PBS (pH 7.2).

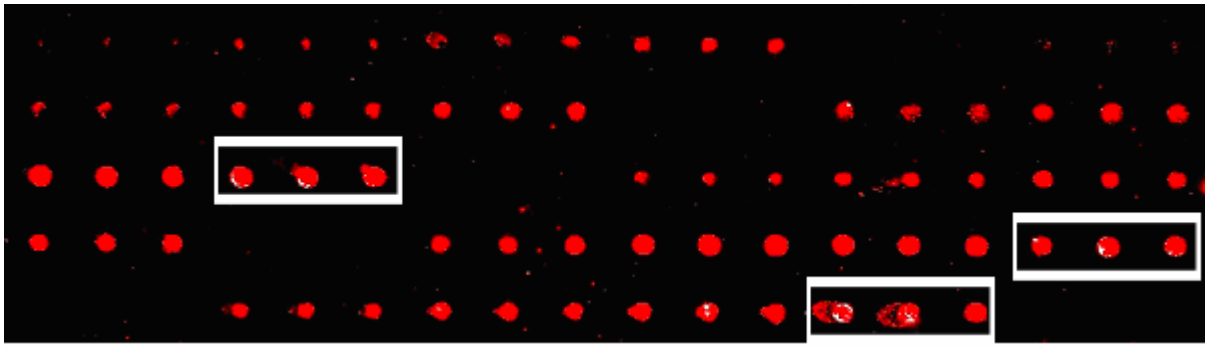


Figure 5 Spot images of IgG spotted in various buffers based on 1x PBS (pH 7.2) (Flu: 4280, CV 29.5%). Spots in PBS containing 0.005% Tween-20 (Flu: 7600 a.u., CV 9.5%), 0.01% thioglucopyranose (Flu: 6335 a.u., CV 7.7%) and 0.01% ASB14 (Flu: 8527 a.u., CV 39%) respectively are highlighted.

3.2. Covalent IgG immobilization on modified PVA

0.005 mg/ml IgG in 1x PBS was spotted onto PVA surfaces, PVA surfaces activated with HIO_4, and surfaces activated and crosslinked with amino-functional linkers of various lengths (ADA; 2EA; 1,6 HMA); furthermore, onto glass slides covered wtih PVA/chitosan and PVA/oligo-chitosan. Fig. 6 shows the percentage of signal increase for each modified surface as compared with the plain PVA4 surface. As is evident from the figure, only modification with adipic acid dihydrazide (ADA) and activation with HIO_4 led to significantly enhanced signals. Modification with ADA addresses the thiol-groups in the cystein units, whereas activated PVA can bind both amino and thiol groups present in the antibody. Functionalization of the chip surface by the addition of oligochitosan to PVA results in similar loading capacity, whereas all other tested modifications led to decreased IgG immobilization.

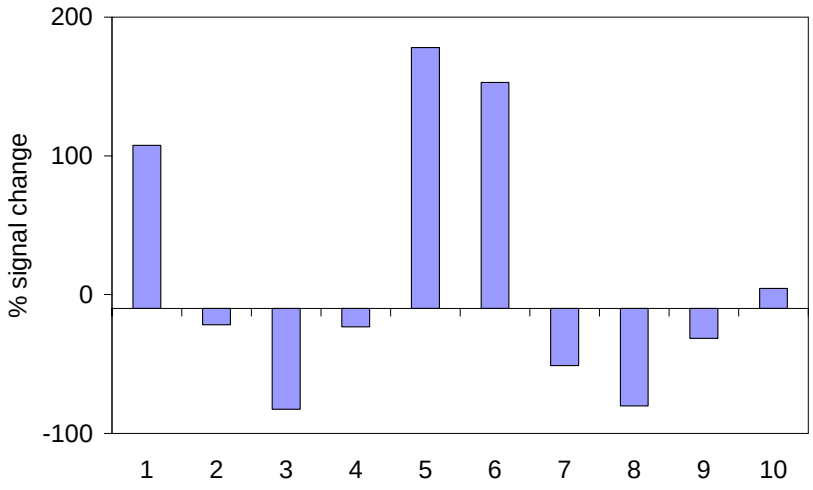


Figure 6 % signal increase for 0.5 mg/ml spotted IgG on surfaces made of PVA4 tailored with additives: 1) 2% chitosan, 2) 4% chitosan, 3) 2% chitosan-oligo, 4) 4% chitosan-oligo; and PVA4 activated with 5) periodate and further crosslinked with 6) ADA, 7) 2EA, 8) 1, 6 HMA, 9) chitosan and 10) chitosan-oligo in comparison with unmodified 4% PVA4.

3.3. Covalent, site-specific immobilization of modified IgG

In order to attach IgG site-specifically to the surface, IgG was activated with $NaIO_4$ as described in *Materials and methods.* Three types of surfaces were employed: plain 4% PVA4, activated 4% PVA4 crosslinked with adipic acid dihydrazide and 4%PVA//4% Chitosan. Fig. 8 compares the fluorescence signals obtained with 0.125 mg/ml, 0. 25mg/ml, 0.5 and 1 mg/ml spotted IgG and IgG-CHO respectively after processing with 4 ng/µl Dy633-labelled anti-Rabbit IgG. As is obvious from the figure, no improved loading was achieved with oriented immobilization using

activated IgG. Moreover, no improvement in data reproducibility was obtained. This agrees well with Kusnezow et al. [19] who reported similar signal-to-noise ratios when using activated and non-activated antibodies and a loss of about 40% of antibody due to activation and purification steps (in our studies the loss of IgG was 33%).

