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SARI VIITALA

NEW APPLICATIONS FOR IMMUNOASSAY BASED PROTEIN MICROARRAYS

New Applications for Immunoassay Based Protein Microarrays

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ABSTRACT

The protein microarray (protein-chip) is a comprehensively miniaturized and totally parallelized assay system that contains a large number of different molecules, such as proteins, peptides or DNA strains, which have been immobilized as an array of microspots which are formatted onto a small area of a solid support. These well-defined arrays represent a versatile platform, which allows a parallel analysis of hundreds or even thousands of proteins or other molecules while requiring only tiny amounts of sample and reagent volumes. Microscale assays of biomolecules are recognized as potentially powerful tools for medical investigations and research since they enable the screening of a wide variety of biologically important binding events in a parallel and high-throughput fashion.

Since the protein microarrays are solid-phase assays, one major issue for successful microarray fabrication is the surface activation of the solid support on which the molecules are immobilized. In this study, a new glutaraldehyde polymerization based method was examined for the chemical activation of both plastic and glass surfaces. Various kinds of biomolecules (antibodies, proteins, peptides, whole viruses and oligonucleotides) immobilized as array formats onto the activated surfaces were used in immunoassay based analyses to evaluate the functionality of the new surface activation method.

In the analyses, the arrays printed onto the bottoms of 96-well plate wells were utilized in serological studies to: 1) detect antibodies, 2) quantify antigens and 3) evaluate potential antigens for diagnosing microbial infections. In addition, a large set of mutated oligonucleotide sequences arrayed onto microscope glass slides were used to study the effects of DNA mutations on the binding affinity of transcription factor proteins.

The results obtained with these protein microarray assays displayed an excellent correlation to the results from reference methods used in a routine diagnostic and research laboratories. The use of the polymerized glutaraldehyde to the chemical activation of the surfaces represents a promising method for the immobilization of various molecules onto solid supports. This simple and low-cost new surface activation method is compatible with different kind of solid supports and can be readily exploited in a wide variety of protein microarray applications.

National Library of Medicine Classification: QU 25, QU 57, QW 525.5.I3
Medical Subject Headings: Serology/methods; Immunoassay; Microarray Analysis; Protein Array Analysis; Surface Properties; Glutaral; Polymerization; Antibodies/analysis; Antigens/analysis; Infection/diagnosis; Oligonucleotide Array Sequence Analysis; DNA Mutational Analysis; Transcription Factors

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TIIVISTELMÄ

Proteiinisiru on kokonaan miniatyrisoitu ja täysin rinnakkaistettu laboratorioanalyysisysteemi, jossa kiinteälle alustalle on kiinnitetty suuri määrä molekyylejä pieniksi pisteiksi matriisimuotoon. Näiden molekyylimatriisien avulla on mahdollista tehdä yhtäaikaisesti proteiineille ja muille molekyyleille satoja tai jopa tuhansia rinnakkaisia analyysejä käyttäen vain pieniä näyte- ja reagenssimääriä. Nämä mikromittakaavaiset biomolekyylien määritysmenetelmät ovat nousemassa tehokkaiksi lääketieteen ja tutkimuksen työkaluiksi, sillä ne mahdollistavat monenlaisten biologisesti tärkeiden sitoutumistapahtumien rinnakkaisen ja tehokkaan analysoinnin.

Proteiinisirun valmistuksessa merkittävin asia sirun tarkoituksenmukaisen toimivuuden kannalta on molekyylien kiinnitys alustaan. Tässä tutkimuksessa käytettiin uutta glutaraldehydin polymerisaatioon perustuvaa menetelmää muovi- ja lasipintojen kemialliseen aktivointiin molekyylien kiinnittämistä varten. Menetelmän toimivuus arvioitiin kiinnittämällä aktivoituun pintaan erilaisia molekyylejä ja testaamalla niiden tarkoituksenmukainen toiminta immunomääritykseen perustuvien analyysien avulla.

Analyyseissä 96-kuoppalevyn kuoppien pohjiin printattuja molekyylimatriiseja käytettiin serologisissa tutkimuksissa havaitsemaan vasta-aineita, mittaamaan antigeenipitoisuuksia ja arvioimaan potentiaalisia antigeeneja mikrobi-infektioiden diagnosoimiseksi. Lisäksi käytettiin mikroskooppilasilevylle matriisimuotoon kiinnitettyjä mutatoitujen oligonukleotidisekvenssien sarjoja analyyseissä, joissa tutkittiin DNA-mutaatioiden vaikutuksia säätelytekijäproteiinien sitoutumisvoimakkuuteen kohdegeeniinsä.

Proteiinisiruilla tehtyjen analyysien tulokset korreloivat vahvasti referenssimenetelmillä saatujen tulosten kanssa. Polymerisoidun glutaraldehydin käyttö pintojen kemialliseen aktivointiin on lupaava menetelmä erilaisten molekyylien kiinnittämiseksi kiinteälle alustalle. Tämä yksinkertainen ja edullinen pinta-aktivointimenetelmä sopii erilaisille alustoille ja on helppo hyödyntää monenlaisissa eri proteiinisirusovelluksissa.

Luokitus: QU 25, QU 57, QW 525.5.I3
Yleinen suomalainen asiasanasto: serologia; immunokemia; mikroanalyysi; proteiinit; pintakemia; pintakäsittely; aktivointi; polymerointi; vasta-aineet; antigeenit; infektiot; diagnoosi; diagnostiikka; oligonukleotidit; DNA-analyysi; mutaatiot; transkriptiotekijät

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Science is not only work you do to make a living, it is a way of life. The working day in the world of science does not just stop when you leave the workplace. It is always with you, wherever you are and wherever you go. It takes a lot, but it will give you much more, sometimes at the expense of family and friends. Thus I am deeply thankful to my family for their understanding, love, sympathy and support. I am forever grateful to my mother Eija and to my brother Marko, who with their unlimited love, taught me to believe in myself. I am thankful also to my beloved fiancé Ukko for his endless and unselfish love and support. Finally and most importantly, I want to express my deepest love and gratitude to my dearest ones: my daughters Sanna, Tessa and Nitta, who bring so much love and joy into my life. I promise that we will spend more time together with all of you after finishing this scientific marathon and I promise not to undertake another thesis!

Kuopio, November 2016 Sari Viitala

List of the original publications

This dissertation is based on the following original publications:

I Viitala SM, Jääskeläinen AJ, Kelo E, Sirola H, Moilanen K, Suni J, Vaheri A, Vapalahti O and Närvänen A. Surface-activated microtiter-plate microarray for simultaneous CRP quantification and viral antibody detection. *Diagnostic microbiology and infectious disease, vol. 75, no. 2, pp. 174-179, 2013.*

II Jääskeläinen AJ, Viitala SM, Kurkela S, Hepojoki S, Sillanpää H, Kallio-Kokko H, Bergström T, Suni J, Närvänen A, Vapalahti O and Vaheri A. Performance of a multiplexed serological microarray for the detection of antibodies against central nervous system pathogens. *Journal of microbiological methods, vol. 100, pp. 27-31, 2014.*

III Kuosmanen SM, Viitala S, Laitinen T, Peräkylä M, Pölönen P, Kansanen E, Leinonen H, Raju S, Wienecke-Baldacchino A, Närvänen A, Poso A, Heinäniemi M, Heikkinen S and Levonen AL. The Effects of Sequence Variation on Genome-wide NRF2 Binding-New Target Genes and Regulatory SNPs. *Nucleic acids research, vol. 44, no. 4, pp. 1760-1775, 2016.*

Contents

Abbreviations

2-D	Two dimensional
3-D	Three dimensional
APTES	(3-Aminopropyl)triethoxy-silane
BSA	Bovine serum albumin
CEMA	2-Cyanoethyl methacrylate
ChIP-seq	Chromatin immune-precipitation coupled with deep sequencing
CNS	Central nervous system
CRP	C-reactive protein
DMA	N,N-dimethylacrylamide
EDC	1-Ethyl-3-(3-dimethyl-aminopropyl)carbodiimide
EDMA	Ethylene dimethacrylate
EGMA	(ethylene glycol)methacrylate
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
GA	Glutaraldehyde
GDMA	Glycerol dimethacrylate
GMA	Glycidyl methacrylate
HEMA	2-Hydroxyethyl methacrylate
HIV-1	Human immunodeficiency virus type 1
HPIEAA	N-hydroxyphthalimide ester of acrylic acid
HSV-1	Herpes simplex virus 1
HSV-2	Herpes simplex virus 2
IgG	Immunoglobulin G
LC	Liquid chromatography
MAPS	N,N-acryloyloxysuccinimide
MS	Mass spectrometry
NAS	3-(Trimethoxysilyl)propyl methacrylate
NHS	N-hydroxy succinimide
NRF2	Nuclear factor, erythroid 2 like 2
OEGMA	Oligo(ethylene glycol) methacrylate
PBS	Phosphate buffered saline
PBST	Tween 20 in phosphate buffered saline
PC	Polycarbonate
PEG	Polyethylene glycol
PEG-DA	Poly(ethylene glycol) diacrylate
PET	Polyethylene terephthalate
PIII	Plasma ion immersion implantation
PMMA	Poly(methyl methacrylate)
POC	Point-of-care
PS	Polystyrene
TCF7L2	Transcription factor 7-like 2
TF	Transcription factor
PUUV	Puumala virus
SNP	Single nucleotide polymorphism
SINV	Sindbis virus
TIFF	Tagged image file format
UV	Ultraviolet
VZV	Varicella-zoster virus

1 Introduction

Every measurable biomolecule, which can be used as indicators for the disease or biological condition, is called a biomarker. Several biomarkers need to be often assayed in order to make a precise disease diagnosis or to determine status of the disease and prognosis as well as to monitore the responses to therapies, drugs and vaccinations. Thus the parallel assays of the multiple biomarkers have many potential applicationsin medicine and research. These kind of applications are i.e. allergen chips (Santosa et al. 2015, Feyzkhanova et al. 2014), arrays developed to discovery new potential biomarkers (Matarraz et al. 2011) or to screen immune responses against different antigens (Henjes et al. 2014, Hong et al. 2015, Madi et al. 2015). In addition to their exploitation in high throughput screening methods in medical diagnostics, the multiple analyte assays also have many applications in basic research. The background to several diseases remains to be clarified and there are even many physiological and biological mechanisms which are not fully understood and would benefit from parallel screening. By clarifying the interactions between different pathways and the expressions of the various molecules could provide not only a better understanding about disease susceptibility, occurrence and progression, but also be beneficial in the development of the new vaccines, drugs and therapies (Henjes et al. 2014, Matarraz et al. 2011, Natesan and Ulrich 2010).

The protein microarray is a miniature platform which which allows the study of a large number of the molecule interactions. It is a versatile tool for screening, identifying and analysing many molecules and samples simultaneously. Nowadays, protein microarrays are being widely used to measure the presence or absence of certain proteins or other molecules of interest as well as their concentrations from complex samples; not only can this make it possible to identify and study the protein structure but one can also investigate their interactions with other proteins or molecules (Desbien et al. 2013, Feron et al. 2013, Köhler and Seitz 2012, Ellington et al. 2010, Domnanich et al. 2009, Hartmann et al. 2009, Beare et al. 2008).

Traditionally, studies of proteins and their interactions with other proteins or molecules have been performed with enzyme immunoassay (EIA, ELISA Lequin 2005), gel electrophoresis (El Khoury et al. 2010, Issaq and Veenstra 2008, Kaushansky et al. 2008), liquid chromatographic (LC Denoroy et al. 2013, Hage et al. 2012) and mass spectrometric (MS Lee et al. 2013, Kwak et al. 2014, Kaushansky et al. 2008) techniques. These conventional methods are usually laborious and time-consuming, and often require relatively large amounts of biological products, such as serum samples or cell extracts, and other reagents, required for assay performance. These features limit their application for mass screening. The miniaturization of protein studies by the protein microarray introduces several advantages - not only does it become possible to analyse a high number of parallel samples, but one can achieve a reduction in the assay time, sample volume and reagent costs (Shi et al. 2016, Pratsch et al. 2014, Feron et al. 2013, Desbien et al. 2013, Sutandy et al. 2013, Köhler and Seitz 2012, Haab and Yue 2011, Kwak et al. 2014, Weinrich et al. 2009, Seurynck-Servoss et al. 2007).

The concept of protein microarray traditionally refers to a “protein-chip” on which the proteins are immobilized in an array format on the top of a planar solid surface and then one screens for the molecules of interest in the sample. Today, the protein studies using the microarray platforms are also being performed using other molecules, such as lectins

(Huang et al. 2014, Shinzaki et al. 2013) or peptides (Stephenson et al. 2015, De-Simone et al. 2014), immobilized onto a solid support. In fact, most of the microarray formats which are available for studying proteins are still usually called protein microarrays, although they are really not the same kind of protein chips as originally described (Hartmann et al. 2009).

The protein microarray was originally and typically still is today an immunochemical method based on the specific interactions between the antigens and the antibodies to which they bind. Any molecule which is known to bind to a specific antibody can act as antigen. In the basic immunoassay based protein microarray method, one of the antigen-antibody pairs is immobilized onto the solid support as a capture agent, which is then probed by the other member of the pair. Subsequently, the captured molecules or complexes are detected using the labeled molecules, usually the antibodies. Depending on whether it is the antigen or the antibody which has been immobilized as the capture agent, the assays are called either antigen or as antibody arrays. It is also possible that both molecule types are incorporated into the same arrays (Buchegger and Preininger 2014, Pratsch et al. 2014, Sutandy et al. 2013, Sokolove et al. 2012, Ardizzoni et al. 2011, Sanchez-Carbayo 2010, Borrebaeck and Wingren 2009, Burgess et al. 2008).

The immobilization of the capture proteins (or the other capture agents, such as peptides) onto the solid support is a major factor in the successful protein microarray fabrication. This is because the functionality of the entire assay is based on the ability of the arrayed agents to bind or capture their target molecule. A high binding capacity, as well as the prevention of protein denaturation, minimal non-specific adsorption and high signal-to-noise ratio, is all important issues in the immobilization and therefore these need to be assessed during surface fabrication. In addition, the surface fabrication method should be repeatable and result in the production of a stable surface (Lee et al. 2013, Köhler and Seitz 2012, Hartmann et al. 2009, Rusmini et al. 2007, Oh et al. 2006).