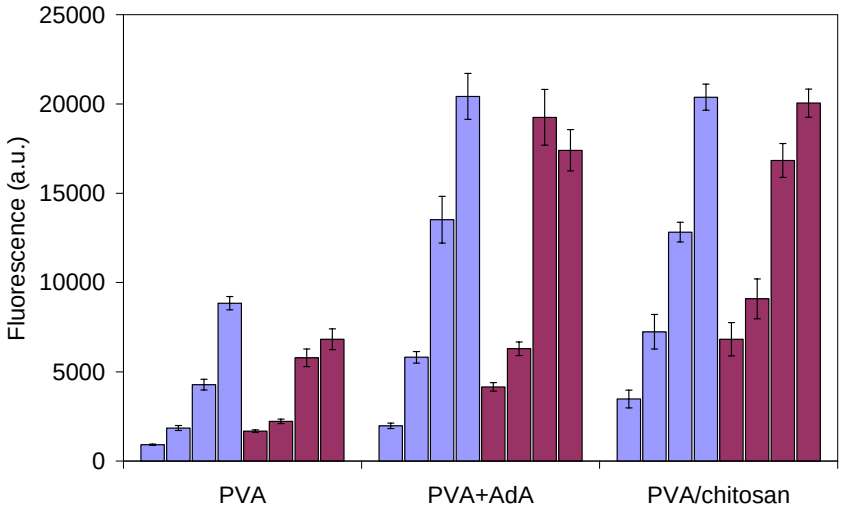


Figure 8 Comparison of 0.125 mg/ml, 0. 25mg/ml, 0.5 and 1 mg/ml spotted IgG and IgG-CHO on plain PVA4 surfaces, PVA4 crosslinked with adipic acid dihydrazide and PVA4/chitosan surfaces.

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Chapter 4

Reproducibility of hydrogel slides in on-chip immunoassays with respect to scanning mode, spots circularity, and data filtering

***Submitted to Anal. Biochem**:* Katarzyna Derwinska, Ursula Sauer, Claudia Preininger. Reproducibility of hydrogel slides in on-chip immunoassays with respect to scanning mode, spot circularity and data filtering.

Abstract

The reproducibility of 3D hydrogel surfaces based on polyurethane (PU), poly(vinyl alcohol) (PVA) and polyacrylamide (HydroGel) was investigated with respect to scanning mode (confocal vs. non-confocal), circularity (circular vs. irregular spots) and the influence of fluorescent background. It is demonstrated that, if even probe spots are provided, fluorescence intensities measured at the same PMT gain are similar for confocal and non-confocal scanning modes. Uneven probe spots, however, cause reduced fluorescence with confocal scanners as well as greater spot-to-spot variation and a higher degree of intra- and inter-experimental variability (%CV among three experiments). By using irregular instead of circular spot alignment, reproducibility (%CV) is improved for good- and bad-quality spots; in the latter case by up to three times as much. In addition, circularity can be used together with the mean-median correlation of pixel intensities as a quality measure.

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1. Introduction

Protein arrays used for quantitative analyses in medical diagnostics demand a great deal of specificity, sensitivity and precision in order to be capable of competing with conventional methods, such as ELISA, in clinical and research applications. To meet these demands, highly specific antibodies as well as powerful, sensitive detection techniques and proper surface chemistry are required. The latter aspect is quite challenging, since proteins, in contrast to DNA, are complex with regard to structure and behaviour on solid surfaces. A variety of chip surfaces has been reported [1-5], including 1D or 2D surfaces, mainly derived from DNA chip technology, and 3D surfaces consisting of dendrimeric or branched polymers [6] or hydrogels. 3D hydrogel surfaces for protein immobilization are reported to have advantages over 1D and 2D surfaces with respect to loading capacity and ability to maintain the protein structure, while offering low autofluorescence, improved accessibility for the target and low non-specific adsorption. Due to the quasi-liquid, hydrophilic microenvironment of 3D hydrogels, proteins are maintained on the surface in a stable, hydrated condition, whereas they can easily denaturate on hydrophobic surfaces such as 2D silanes [1]. Usually this is prevented through the addition of stabilizing agents, such as glycerol [2] or trehalose, to the printing solution.

P. Angenendt et al. [3] evaluated 11 hydrogel and non-hydrogel surfaces with respect to detection limit, inter- and intra-chip variation and storage characteristics. Four different antibodies were compared by arraying 40 fmol to 25 amol per spot in quadruplicate. Stronger signals were obtained on non-hydrogel surfaces. The detection limit was lowest on a commercial polyacrylamide slide (1313 amol/spot) but was higher on homemade polyacrylamide slides (~3000 amol/spot) when compared with non-gel slides (~2000 amol/spot). The mean intra- and inter-field variation was calculated for four to eight replicates, revealing about two times higher coefficient values for polyacrylamide slides. E.W. Olle et al. [2] compared acrylamide, nitrocellulose, aldehyde- and epoxy-silane slides using 25 µg/ml anti-goat IgG and found that the tested epoxy surface ES resulted in the greatest median fluorescence yet with a comparable standard deviation (6%). C. Steinhauer et al. [4] evaluated porous, silicon-based substrates developed in-house along with five commercially available glass substrates (silane slides, Xenoslide N, FAST slides, SpotOn and polyacrylamide slides); the substrates consisting of 3D coatings (silicon-based, FAST and polyacrylamide) and SpotOn slides showed the highest loading capacity and signal intensities. Significantly improved coefficient of variation (CV) calculated from three probe concentrations at 8 nM analyte concentration was achieved for silicon-based substrates (CV 5%) and SpotOn slides (1%), whereas for the other tested slides the coefficient of variation was between 11% and 16%. When comparing 2D aldehyde with 3D acrylamide slides at a capture antibody concentration

of 0.8 mg/ml in a sandwich immunoassay, two times higher signal-to-noise ratios were achieved for the 3D slides yet with comparable standard deviation (8%). Though the hydrogels that have been reported in literature make use of agarose [5], polyurethane [7], polyacrylate [8] and dextran [9], published work most often cites functional polyacrylamide or copolymers of acrylamide as the immobilization matrix for proteins. In the wake of the fundamental works of Mirzabekov et al. [10] in the late 90s (now commercialized as HydroGel slide by Perkin Elmer), new and promising acrylamide materials have been reported [10-14], e.g. for quantitative immunoassay of plant and bacterial toxins [14].

Though protein chip surfaces in general, and hydrogel surfaces in particular have been widely described with regard to surface chemistry and assay conditions, there are only a few detailed studies that investigate protein chip reproducibility in greater detail. While all of the papers cited here report assay reproducibility by providing error bars in the data graphs and number of replicates and standard deviations, only few authors [2-4] disclose in detail how data analysis was done; whether raw data, normalized or filtered data and whether mean or median fluorescence intensities were employed; whether the fluorescence signal, if not given in terms of signal-to-noise ratio (SNR), was background-corrected; nor do they indicate the degree of inter- and intra-variation among spots, arrays, slides and experiments to be expected etc.

In order to contribute to research on this, in our opinion, crucial topic in the area of protein chips, we investigated the assay performance of hydrogel surfaces made of polyurethane (PU), poly(vinyl alcohol) (PVA) and polyacrylamide (HydroGel) with regard to data reproducibility to determine whether confocal and non-confocal scanning mode, spot circularity and data normalization affect data reproducibility and reliability and, if so, in what manner.