Since the surface functionality, including the immobilization of the protein, is crucial when developing a robust protein microarray platform, a large variety of the surface modifications methods have been developed. These range from the direct adsorption onto the planar surface to covalent binding onto a chemically activated complex of 3-D polymers. Although there are several platforms based on high quality slides with the pre-activated surfaces for the protein immobilization and even ready-to-use arrays with the pre-immobilized proteins commercially available (Santosa et al. 2015, Seurynck-Servoss et al. 2007), many researchers still fabricate their own surfaces and arrays. Thus, new coating methods based on well-defined protocols are still needed in protein microarray technology and applications (Shi et al. 2016, Hu et al. 2011, Rabe et al. 2011, Rusmini et al. 2007, Oh et al. 2006).

This thesis focuses on protein microarray technology, especially on the development of novel protein microarray platforms for serology, antigen detection and for studying protein-DNA interactions.

2 History of Protein Microarrays

In 1989, Roger Ekins and his co-workers described the fundamental principle behind microarray technology (Ekins and Chu 1999, Ekins 1989). In these early microarrays, antibodies which had been "printed" robotically in an array format onto a solid support, were used to screen drugs and hormones. Their ambient analyte theory represented the start of protein microarray technology development. While the research group of the Roger Ekins is often considered as the founder of this technology, there are also a few earlier publications which can be considered to be describing microarrays.

The first microspot-based protein immunoassays were developed in the early sixties by Feinberg and his colleagues (Feinberg and Wheeler 1963, Feinberg 1961). This group used autoantigens immobilized in microspots on a solid support to screen for autoantibodies from human serum samples. The concept of protein microarrays was first introduced in the 1980s by Chang (Chang 1983) who used arranged immobilized antibodies as orderly spots on a solid support to allow the isolation of specific cells.

Although the first microarray systems were developed with proteins, a much more rapid development in this technology occurred with the appearance of DNA microarrays. This is due to the easier manufacturing, handling and storing associated with the DNA based microarrays, partly because of the more homogenous physicochemical properties of nucleotides in comparison with proteins (Köhler and Seitz 2012, Hartmann et al. 2009).

Microarrays have been used widely for gene expression profiling; the platforms are generally called gene chips or DNA chips. DNA microarrays are based on the ability of the probe to bind a complementary DNA strand from the analyte. The complementary gene fragments from human, animal, plant or micro-organism on a single chip enables the specific analysis and quantification of gene expression from any cell or tissue (Sutandy et al. 2013, Hartmann et al. 2009, Oh et al. 2006). The first DNA microarrays were described in 1995 by Schena et al. (Schena et al. 1995). Although DNA microarrays provide information about the gene expression profiles, including parallel analyses of DNA sequences and genetic variation, they cannot be used directly to analyze the gene products, proteins, or their interactions. The measurement of the gene expression levels does not directly reflect the protein expression, since transcribed mRNA is not necessarily expressed proportionally as the corresponding protein. In addition, the post-translational modifications of proteins cannot be investigated with DNA microarrays (Lee et al. 2013, Sutandy et al. 2013, Zhu and Qian 2012, Hu et al. 2011, Hartmann et al. 2009, Rusmini et al. 2007).

3 Protein Microarray Assay

Numerous molecule configurations are available for conventional enzyme immunoassays (EIA) or enzyme-linked immunosorbent assays (ELISA) (Lequin 2005); most of these can also be incorporated into protein microarrays formats. Thus the immunoassay based protein microarrays can be considered as miniaturized EIA methods which can be combined with various detection methods. As well as the configurations, the assay steps, such as immobilization, bocking and washing, are basically the same in protein microarrays as in the EIA systems.

3.1 MOLECULAR CONFIGURATIONS

In the basic immunoassay based protein microarray technique, one of the antigen-antibody pairs is immobilized onto the solid support and this is probed by the other member of the pair, with the captured molecules being detected using labeled secondary antibodies (Figure 1, Madi et al. 2015, Ardizzoni et al. 2011, Gonzalez et al. 2011). Any molecule that can be detected by an antibody, can act as an antigen. A method, which uses the antibodies for both capturing and detection, is traditionally called a sandwich immunoassay since it is based on the antigen being "sandwiched" between the antibodies (Figure 1, (Gonzalez et al. 2011, Haab and Yue 2011, Lian et al. 2010, Domnanich et al. 2009); it requires the antibody recognition of different epitopes, the specific binding sites, in the protein. These kinds of systems are indirect assays since they involve a secondary detection method. The recognition of captured probes can also be made via direct detection using labeled probes which bind to the immobilized capture agents (Figure 1, Dobosz et al. 2015, Lee et al. 2013, Ardizzoni et al. 2011, Kwak et al. 2014). Depending on which of the antigen-antibody pairs was immobilized as the capture agent, the assays are either called antigen or as antibody arrays (Figure 1, Pratsch et al. 2014, Sutandy et al. 2013, Sokolove et al. 2012, Sanchez-Carbayo 2010). In addition, both of the antigens and the antibodies can be incorporated into the same arrays (Buchegger and Preininger 2014, Ardizzoni et al. 2011, Burgess et al. 2008).

If the antigen to be assayed is small in size or has only one epitope for antibody binding, it will be necessary to utilize competitive methods. These kinds of systems are performed either with the labeled antigen which competes with the unlabeled sample antigen for the binding sites of immobilized antibody (competitive direct antibody array, Figure 1, Dobosz et al. 2015) or the antibody is labeled and competes for the immobilized antigen and antigen in the sample (competitive direct antigen array, Figure 1, Dobosz et al. 2015). These types of systems are direct methods, but the indirect methods are also used in competition approaches (competitive indirect models, Figure 1, Hu et al. 2013, Desmet et al. 2012, Jia et al. 2012, Zuo et al. 2010). In the indirect methods, the formed antigen-antibody complex is detected via a secondary detection method, such as the avidin-biotin technique in the antibody assays (Jia et al. 2012) or labeled secondary antibodies in the antigen assays (Hu et al. 2013, Desmet et al. 2012).

The array quantification is performed by measuring the labels, such as enzymes. In non-competitive systems, the intensity of labels on the assayed array is directly proportional to the captured probe. Competitive assays give inversely proportional results for the bound probes, based on the competition between molecule of interest and corresponding labeled molecule; the greater the amount of the bound non-labeled probes, the smaller is the intensity of the label (Pratsch et al. 2014).

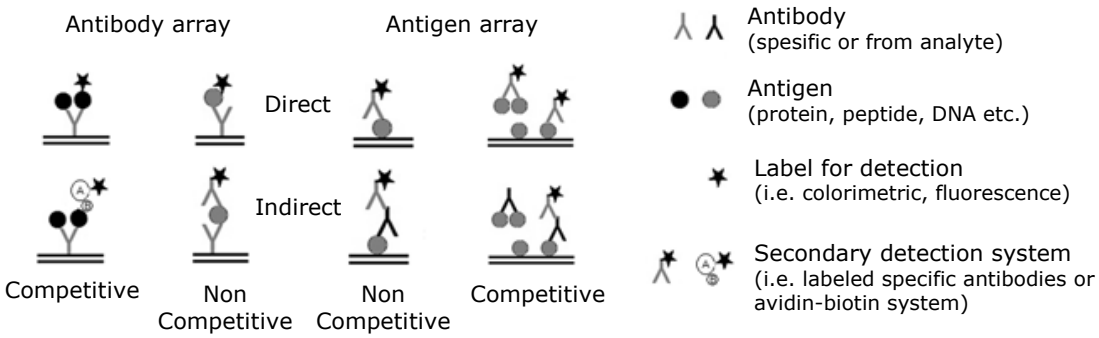

Figure 1. The basic molecule configurations of the immunoassay based protein microarray assays.

The most extensively used labels in the protein microarrays are enzymes (Hong et al. 2015, Desmet et al. 2012, Sauceda-Friebe et al. 2011) and fluorescent chemical compounds (Madi et al. 2015, Lee et al. 2013, Lu et al. 2012), but also other methods such as various detectable particles have been described (Dobosz et al. 2015, Ylihärsilä et al. 2015, Päkkilä et al. 2012, Lian et al. 2010). The biotin-avidin technique has been widely exploited as the secondary detection method. The probe is conjugated with biotin and the detection is performed using labeled avidin/streptavidin/neutravidin (Shi et al. 2016, Lee et al. 2013, Jia et al. 2012, Lu et al. 2012). Alternatively, biotin, linked to the secondary detection molecule, usually the antibody, is used to bind the labeled avidin/streptavidin (Shi et al. 2016, Selvarajah et al. 2014, Lee et al. 2013, Lian et al. 2010).

3.2 ASSAY PROTOCOL

Various steps are involved in the protocol of a protein microarray assay, depending of the molecule configuration being used in the assay. The general procedure of the assay experiment usually includes a few main steps (Figure 2). A set of capture agents is arrayed onto the solid support, and then the array is washed to remove the unbound capture agents and blocked to eliminate the unoccupied binding sites of the solid support. These are necessary steps before the probing, to minimize the unspecific binding of analytes or detection molecules and they help to achieve high signal-to-noise ratios. After these steps, the array is probed with an analyte containing the counterparts of the molecular recognition events being evaluated. Depending on the assay configuration, the probe is labeled or the assay may require the adoption of some secondary detection method. After the wet laboratory procedure, the assayed array is screened with an appropriate method, such as its evaluation in a microarray scanner or a confocal microscope, depending of the label being used. The images of detected arrays are subsequently analyzed with the appropriate software to measure the quantity of the signal intensity (Lee et al. 2013, Köhler and Seitz 2012, Hartmann et al. 2009).

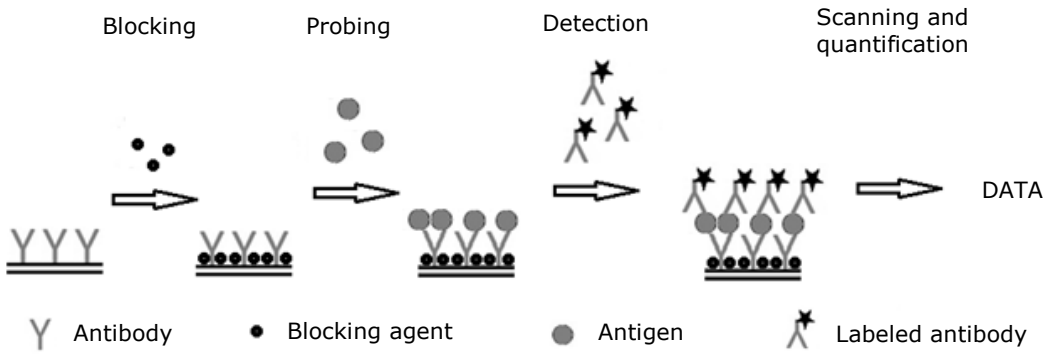

Figure 2. The sandwich assay as an example of the basic protocol of the protein microarray assay. The arrays are washed after every step to remove the unbound molecules.

3.3 TYPES AND APPLICATIONS

The protein microarray formats can be classified into two types: forward-phase and reversed-phase arrays, depending on which molecule of interest is involved in the assay; the capture or the probe. Furthermore, the assay formats can be divided into analytical and functional categories, depending on the assay being used. Analytical protein microarrays are mainly used to evaluate protein or other molecule expression levels, binding affinities and specificities whereas the functional protein microarrays are exploited to analyze protein activities, binding properties and post-translational modifications. The basic issues, such as array fabrication, the assay protocol and the detection methods, are mainly the same for both classes of microarray (Sutandy et al. 2013, Zhu and Qian 2012, Hu et al. 2011, Hartmann et al. 2009).

3.3.1 Forward-phase arrays

The forward-phase arrays use well-characterized molecules, with known target specificities, immobilized onto the solid support as capture agents. The arrays are probed with a water (Desmet et al. 2012) or complex sample solution such as serum (Madi et al. 2015, Ott et al. 2014, Desbien et al. 2013, Price et al. 2013), plasma (Rosen et al. 2011, Suwannasaen et al. 2011), milk (Madi et al. 2015, Lian et al. 2010), cerebrospinal (Rosen et al. 2011) or follicular (Ardizzoni et al. 2011) fluids, and cell lysate (Zeng et al. 2014) with the goal being to measure the presence, absence or concentration of the molecule of interest. The analysis of the captured specific molecules provides information about the expression levels of the protein or other molecule, such as toxins, as well as the binding affinities and specificities of the analyte molecules and thus the immunoassay based forward-phase protein microarrays are widely exploited for clinical diagnostic and research use.

3.3.2 Reversed-phase arrays

The most widely applied approach in the reversed-phase formats of the immunoassay based protein microarray assays is to use the antibodies present in serum or plasma samples to probe a large number of investigated proteins immobilized in the array format. The analysis of the resulting binding profile can help to identify new biomarker candidates for diagnostic and vaccination development (Fernandez et al. 2011, Crompton et al. 2010). The reversed-phase arrays also have other applications i.e. to screen for the specificity of

antibodies (Feron et al. 2013) and to study the post-translational modifications of proteins (Kwak et al. 2014, Lu 2012, Haab and Yue 2011).

4 Technology Exploited in Protein Based Microarrays

Standard equipment developed for the DNA microarray studies, such as array printers, scanners and software, are often suitable also for protein microarray production and analysis. Unfortunately, the immobilization methods used in the DNA microarrays are not still directly adaptable for protein microarrays. In contrast to DNA, proteins are a large group of heterogeneous molecules with very distinctive physicochemical properties, i.e. they are both chemically and structurally more complex than DNA molecules. Unlike the predictable sequence-specific hybridization chemistry inherent in the DNA arrays, proteins exhibit extraordinary conformational diversity and binding properties. Proteins may easily lose their native conformation and biochemical activity due to denaturation, dehydration or oxidation, issues not relevant in the immobilization and assays of the DNA (Köhler and Seitz 2012, Rabe et al. 2011, Weinrich et al. 2010, Wu et al. 2008, Oh et al. 2006).

The immobilization of the capture and carrier proteins onto the solid support is a major issue for protein microarray fabrication, since the quality of the entire assay is based on the functionality of the array. In addition to the properties of the protein, the binding affinity of protein to the surface of solid support also depends on the surface functionality. The high binding capacity, as well as the prevention of protein denaturation, minimal non-specific binding and high signal-noise ratio, is all important issues to be tackled in the fabrication of the solid surface (Lee et al. 2013, Köhler and Seitz 2012, Hartmann et al. 2009). The surface fabrication method should also be repeatable and produce a stable surface. In addition, the conditions, such as temperature, pH and buffer composition, under which the immobilization is conducted, can alter the success of the array. For example, temperature can influence the kinetics of protein adsorption and thus an elevation in the temperature may increase the amount of surface adsorbed proteins (Mujawar et al. 2012, Rabe et al. 2011, Rusmini et al. 2007, Oh et al. 2006).