2. Experimental

Materials

Hydrophilic polyether polyurethane hydrogels PU2 and PU8 were purchased from Cardiotech International and coated onto Silane Prep™ slides (25 x 75mm) (Sigma, S4651). PVA (99+% hydrolysed, No. 9002-89-5) was from Aldrich and coated on plain glass slides (25 x 75 mm) (Sigma, 8902). Phosphate buffered saline (PBS) was purchased from Gibco. Dy633-labelled anti-rabbit IgG (DyAB) was purchased from Dyomics. Rabbit IgG (technical grade) was from Sigma. All other reagents were analytical grade.

Chip fabrication

Glass slides were dip-coated, using the dip coater from KSV Instr. Ltd, from plain hydrogel solutions of 2% PU1 and PU8 in EtOH/H_2O (95/5) and 4% aqueous PVA. HydroGel® slides from Perkin Elmer were employed as a reference. Layer thickness of the PU, PVA and HydroGel slides was measured after 3 h incubation (typical assay time) over 2.5 x 2.5 mm^2 surface areas using the Wyko NT1100 optical profiling system (Veeco) and Vision32 Veeco software.

Microarray printing

Three replicates of 0.007-1 mg mL^{-1} (4.2 pg- 600 pg) rabbit IgG in 1x PBS (pH 7.2) were arrayed onto the chip surfaces using the OmniGrid contact spotter from GeneMachines (pin SMP3). The spot-to-spot distance was 400 µm, spot volume was 0.6 nl.

Postarraying & blocking

After arraying the slides were incubated in a humid chamber at 4 °C overnight to complete probe immobilization. Surface blocking was performed in 1x PBS (pH 7.2)/0.1% Tween-20 to rinse off any unbound protein and deactivate reactive surface groups. Finally, the slides were washed twice in 1x PBS (pH 7.2), blow-dried with compressed air or spin-dried using a centrifuge (900 rpm for 3 min).

Direct immunoassay

Protein slides were processed with 4 ng/µl Dy633-labelled anti-Rabbit IgG (λ_{ex}=637 nm, λ_{em}= 657 nm) in 1x PBS (pH 7.2)/0.1% Tween-20 at 4 °C for three hours, then washed twice in 1x PBS (pH 7.2) and blow-dried with compressed air or spin-dried using a centrifuge (900 rpm for 3 min).

Fluorescence detection

Slides were stored in the dark and scanned at fixed wavelengths of λ_{ex}=635 nm, λ_{em}= 670 nm) on the same day the immunoassay was performed. Fluorescence measurements were taken using the Genepix^{TM} 4000B non-confocal scanner from Axon Instruments and Tecan LS Reloaded Scanner with confocal scanning mode. Data analysis was done with the Genepix 6.0 software (Axon Instruments, Foster City, CA)

3. Results and discussion

Assay performance and scanning mode

In biochip technology confocal and nonconfocal fluorescence detection techniques are widely used and well established [15]. There are pros and cons with both methods: In general, confocal methods produce greater signals. However, brighter images are not necessarily better images. In most cases the reached detection limits are comparable. Confocal methods effectively measure the fluorescence in a certain plane of the surface thereby ignoring fluorescence coming from neighboring surface areas. Fluorescence background that may occur is therefore reduced. By contrast, non confocal methods collect all fluorescence reflected from the surface. Using confocal methods the correct focus plane is critical, since the surface depth is small, thus requiring very flat surfaces that allow to keep the surface in focus during the scanning process. Non confocal methods provide larger focus depths and are thus less prone to surface variations and uneven spots.

Confocal and nonconfocal scanning modes are evaluated with respect to measured fluorescence intensities, background (BG) influence and reproducibility of data using an IgG immunoassay on PU2, PU8, PVA and HydroGel slides. For analysis fluorescence intensity data were calculated from 18 spots per probe concentration (2 slides per hydrogel type, three subarrays per slide, and each probe in triplicate). Spots that were flagged bad by the scanner software were not taken in account for the calculation (on average 2% of all spots in all performed scans). Fluorescence signals were not background (BG) corrected. PMT gain was set to 50 % (non confocal and confocal) of maxium. As shown in Fig. 1 up to two times greater signals were obtained for PU8 and HydroGel with the non-confocal than with the confocal scanner, whereas the signal intensities measured for PU2 and PVA slides are similar for both scanning modes. Since in confocal scanning the proper focus plane is critical, the diminished fluorescence on PU8 and HydroGel might be due to scanning out of the optimum focus plane. This could be caused by spot roughness as is likely for PU8 (see *consequences of circular versus irregular spot alignment*) or increased layer thickness as it is the case for HydroGel (~2µm) (PU2, PU8: ~200 nm; PVA: 250 nm).