4.1 PROTEIN IMMOBILIZATION

Proteins are a large group of heterogeneous molecules, which display extensive diversity in their conformations and binding characteristics; these are attributable to their complex chemical and structural properties. The proteins must be protected from denaturation, dehydration and oxidation since there are processes that may change their native conformation resulting in a loss of their biochemical activity. These features influence how the immobilization process can proceed and subsequently introduce limitations in the choice of the surface which can be used. The proteins are bound to the solid support in varying concentrations. In addition to the protein properties, the levels can depend on various other protein-related factors e.g. their hydrophobicity and charge differences (isoelectric point), as well as the immobilization method used and the properties of the surface. The protein immobilization methods can be divided into physical and chemical categories, depending on the protein attachment mechanism (Shi et al. 2016, Lee et al. 2013, Rabe et al. 2011, Hartmann et al. 2009, Rusmini et al. 2007, Oh et al. 2006).

4.1.1 Physical immobilization

The physical binding process is based on the direct adsorption or entrapment of the proteins; i.e. they can be passively attached onto the surface through non-specific interactions. This is the simplest immobilization method but it has a limited binding

capacity due to the different physicochemical properties of the proteins. There exist wide variations in the hydrophobic and charged domains all over the protein structure, which will affect the immobilization and thus the binding will tend to occur in a random orientation. The excessive interactions between the protein and the solid surface have often resulted in the denaturation of the protein's 3-D structure with a partial or even complete loss of activity (Liu et al. 2014, Lee et al. 2013, Wong et al. 2009, Ajikumar et al. 2007).

The immobilization by direct adsorption is mainly based on hydrophobic, hydrophilic and electrostatic binding interactions between the protein and the solid surface. Hydrophobic proteins undertake good adsorption onto hydrophobic surfaces whereas proteins with many hydrophilic moieties, such as glycoproteins based on their saccharide chains, are well adsorbed onto hydrophilic surfaces. The adsorption via electrostatic forces is based on interactions between the opposite charges of the immobilized molecules and the solid surface. The electrostatic state of the proteins is determined by the pH, thus the numbers of the negative and positive charges of the proteins exist in balance when the pH equals the protein's isoelectric point (pI), resulting in a molecule with a net neutral charge. The protein adsorption rate onto the charged surface depends on the opposite charges of the protein since electrostatic attractions accelerate the migration towards the surface. Thus, an increase in the pH above the pI of the protein increases the net negative charges on the protein's surface and thus increases its adsorption onto positive charged surfaces and *vice versa* (Lee et al. 2013, Mujawar et al. 2012, Rabe et al. 2011, Hartmann et al. 2009, Messina et al. 2009).

Immobilization by the physical adsorption onto smooth surface achieves a close proximity between the protein and the surface. Nonetheless, the binding of a protein with diverse local domains will occur in a random manner, resulting in its partial denaturation and with a reduction in affinity of the target molecule due to the unfavorable position of the reactive sites. Surface modification methods which prevent proteins from making a direct contact with the surface increase the protein's biological activity compared with the direct adsorption. The patterning the solid support by spacers with charged, hydrophilic or hydrophobic groups represents one way to enable the binding to occur without the need for any direct solid support interactions. In addition, the binding affinity of the proteins has been increased by the exploitation of direct adsorption, or by use of porous or other 3-D surface structures. In these cases, the adhesive area is larger than can be achieved on a smooth surface, resulting in a high areal density of the immobilized molecule (Liu et al. 2014, Mujawar et al. 2013, Rabe et al. 2011, Wong et al. 2009, Ajikumar et al. 2007).

4.1.2 Chemical immobilization

Immobilization by chemical methods will also occur randomly, but one can achieve a more specific orientation than possible with non-specific physical adsorption due to the covalent binding with the chemically-active moieties of the proteins. In the chemical attachment, new molecular bonds are formed between the protein and the surface. Covalent bonds are more effective and reproducible than the binding forces involved in physical absorption, because only certain reactive groups on the protein and the support surface are involved in the immobilization. The most commonly utilized reactive groups in proteins are primary amines present in lysine and arginine which provides stable amide bonds. Almost all proteins have side chains with these amines and thus they can generally be immobilized by amine-reactive chemistry (Lee et al. 2013).

In covalent immobilization, the solid surface is patterned with chemically active groups like a primary amine, carboxylic acid, aldehyde or epoxide. The aldehyde and epoxy groups bind amino groups of the proteins covalently via carbon-nitrogen bonds. Both amine and carboxylic acid functionalized supports bind proteins via electrostatic

interactions but they are also widely used for covalent binding after modifications i.e. by cross-linkers such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxy-succinimide (NHS). The EDC-NHS chemistry is better known for its use in a variety of protein conjugation applications but it may also be exploited in the immobilization of the protein. The EDC reacts with the carboxyl groups and with the NHS, it forms an EDC mediated amine-reactive NHS ester which further reacts with primary amines forming an amide bond (Shlyapnikov et al. 2014, Lee et al. 2013, Moschallski et al. 2010).

4.2 PROTEIN MEDIATED IMMOBILIZATION

Proteins are widely used as carriers for antibodies as a way to increase their binding affinity and they can also be used in the assay of other molecules requiring some kind of site-specific immobilization because they only have one antigenic site. If the immobilization methods underpinning the molecular binding occur in a random orientation, this may reduce the biochemical activity of the immobilized capture molecules due to blocking of either the binding or the antigenic sites**.** In contrast, immobilization via a carrier protein system provides a site-specific orientation of the capture agents. The oriented immobilization will maintain or even increase the activity of the capture molecules often by increasing the number of the expressed active sites of the capture agents. It also allows for the immobilization of the proteins or other molecules which might lose their activity when using conventional adsorption methods or if subjected to covalent conjugation (Liu et al. 2014, Wong et al. 2009). The use of the protein mediated immobilization requires a bond formation between the carrier protein and the capture agent. This can be performed either via direct conjugation of the capture agent onto the carrier protein by covalent linking or via affinity binding by non-covalent, specific recognition.

4.2.1 Direct conjugation

Attachment of capture agents to the carrier proteins via covalent conjugation can be improved by a variety of different chemical approaches. The most widely used carrier is serum albumin obtained from either cows or humans (Dobosz et al. 2015, Hu et al. 2013, Desmet et al. 2012, Li et al. 2010, Zuo et al. 2010) but also other proteins have been exploited (Dobosz et al. 2015, Oberli et al. 2011). The requirement to use a carrier protein is often related to the need to elicit an immune response i.e. in the development of antibodies and vaccines, and thus it is necessary to analyse the haptens (Oberli et al. 2011, Li et al. 2010). The protein carriers are also widely used for the immobilization of small molecular weight chemical compounds, such as toxins (Hu et al. 2013, Desmet et al. 2012) and drugs (Zuo et al. 2010), which are not able to bind onto a solid support without losing their antigenic properties.

4.2.2 Affinity binding

Affinity binding refers to a non-covalent specific recognition based bond between two different molecules. The most extensively used affinity binding methods are the exploitation of the specific binding properties of antibodies and the biotin–avidin (or streptavidin/neutravidin) interaction.

Antibody mediated affinity binding is mainly used for the immobilization of antibodies in an oriented form. Antibodies have two antigen-binding domains which are located at the top of their two outstretched arms and thus the oriented immobilization of the capture antibody may entail the use of low binding affinity antibodies. Oriented immobilization exposes the antigen-binding sites, thus significantly improving the target binding efficiency

and detection sensitivity. Antibodies can be utilized as capture agents also by non-oriented immobilization, especially monoclonal antibodies which possess a certain type of binding sites. If antisera or polyclonal antibodies, which bind to multiple epitopes, are used as capture agents, the affinity binding via a secondary antibody system can be applied to increase the binding affinity of specific antibodies towards the antigens of interest (Liu et al. 2014, Lee et al. 2013, Wong et al. 2009, Seurynck-Servoss et al. 2007). The antibodies can also bind other capture agents, such as glycoproteins (Lu et al. 2012, Haab and Yue 2011), as is done in protein-ligand complex interaction studies.

Avidin, streptavidin and neutravidin are homologous tetrameric proteins consisting of four identical subunits each with a single biotin-binding site. The interaction between avidin/neutravidin/streptavidin and biotin has been widely utilized in immunoassay constructions since their binding affinity is the strongest of any known non-covalent bond (Nguyen et al. 2012, Marttila et al. 2000). Immobilization via the interaction between biotin–avidin (including streptavidin or neutravidin as alternatives to avidin) can be achieved by attaching the biotinylated capture molecules onto the avidin/streptavidin/neutravidin coated solid surface. This approach requires that immobilized capture molecules must be linked with biotin either by fusion (Gnjatic et al. 2009) or chemical attachment (Ylihärsilä et al. 2015, Päkkilä et al. 2012, Gehring et al. 2008) and the solid surface has to be coated with avidin, streptavidin or neutravidin. The biotin-avidin approach has also been modified by binding some of the biotin-binding molecules to the biotinylated capture molecule to act as the carrier before the immobilization (Andresen et al. 2006). The biotin-avidin approach is widely used for creating site-specific orientations of antibodies (Päkkilä et al. 2012, Gehring et al. 2008) and small weight molecules (Andresen et al. 2006), and it has also been applied to separate the capture proteins from some complex solution, such as a cell lysate (Gnjatic et al. 2009), as well as immobilizing different types of antigens onto the same surface (Ylihärsilä et al. 2015).

4.3 PROTEIN MICROARRAY PLATFORMS

Most commonly protein microarrays adopt the same kind of arraying equipment and scanners incorporated into DNA microarray technology i.e. the most widely used platform in protein microarray is the conventional microscope slide with dimensions of 25 mm x 75 mm and about 1 mm thick. The conventional glass and plastic slides are the most widely used platforms since they are usually less expensive and more compatible with commercial microarray instrumentation than any other platforms (Mujawar et al. 2012, North et al. 2010). The 96-well plate and other multiwell plate formats, used in conventional immunoassays for decades, have also been exploited in protein microarrays. The arrays are printed onto the bottom of the wells and can be processed using standard laboratory equipment such multichannel pipettes and plate washers, i.e. equipment which is readily available in the most industrial and clinical laboratories (Ylihärsilä et al. 2015, Päkkilä et al. 2012, Gehring et al. 2008, Kwon et al. 2008). The slides and the multiwell plates are typically made of glass or polystyrene, but also other materials can be used.

4.3.1 Glass supports

The conventional microscope glass slides (sodium borosilicate or soda-lime glass) and multiwell plates have silicon dioxide (silica, SiO_2) as their main component (North et al. 2010). Direct adsorption is not an efficient method of immobilizing proteins to the surfaces of the silica materials because of their low or unstable binding profile (Falipou et al. 1999). Thus, glass platforms are mainly used with a chemically activated surface or with a special 3-D functional layer designed for protein arrays (Ma et al. 2012, Malainou et al. 2012).

4.3.2 Plastic supports

Polystyrene (PS, Mujawar et al. 2012, Gehring et al. 2008), polycarbonate (PC, Main et al. 2014, Souplet et al. 2009, Morais et al. 2008, Bora et al. 2006), poly(methyl methacrylate) (PMMA, Liu et al. 2011, Tsougeni et al. 2010) and polyethylene terephthalate (PET, Liu et al. 2009) are some of the most commonly utilized plastic solid support materials in protein microarrays. Large proteins, such antibodies, are capable of being immobilized onto PS or PC surfaces by direct adsorption, because of their hydrophobic characteristics. Nonetheless, surface modifications are still generally used to improve the binding capacity of the plastic support in the fabrication of the protein microarray.

4.4 SURFACE FABRICATION

Although some of the solid support materials are capable of binding proteins without any pre-activation, most arrays are performed on functionalized surfaces. The surface modifications can be divided into two types; two dimensional (2-D), including i.e. surface activation via plasma treatment, irradiation or chemical treatment, and three dimensional (3-D) where a chemically activated porous layer is fabricated onto a solid support by physical or chemical methods. The 3-D surface layers are typically polymers which are capable of forming membranes, hydrogels, layers or brushes. The 3-D surfaces have an increased binding capacity versus the 2-D surfaces due to their porous nature and thus their higher binding area. However, the fabrication processes for 3-D models are usually more complicated than required for 2-D surfaces and furthermore, they tend to exhibit a higher background level (Sinitsyna et al. 2009, Seurynck-Servoss et al. 2007, Zuptsov et al. 2007).

4.4.1 Plasma treatment and irradiation

Irradiation and plasma treatments with or without additional chemicals are widely used methods of modulating the physical and chemical properties of the plastic surfaces or polymer layers attached to the solid support. These techniques have been used to enhance protein binding since they modify the charge and the chemically reactive groups of the surface or they may change the smooth support into a 3-D form or both. Both methods have often used also as pre-activation methods before the final surface fabrication.

The plasma treatment of the polymer surfaces, such as plastic slides (Tsougeni et al. 2012, Tsougeni et al. 2010) or the plastic thin films on top of glass slides (Malainou et al. 2012, Vlachopoulou et al. 2011) involve both the physical and chemical modifications. The physical ion bombardment considerably increases the surface area due to the formation of pores. In addition, the plasma (i.e. O_2 or SF_6) modulates the chemical nature of the surface by elevating the hydrophilicity and increasing the formation of the charged groups like OH, O^- and H^+ by partially breaking the polymer structure of the plastics.

One plasma treatment method, called a plasma ion immersion implantation (PIII), is simple and a compatible method for the modulations of any polymeric surface (Main et al. 2014, Kosobrodova et al. 2012, Nosworthy et al. 2009). The proteins bind covalently to PIII-treated surfaces, forming a continuous layer covering the entire surface without using any linker chemistry. In the PIII method, the plasma treatment involves the immersion of polymers in a nitrogen plasma which induces the chemical activation of the polymeric surface. The energetic ions interact with the polymer surface, breaking polymer bonds and forming new bonds in an amorphous carbon surface layer producing radical groups which are then available for the covalent immobilization of the proteins (Kosobrodova et al. 2014, Main et al. 2014, Nosworthy et al. 2009).

In addition to plasma treatment, the UV-based irradiation methods have been used to modify the surfaces of the solid supports. UV-initiated photochemical activation is performed by photo-activated cross-linking between the protein and the photosensitive polymer attached onto solid support. The advantage of this method is that protein immobilization can be performed simultaneously when the polymer is being formed, as will be described later.