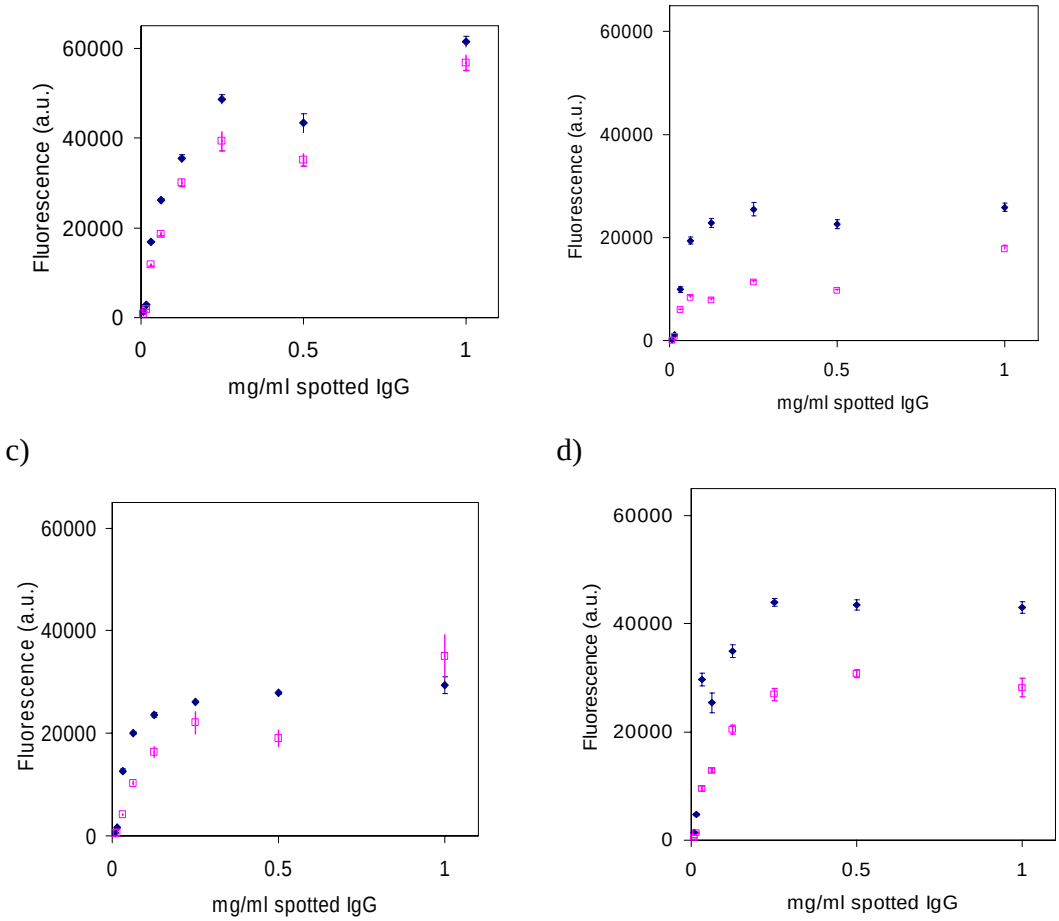


Figure 1 Loading curves for 0.007 to 1.0 mg/mL immobilized IgG on surfaces: a) PU2, b) PU8, c) PVA d) HydroGel scanned with confocal (□) and nonconfocal scanner (♦) Error bars: SE

Linearity of the standard curve was determined as the coefficient of determination (R^2) of a linear regression. Values >0.8 were obtained for all tested IgG concentrations with PU2 and PVA with the non-confocal scanning mode, and for PU8 (non-confocal) and Hydrogel (confocal) a linear range was observed only up to 0.125 mg mL^{-1}. Compared with PU2 and HydroGel surfaces, PVA and PU8 surfaces get overloaded at concentrations as low as 0.1 mg mL^{-1}. However, it needs to be pointed out that the PMT chosen for slide comparison was not the optimum for the PVA slides. In order to achieve stronger signals and increased linearity between fluorescence intensity and IgG concentration[16] a higher PMT gain is required.

Influence of fluorescence background

One of the major issues in microarray development is fabricating a surface that apart from excellent signals gives as low as possible background. Usually signal fluorescence (flu) is background (BG) corrected either by substraction (flu-BG) or calculation of the signal-to-noise ratio (SNR). Clearly, the impact of the background noise is greater when using SNR than fluorescence minus background. In order to examine the effect of BG, in Fig. 2 the signal-to-noise ratio is correlated with the not background corrected fluorescence signal obtained for hydrogel slides imaged with the confocal and nonconfocal scanner. Thereby the slope of the curves indicates the influence of the background noise: the steeper the slope, the less the signal is affected by the BG. The more the points close to the trend line the more homogeneous BG within the experiments [17].

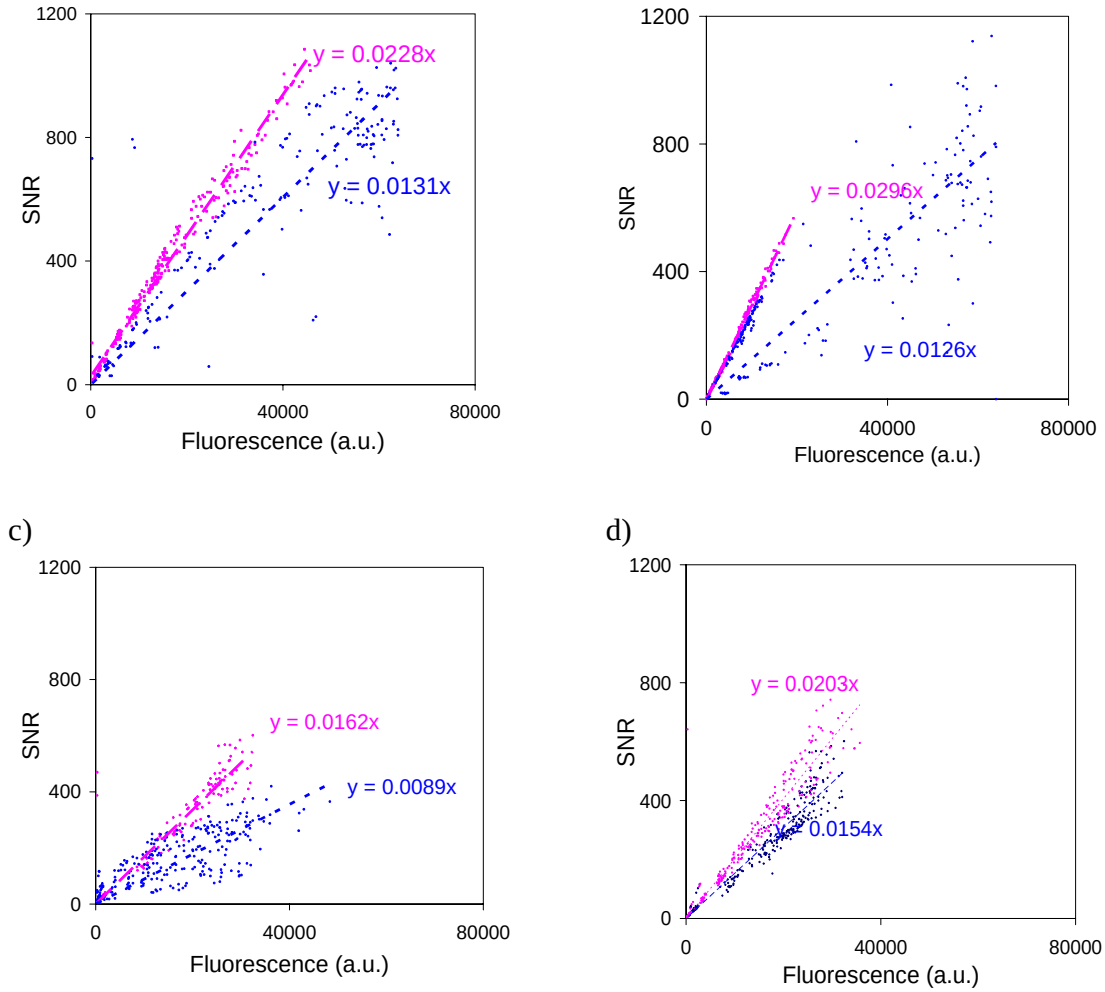


Figure 2 Plot of SNR vs fluorescence (a.u.) for Experiment 1: a) PU2, b) PU8, c) PVA and d) HydroGel scanned with confocal (■) and nonconfocal scanner (■).

A narrow distribution of data points and steep curve slope, as shown in Figure 2 a) for the non-confocal scanning mode, indicate a homogenous BG at a low background level for PU2. Variation of BG is higher for all surfaces when scanning confocally. For PU2, PU8 and PVA, the curves show good results with both scanning modes, with non-confocal scanning being superior with respect to BG influence. The non-confocal scanner detects fluorescence from different planes, which is why the fluorescence results are influenced by BG as well as by neighboring spots. Confocal scanning, detecting only from a single plane, results in weaker signals; this is another indication that confocal scanner is the best for more planar, thin surfaces or surfaces suffering from high BG. In the case of the HydroGel slide (Fig. 2 d), the curves are almost overlapping, indicating that SNR and BG influence is of the same magnitude regardless of the scanning mode, which is also true for the previously described loading curves. A possible explanation is that antibody adsorption and distribution within the spots on HydroGel are more even and more homogeneous, resulting in reduced spot roughness. Reduced spot roughness favors confocal scanning, since it allows the surface to be kept in focus during the scanning process at all times [15]. In accordance with this assumption, spot roughness is highest on PU8 (Fig. 2 b), and this is also reflected in the high standard deviations of the fluorescence intensity values and the high coefficient of variation (see Table 1).

Intra- and interexperiment variation

- Spot-to-spot variation

Reproducibility of the on-chip immunoassay was determined using the procedure outlined in the following. For analysis, the slides were divided into three groups: two groups were processed on the same day with target that was freshly prepared for each group (Experiment 1, Experiment 2), whereas the third group was processed 2 days later (Experiment 3). The coefficient of variation (CV) was calculated from the fluorescence signals from 18 spots (2 slides per slide type, 3 subarrays per slide and spots in triplicate) from single probe concentrations as well as averaged over all concentrations. As seen in Table 1, the % CV varies depending on the scanning mode: PU2, PU8 and Hydrogel showed generally improved reproducibility when using the non-confocal scanner. Raw data from PVA slides revealed lower CVs with data derived from confocal scanning, while normalized data in contrast is more reproducible with non-confocal scanning. Yet it needs to be pointed out that data reproducibility can be also affected by spotted protein concentration. This is true for PU8 and HydroGel slides regardless of the scanning mode used. At the lowest spotted IgG concentration, PU8 surfaces showed the lowest CV by far among the surfaces tested, whereas reproducibility was poorest at the highest

concentration. This correlates well with the binding reproducibility behavior shown by glutathione S-transferase (GST) – green fluorescent protein (GFP) on acrylamide gel pads as reported in [11]. This might be due to increasing spot roughness as a result of increased surface coverage (homogeneous, fully covered spots at any concentration, but thicker spots and increasing amount of IgG within the spot) as reported for microcontact-printed IgG [18]. Quite the contrary was seen with Hydrogel slides: CV increased from about 40% at the lowest probe concentration to about 20% at the highest. Similar results were obtained by Cretich et al. [12], who reported improved reproducibility for highly immobilized rabbit IgG concentrations. The poor reproducibility at low IgG concentrations may be due to incomplete coverage of the spot area with IgG. However, the spot area is filled up with increasing IgG concentration, thereby decreasing spot roughness and improving assay reproducibility. The other surfaces tested exhibit the same high degree of reproducibility (CV<25%) at all spotted protein concentrations.

The situation is different when globally normalized data is processed instead of raw data. Global normalizing results in more evenly distributed signals. This is true, for example, with PU2, in which case the mean CV for experiment 1 was 14, 9 for experiment 2 and 35 for experiment 3; an improvement was achieved as compared with non-normalized data.

Table 1 Inter- and intraexperimental variabilty (% CV) of the tested hydrogel surfaces

		% CV							
		PU2		**PU8**		**PVA**		**HydroGel**	
	Signal (a.u.) (raw data)	intra	inter	intra	inter	intra	inter	intra	inter
Over all spotted IgG concentrations	confocal	19	30	48	28	17	50	21	20
	non confocal	14	29	37	8	24	59	20	12
	Signal (a.u.) (normalized)								
	confocal	14	23	23	28	13	32	15	11
	non confocal	12	18	19	7	12	29	16	24
At 0.2 mg/mL Spotted IgG	**Signal (a.u.) (raw data)**								
	confocal	18	33	61	37	22	48	19	63
	non confocal	16	36	53	21	29	73	15	10
	Signal (a.u.) (normalized)								
	confocal	16	44	23	19	13	59	9	38
	non confocal	11	25	19	38	13	37	10	30

- Processing data from different PMTs

In a common microarray experiment, different kinds of slides require different optimum photomultiplier tube (PMT) settings, and this makes difficult a comparison of results obtained from different slides and experiments. In DNA chip technology this problem is solved by using a control for data normalization (e.g. external reference RNAs - spikes [19] or invariant genes for the expression studies [20]). Due to the complexity of proteins and the specificity of protein assays, this approach is

not used with such arrays. Instead, global normalization of data is suggested for the comparison of fluorescence signals deriving from slides scanned at different PMTs. Global normalization was achieved by dividing the (background-corrected) fluorescence signal values obtained within one slide by the mean fluorescence value for all spots present on the slide. The spots flagged as bad by the software were excluded from calculations. The mean globally normalized signal value for one probe was calculated from 18 spots (2 slides per slide type, 3 subarrays on each slide and spots in triplicate). As shown in Fig. 3, fluorescence signals vary by more than 100% when scanning the slides at different PMTs; even a change of 5% in PMT intensity produces at least twice as stronger signals. Examination of the PU2 calibration curves reveals that the curves of globally normalized data are closely overlapping regardless of PMT. Global normalization therefore is an effective method for comparing different data sets measured at PMTs that vary due to signal variation or from different experiments using the same type of chip surface. This outcome is the same for globally normalized data coming from the confocal and non-confocal scanners. However, when using a variety of chip surfaces, this approach is attractive only for very low probe concentrations (data not shown). As a consequence, to compare results derived from different types of slides methods, such as global normalization cannot be used and slides comparison should be done using fluorescence signals or signal-to-noise ratios.