4.4.2 Direct chemical surface modification

The most widely used and the simplest method of modifying inert supports, such as the glass slides, is their surface activation with chemically active polymer reagents. Two common methods are the silanization and the poly-L-lysine coating (Lee et al. 2013); both of these methods are based on the surface interactions with the added polymers. The methods make it possible to achieve protein immobilization due to the electrostatic or the covalent interactions between the protein and the modulated surface. Direct chemical surface modifications are also widely used as a pre-activation method for protein immobilization via cross-linkers (North et al. 2010) and also in the fabrication of the 3-D surfaces (Feyzkhanova et al. 2014, Shlyapnikov et al. 2014, Ajikumar et al. 2007).

Activated poly-L-lysine is a positively charged, natural amino acid polymer which has been modified such that approximately one hydrobromide (HBr) group is included in each lysine residue. The HBr groups undergo hydrogen bonding with the amino and carboxyl groups of the surface proteins, as well as with their nitrogen or the oxygen containing moieties. The poly-L-lysine makes it possible to have a non-specific attachment to the solid support via an electrostatic interaction between the negatively charged side-chains of proteins (Lee et al. 2013, Haab and Yue 2011, Kusnezow and Hoheisel 2003).

The silanization is generally performed by covering the surface with organosilanes. Glass or other silica containing platforms naturally have an oxide layer (SiO_2) on their surface (Falipou et al. 1999). Hydroxylation of the oxide surface of the glass or other silica supports produces a surface terminated with silanol groups (Si-OH) (North et al. 2010, Falipou et al. 1999) which is utilized in a subsequent silanization process (Falipou et al. 1999). The organosilanes are a group of silicon derivatives with at least one carbon-silicon bond. Monomeric or dimeric silicon molecules contain at least one hydrolyzable group, such as methoxy, ethoxy, or acetoxy, and one non-hydrolyzable group. The hydrolyzable groups react with the silanol groups of the solid surface forming a covalent -Si-O-Si- bond between the surface and the silyl groups. The non-hydroxylatable groups include alkyl and/or organofunctional groups, such as amino, methacryloxy, epoxy, thiol or octyl which can be involved in the protein binding. The alkyl groups convert a hydrophilic surface into a hydrophobic one and thus bind proteins via hydrophobic interactions. The organofunctional groups are able to react with proteins via either electrostatic or the covalent interactions (Sapsford and Ligler 2004, Falipou et al. 1999). They are also able to react with other chemical reagents, such as glutaraldehyde or other cross-linking agent (North et al. 2010, Kusnezow and Hoheisel 2003), for re-activation of the surface. In addition, the silanization technique can be used as a pre-activation method for coating the solid support with some membrane or polymer layer materials (Feyzkhanova et al. 2014, Shlyapnikov et al. 2014). The most widely used silane found under the polymer layers is called Bind Silane which usually patterns the surface with amine groups.

4.4.3 Hydrogels

Hydrogels are hydrophilic polymeric networks that absorb large quantities of aqueous solutions through hydrogen bonding with the water molecules due to the chemical or physical crosslinking of the individual polymer chains forming the gel matrix (Kim et al.

2008, Derwinska et al. 2007, Lin et al. 2006). Hydrogels have a 3-D structure which increases their loading capacity and ensures that the aqueous environment has near-physiological conditions for immobilized proteins (Kim et al. 2008, Rubina et al. 2008, Derwinska et al. 2007). This semi-liquid environment allows the proteins to maintain their structure and biological activity (Feyzkhanova et al. 2014). Hydrogels are usually deposited as layers but it is also possible to apply gel droplets as array formats (Li et al. 2011).

The fabrication of the hydrogel layers and droplets can be performed on either platforms made from plastic polymers (Moschallski et al. 2013, Moschallski et al. 2010) or glass (Feyzkhanova et al. 2014, Rubina et al. 2010, Marsden et al. 2009, Kim et al. 2008, Zuptsov et al. 2007). The hydrogel forming components are mainly chemically inert and require the activation of the solid support. The pre-activation is usually performed by Bind Silane (i.e. APTES, (3-aminopropyl)triethoxysilane) which can provide a binding site for the hydrogel components. The hydrogels, deposited as layers or droplets onto solid support, are polymerized by drying or UV-light, depending on which hydrogel components are being used. Some hydrogels are capable of immobilizing the proteins via physical adsorption (Derwinska et al. 2008, Derwinska et al. 2007) but usually the immobilization requires a chemical activation of either the hydrogel (Marsden et al. 2009, Derwinska et al. 2008, Derwinska et al. 2007, Kim et al. 2008, Lv et al. 2007) or the proteins (Feyzkhanova et al. 2014, Rubina et al. 2010, Derwinska et al. 2008). It is possible also to use cross-linkers between the proteins and the hydrogel (Moschallski et al. 2013, Moschallski et al. 2010, Marsden et al. 2009, Kim et al. 2008).

Polyacrylamide and agarose gels, common reagents in gel electrophoresis, are the most widely used materials in protein microarrays for producing hydrogel surfaces (Feyzkhanova et al. 2014, Rubina et al. 2010, Lv et al. 2007, Wei et al. 2004). In addition, natural polymers, such as alginate (Meli et al. 2012, Li et al. 2011, Fernandes et al. 2009), and synthetic polymers, such as modified polyacrylamide (Moschallski et al. 2013, Moschallski et al. 2010), poly(ethylene glycol) diacrylate (PEG-DA, Marsden et al. 2009, Kim et al. 2008), poly(vinyl alcohol) (Derwinska et al. 2008), and polyurethane (Derwinska et al. 2007) have also been used in the fabrication of the hydrogels.

Agarose and the alginate are hydrophilic natural polysaccharides which form highly hydrated hydrogels when they come into contact with water. They express hydroxyl groups on their surfaces which are oxidized to form the hydrogel on a pre-activated solid support (Meli et al. 2012, Li et al. 2011, Fernandes et al. 2009, Lv et al. 2007, Wei et al. 2004). The synthetic polyacrylamide and poly(ethylene glycol) based hydrogels are formed using photoactive monomers which have been exposed to ultraviolet light. The UV-initiated free radical polymerization induces the formation of a water insoluble gel allowing gel fixation to the surface (Moschallski et al. 2013, Moschallski et al. 2010, Marsden et al. 2009, Kim et al. 2008). The poly(vinylalcohol) and the polyurethane based hydrogels are typically dip-coated onto the solid support to form a surface covered with a thin layer hydrogel. They are able to achieve direct adsorption via physical interactions between proteins and hydrogels but the chemical activation of the hydrogel before printing can provide a more stable immobilization of proteins (Derwinska et al. 2008, Derwinska et al. 2007).

4.4.4 Membrane

The nitrocellulose is a natural cellulose polymer activated by a nitric acid. It creates a microporous polymeric surface that binds the proteins in a non-covalent and irreversible manner (Steinhauer et al. 2005, Stillman and Tonkinson 2000). The adhesive nitrocellulose membranes are used in traditional blotting and binding applications (Stillman and Tonkinson 2000) and in the lateral flow strips in the point-of-care (POC) diagnostic tests. The relatively thin surface coating creates the three-dimensional structure to adsorb and

bind the arrayed proteins. The proteins adsorb inside the cellulose network and are attached to the nitrate groups by the hydrophobic interactions (Moschallski et al. 2010). The nitrocellulose has autofluorescense properties, which may exhibit the high background in the fluorescence imaging (Nijdam et al. 2007). The nitrocellulose surface can be formed either with an adhesive membrane (Stillman and Tonkinson 2000), by coating of the solid surface with the nitrocellulose polymer (Steinhauer et al. 2005) or by printing colloidal nitrocellulose with the proteins in a particle form (Fici et al. 2010). The carboxymethyl cellulose, in which the cellulose is first derivatized with the carboxymethyl groups and then activated by the EDC-NHS chemistry, is also used in the fabrication of the surface (Shlyapnikov et al. 2014).