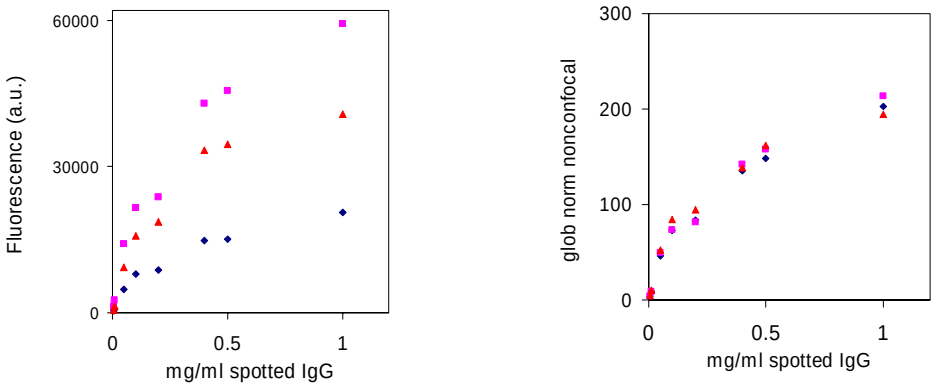


Figure 3 Global normalized data and fluorescence signals obtained for PU2 slides scanned at 3 different PMTs: ♦500, ■550 and ▲600 V.

Circularity

A critical feature of high quality substrates for microarrays is good spot morphology. An ideal substrate exhibits regularly shaped spots, with fluorescence intensities distributed uniformly throughout the spot and without features such as tails or a donut shape. Because of irregularities in shape, coverage and distribution of probe molecules as well as background pixels being included in the spot or foreground intensities being classified as background, erroneous signal intensities can result.

Nevertheless, even the best surface chemistry cannot warrant ideal spot morphology. The crucial role

of image segmentation in downstream analysis has been outlined previously [21]. Algorithms for image analysis have an immense impact on both accurate signal quantification and reproducibility between spots, which is essential for data interpretation. Petrov et al. [22] compared circular, i.e. spatial, segmentation and hybrid segmentation based on spatial and intensity information and found the latter method to be superior when dealing with contaminated spots.

Methods for classifying spot morphology include: the composite quality score introduced by Wang et al. [23]; the spot-related coefficient of variation [24]; the mean-median correlation [25]; and circularity [26]. Up to now, not much attention has been paid to the issue of spot circularity in protein arrays. Most of the research on image analysis [27] and data quality is done in the DNA micorarray field [22, 28]. As protein arrays are used not only for the qualification but also for the quantification of analyte, and furthermore because hydrogel slides especially show extremely homogeneous but nevertheless irregular spots, spot circularity is discussed in more detail in the following.

Consequences of circular versus irregular spot alignment

Fig. 4 presents the circularity (%) for spots of 0.005 to 1 mg/mL IgG on PU2, PU8, PVA and HydroGel slides. Best circularity was achieved for HydroGel surfaces (~92%), whereas PU2 and PVA slides revealed similar values (~89%). The lowest spotted concentrations were not considered as circularity drops with decreasing concentration for PVA and HydroGel slides. Exactly the opposite is the case with PU8 surfaces which showed improved circularity at very low concentration. This might be due to the fact that PU8 has low loading capacity (see Fig. 1) and at higher concentration (>0.05 mg/mL) the overload of spotted IgG causes loss in spot homogeneity and spot shape resulting in irregular spots with increased roughness. The principle of fitting circular versus irregular features is shown in Fig. 5. For surfaces giving nearly perfectly round spots without tails, the difference in fitting the spots with either of the modes seems to be neglectable, but the positive effect on reproducibility is obvious. In Table 2 the variabiltiy of signals, and in Fig. 6 the signal intensities, derived from circular and irregular alignment are compared. The percentage of change of CVs and signals were calculated by setting the CV and signal of circular alignment to 100%. Negative values in Table 2 indicate that circular alignment gave lower variability. Negative values in

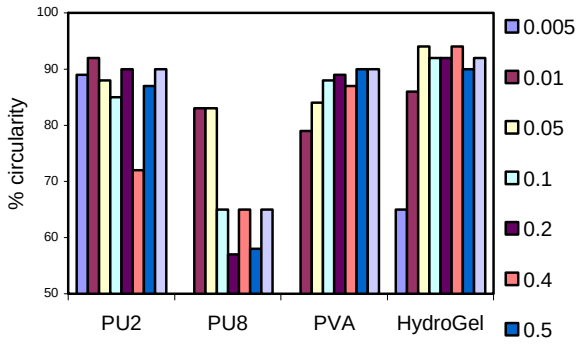
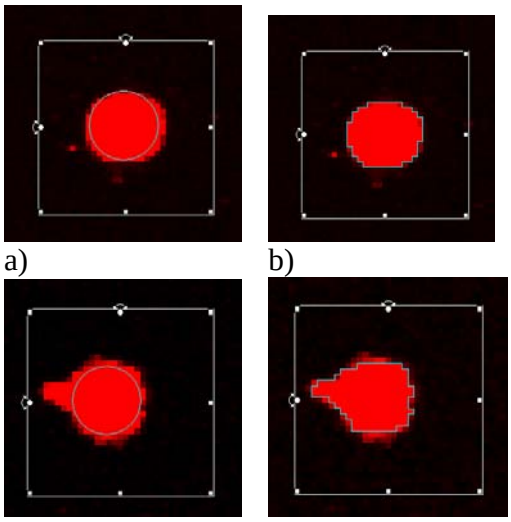


Figure 4 % circularity at various probe concentrations using PU2, PU8, PVA and HydroGel surfaces.



a) b) c) d)

Figure 5 Fitting the grid to the spots with circular mode(a and c) and irregular mode(b and d). Circularity for a and b: 95%, for c and d: 67%

Fig. 6 stand for higher signals applying circular alignment. For PVA and Hydrogel the inter-experiment variation (CV) differs 24% and 16% on average, whereas the signal change is only 5 and 4 %. The circularity of spots on PU8 is low (57-83%), irregular spot alignment improves the reproducibility of data tremendously. The irregular feature mode also allows to improve the reproducibility of results of surfaces that actually presented good spot circularity as PVA - obviously in the case of more round spots still not all pixels are taken for the mean fluorescence calculation of spot signal (see Fig. 5 a) and b) fitting).

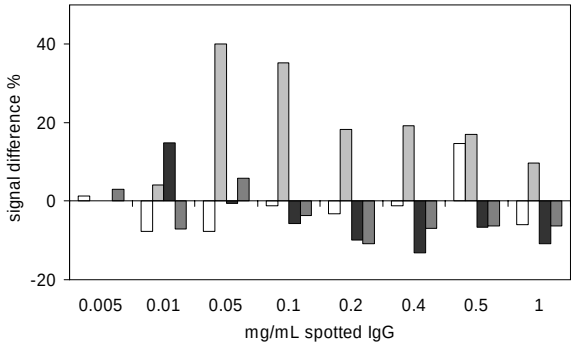


Figure 6 Difference in signal intensities (%) between circular and irregular alignment. Circular alignment was set to 100% for ☐PU2, ▒PU8, ■PVA and ▒ HydroGel

Table 2 Coefficient of Variation (% CV) and its difference (Δ) in % with circular and irregular alignment.

		% CV							
		PU2		**PU8**		**PVA**		**HydroGel**	
Over all spotted IgG concentrations	**Signal (a.u.) (raw data)**	intra	inter	intra	inter	intra	inter	intra	inter
	confocal	19	30	48	28	17	50	21	20
	non confocal	14	29	37	8	24	59	20	12
	Signal (a.u.) (normalized)								
	confocal	14	23	23	28	13	32	15	11
	non confocal	12	18	19	7	12	29	16	24
At 0.2 mg/mL Spotted IgG	**Signal (a.u.) (raw data)**								
	confocal	18	33	61	37	22	48	19	63
	non confocal	16	36	53	21	29	73	15	10
	Signal (a.u.) (normalized)								
	confocal	16	44	23	19	13	59	9	38
	non confocal	11	25	19	38	13	37	10	30

When irregularities exist, the differences are more obvious: irregular spots will result in less circularity (67%) (Figure 5 c and d) and thus a difference in signals.

Surface quality and circularity

Herein, the spot circularity was calculated for all spotted concentrations of IgG in 1x PBS (pH 7.2) from 9 replicates (1 slide per slide type, 3 subarrays on each slide and spots in triplicate). The spot circularity was calculated from the mean value of the circularity data given by the Genepix 6.0 software. Spots of maximum circularity are referred to as 100%.

The correlation of mean and median signal intensity was calculated for each spot in an array by dividing the smaller of the mean or median by the larger. Tran et al. [25] suggested a correlation between mean and median of pixel intensities as a measure for spot quality.

Fig. 7 shows the combination of both measures. High quality spots show a mean-median correlation close to 1, and circularity close to 100%. The more spots fulfill those criteria, gathering in the upper right corner of the graph, the higher the spot quality on the respective surface. PVA shows the highest values for both measures, PU2 has some outliers with lower circularity, Hydrogel shows both ouliers for circularity and for mean-median correlation, while most of the spots on PU8 distribute over the whole range, indicating low spot quality, which is in accordance with low reproducibility data.

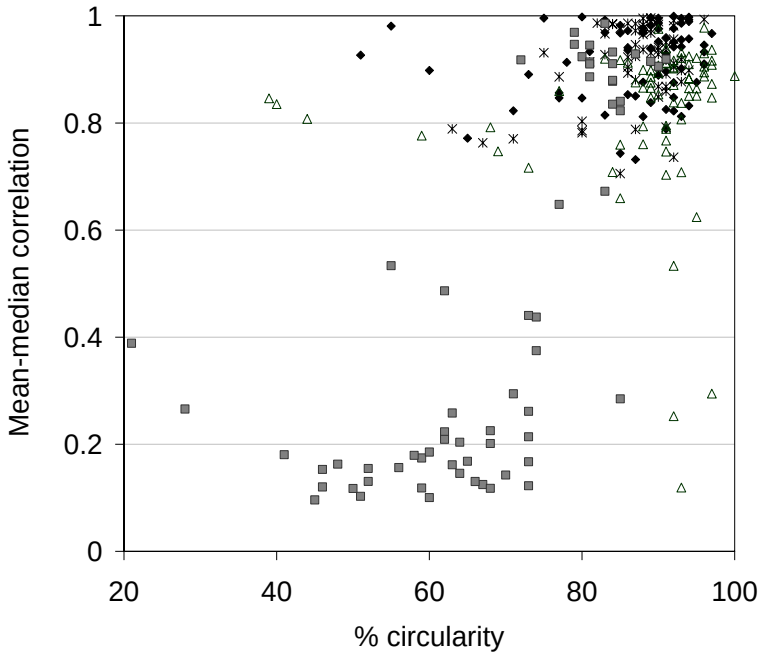


Figure 7 Mean-median correlation (irregular aligned spot) plotted against % circularity of individual spots for ◆ PU2, ■ PU8, ✳ PVA and △ HydroGel

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Final Conclusions

Protein chip technologies have already created new opportunities in medical and environmental diagnostics. Key attributes include parallelism, miniaturization, functional integration, and process automation. To date the technologies are mainly restricted to the research lab due to their high cost, difficult handling procedures and the lack of approved standards. Although small markets are already available beyond the research lab, significant improvements in sensitivity, specificity and high throughput capabilities have to be made to move these technologies from the bench into clinic and analysis laboratories. In order to facilitate widespread use of the protein chip as an analytical tool, certain technical hurdles, such as insufficent sensitivity and unreliability of results still have to be overcome.

Thus, one of the major challenges to further developing protein chip technology is to strengthen the signals produced by probe-target interaction, especially in cases of low probe and target concentrations.

Several approaches have been reported for achieving stronger signals and lower detection limits, one of the most important is the development of more efficient surface chemistries.

Most existing biochips consist of a planar, non-porous epoxy-, aldehyde- or amino-modified polymer layer that is most suitable for immobilization of DNA, but not appropriate for proteins. However, measuring RNA levels does not always give a complete or accurate description of a biological system. Because proteins mediate nearly all cellular activities, the objective of molecular diagnostics is to provide information at the protein level allowing for more-accurate detection and diagnosis of disease.

Though protein chip technology emerges extensively from DNA chips, there are major differences in the physical properties and stability of soluble proteins and DNA at interfaces to be considered:

- Nucleic acid arrays are more robust than protein arrays, partly as a result of intrinsic DNA stability and structure as well as sample preparation in assay designs.
- In contrast to proteins isolated DNA or RNA can be easily amplified at high purity levels.

- Dissolved ssDNA or RNA is homogeneously negatively charged and hydrated, and thus can be conveniently immobilized on cationic surfaces (e.g. poly-L-lysine or aminosilane). By contrast, proteins have tremendous structural variability (metastable native folded states, long- and short-range forces) that dictate and complicate their interactions with biochip surfaces. Very often proteins unfold or denature.
- In contrast to DNA, proteins exhibit strong non-specific surface activity, referred to as non-specific adsorption.
- Though DNA sequences differ from each other, they generally have similar chemical properties and therefore similar affinities. Two proteins, however, may have different size, charge and stability. Thus, the affinity of protein-protein interactions may be extremely different and difficult to control.

Major requirements for protein chip surfaces therefore are:

- maintainance of protein´s structure and activity
- low non-specific adsorption
- high loading capacity

Hydrogels which provide a three-dimensional, solution-like microenvironment fulfill these demands, since they allow protein activity to be maintained and show good spot morphology as well as excellent immobilization capacity. However, hydrogels often have the disadvantages of low mechanical stability and long processing times due to thicker layers (up to 30 µm) and increased diffusion limits. The preparation of some hydrogels, for instance polyacrylamide, is particularly time-consuming.

In this thesis 3D hydrogel surfaces based on polyurethane and poly(vinyl alcohol) have been developed which are only 200 nm thick, are mechanically stable and provide excellent immobilization capacity. Both surface and assay parameters have been optimized and evaluated using commercial hydrogel surfaces. In order to better understand the interaction between chip surface and protein the assay performance is correlated with chip surface parameters, such as water content and expansion, mechanical stability, hydrophilicity, thickness and surface topography. The commercial chip surfaces ARChip Epoxy, Nexterion slide H and HydroGel are used as a reference. A strong correlation between assay sensitivity and physical surface parameters is only found for various hydrogels of the same chemical composition, in which cases assay sensitivity increases with decreasing hydrogel concentration as well as decreasing roughness, water content and expansion. However, as is the case with all hydrogels tested, more hydrophobic layers with low water content are more highly reproducible from one measurement to another.

In general layer thickness has no effect on assay performance. However, the layer thickness does determine assay sensitivity, inasmuch as too low a concentration produces incomplete and inhomogeneous coverage of the slide with hydrogel, whereas too high a concentration results in increased BG noise, reducing the signal-to-noise ratio and thus assay sensitivity.

In most cases an increase in immobilization capacity evokes stronger fluorescence signals. However, the increase in immobilization capacity is not directly proportional to the increase in signal intensity, which is usually smaller.

When comparing material, surface and assay parameters of chip surfaces correlation within parameters is found only for hydrogels of the same origin: spot diameter and roughness increase with increasing water content and expansion, whereas signal intensity and coating thickness decrease. Data reproducibility deteriorates with increasing water content and expansion. This can be understood in that at high hydrogel expansion more peripheral chains penetrate into the solution giving rise to a more diffuse interface, and as a consequence rougher surface which leads to less reproducible spots and data. By contrast, when different hydrogels of different chemistry and modifications are compared, no correlation between surface properties and assay performance is found, except for the assay reproducibility, showing that more hydrophobic layers with low water content are more highly reproducible between different measurements.

It is furthermore demonstrated that, if even probe spots are provided, fluorescence intensities measured at the same PMT gain are similar for confocal and non-confocal scanning modes. Uneven probe spots, however, cause reduced fluorescence with confocal scanners as well as greater spot-to-spot variation and a higher degree of intra- and inter-experimental variability. By using irregular instead of circular spot alignment, reproducibility is improved for good- and bad-quality spots; in the latter case by up to three times as much. In addition, circularity can be used together with the mean-median correlation of pixel intensities as a quality measure.

Curriculum Vitae

Personal details

Name:	**Katarzyna DERWINSKA**
Date of birth:	27 June 1978
Place of birth:	Warsaw
Nationality :	Polish
Maiden name:	Sowinska

Education

2004-2007	Vienna University of Technology
	PhD thesis for Doctor of technical sciences
1997-2002:	Warsaw University, Chemistry Department, Poland
	Master thesis
1993-1997:	Stefan Zeromski High School in Zyrardow, Poland

Professional experience

March'04 - present	PhD research in Austrian Research Centers in Seibersdorf
	Profile: Hydrogel surface development for microarray approach.
Aug '02 – March'04	Young Research Staff, Industrial Chemistry Research Institute, Detergents Department, Warsaw, Poland
	Profile: Biodegradation of surfactants
March'00 – May'00:	Socrates – Erasmus Fellowship in Coimbra (Portugal) ended with thesis: 'Flow injection analysis of L-glutamic acid with electrochemical detection'

Posters and oral presentations:

2006	Diplomanten/Dissertantenseminar des Bereiches Biogenetics Natural Resources, Oral presentation 3D hydrogel surfaces in on-chip immunoassays for C-reactive protein
2006	Nanoparticles 2006, May 13-16 Orlando, Florida, poster presentation: Hydrogels and Nanoparticles in Microarrays for food control
2005	7th Austrian Polymer Meeting, July4-6, Graz, Austria, Oral presentation: 3-dimentional polyurethane-based hydrogels for microarrays
2004	Diplomanten/Dissertantenseminar des Bereiches Biogenetics Natural Resources, poster presentation: 3-dimensional polyurethane-based hydrogels for protein arrays
2002	9th ESEAC conference, June 9-13, Cracow, poster presentation: „Development of Biological Sensor Based on Electrocatalytic Detection of Hydrogen Peroxide at Prussian Blue/Polymer Composite Film"

Publications

2003	Application of Prussian Blue Based Composite Film with Functionalized Organic Polymer to Construction of Enzymatic Glucose Biosensor; Electroanalysis, 2003, 15, 1843-1849

Courses

2005	VII. Annual Linz Winter Workshop on „Single molecule techniques in biophysics and drug discovery", February 4-7
2004	ASCOS 2004 *(Advanced Study Course on Optical Chemical Sensors)*, July 30 – August 10, Erice, Italy