4.4.5 Brushes and monoliths

Methacrylate copolymers are widely used to form the 3-D surfaces of brushes and monoliths on the solid support of protein microarrays. Methacrylates are polymer- forming organic compounds. The methacrylate based copolymers have binding sites for proteins depending on the copolymers prepared from monomers with protein-reactive functional groups in the side-chains. The formation of multi-activated polymers depends on which surface attachment technique has been utilized.

In the surface-initiated polymerization method, the initiator is attached onto the surface and the polymer chains are grown from the surface, forming the brushes. The coating of copolymer brushes via a surface-initiated polymerization and then individual polymer chains are tethered with one of their chain ends to the solid support in a bottom-up fashion. The polymer brushes are synthesized in-situ on the solid support by adding the mixture of the copolymers and the additives onto the pre-activated and initiator functionalized support (Liu et al. 2014, Hu et al. 2013, Liu et al. 2011).

Monoliths have an internal structure representing a system in which the polymer microglobules are interconnected with cross linkers and separated by pores, exposing the functional groups in the structure of the synthesized copolymers. Macroporous monoliths are prepared by undertaking a photo-initiated copolymerization of the mixture of the functional monomers, the cross-linking agent, the porogenic solvent and the initiator. The reaction mixture added on 3-(trimethoxysilyl)propyl methacrylate activated solid support are polymerized by the UV-light (Sinitsyna et al. 2011, 2009, Rober et al. 2009, Kalashnikova et al. 2007).

The common copolymer brushes are made from glycidyl methacrylate monomers (GMA, Liu et al. 2014, Liu et al. 2009) or from heteropolymers created from two different monomers (GMA-co-EGMA, (ethylene glycol) methacrylate, Liu et al. 2011; GMA-co-OEGMA, oligo(ethylene glycol) methacrylate, Hu et al. 2013). Monoliths are polymerized from two (GMA-co-GDMA, glycerol dimethacrylate, Sinitsyna et al. 2009, Rober et al. 2009; GMA-co-EDMA, ethylene dimethacrylate, Rober et al. 2009, Kalashnikova et al. 2007; HEMA-co-GDMA, 2-hydroxyethyl methacrylate, Sinitsyna et al. 2011; EDMA-co-CEMA, 2-cyanoethyl methacrylate, Rober et al. 2009) or three (GMA-co-EDMA-co-HPIEAA, N-hydroxyphthalimide ester of acrylic acid, Rober et al. 2009; EDMA-co-HEMA-co-CEMA, Rober et al. 2009) different polymers.

The polyethylene glycol (PEG) side chains of the EGMA and the OEGMA copolymers are able to suppress non-specific protein adsorption (Hu et al. 2013, Liu et al. 2011) while the GMA copolymers provide side chains with epoxy groups for the covalent immobilization of the amine groups present in the proteins (Liu et al. 2014, Hu et al. 2013, Liu et al. 2011). The HEMA, EDMA and GDMA copolymers possess hydroxyl groups which are able to undergo re-activation or they can form a hydrophilic surface (Rober et al. 2009, Sinitsyna et al. 2009). Depending of which copolymers are being used, the surfaces

coated on the brushes or the monoliths can be utilized for protein immobilization either after the coating (Hu et al. 2013, Liu et al. 2011, Rober et al. 2009) or after the chemical re-activation of the polymer surface (Liu et al. 2014, Liu et al. 2011, Sinitsyna et al. 2011). Alternatively, the proteins can be activated before their immobilization (Sinitsyna et al. 2011).

Functionalization of the solid support can also be performed using copoly-(DMA-NAS-MAPS) which forms a nanometric film of polymer brushes onto the solid support. The DMA-NAS-MAPS polymer consists of three co-monomers with different functions. The N,N-dimethylacrylamide (DMA) creates the polymer backbone and this facilitates polymer adsorption by forming hydrogen bonds with the surface. 3-(Trimethoxysilyl)propyl methacrylate (MAPS) reacts covalently with the free silanols on the surface and the N,N-acryloyloxysuccinimide (NAS) possesses a reactive group which is able to bind to the primary amines of the proteins. During the coating of the surface with the DMA-NAS-MAPS polymer, the silanol group patterned solid support is either immersed (Cretich et al. 2009) or dip-coated into the pre-polymerized polymer solution (Cretich et al. 2009, Marquette et al. 2007).

4.4.6 Dendrimers

Dendrimers are a unique class of synthetic polymers that do not form entangled chains associated with linear polymers. They can be utilized to form a 3-D layer with numerous side chains which can be easily functionalized and used in the surface fabrication of the solid support. The most widely used dendrimers are based on poly(propyleneimine) with either amine or carboxyl terminals (Ajikumar et al. 2007, Ng et al. 2007, Pathak et al. 2004), although other dendrimers have also been examined (Kwak et al. 2014). Dendrimers can be attached to either silanol (Pathak et al. 2004) or amine (Kwak et al. 2014, Ajikumar et al. 2007, Ng et al. 2007) terminated surfaces and they can also be functionalized with NHS groups (Kwak et al. 2014, Ajikumar et al. 2007, Ng et al. 2007).

5 Aims of the Study

The protein microarray has been shown to be a powerful tool for the simultaneous and parallel screening of many different molecular interactions with a short analysis time and low reagent consumption. The use of a polymerized glutaraldehyde to achieve a chemical activation of the surfaces was shown to be a promising method for immobilizing proteins onto solid supports. The general objective of the study was to evaluate the functionality of this simple surface fabrication method by conducting studies in serology, antigen detection and protein-DNA interactions with the protein microarray platforms.

The specific aims were as follows:

1. To undertake a parallel analysis of anti-viral antibodies and inflammatory-mediated antigens in human serum using several different capture agents

2. To perform a simultaneous screening of the human serum antibodies against various central nervous system pathogens using different well-known antigens and potential antigen candidates as the capture agents

3. To evaluate the parallel screening of the binding profile of a transcription factor for a large set of mutated oligonucleotide sequences, immobilized as capture agents via their carrier proteins

6 Materials and Methods

This section describes the methods, laboratory equipment and most widely used reagents in the protein microarray processes. More detailed descriptions of the biomolecules and reference methods used can be found in the published articles mentioned in parentheses.

6.1 SURFACE ACTIVATION OF SOLID SUPPORTS

Conventional 96-well plates (polystyrene; Thermo Fisher Scientific Inc, Vantaa, Finland) (Papers I and II) and microscope glass slides (Menzel Gläser GmbH, Braunschweig, Germany) (Paper III and unpublished study) were used as the solid supports for the protein microarrays.

The surface activation solution was prepared by diluting 25 % aqueous glutaraldehyde (GA) with 10 mM $NaHCO_3$ (pH 9.5) solution to a final 1 % (v/v) GA concentration under alkaline conditions. The pre-polymerization of the GA was performed by incubating the solution for three days with stirring at +37 °C in the dark. After the pre-polymerization of the GA, it was used for the surface activation of both glass slides and microtiter plate wells.

Polystyrene plates: 100 µl of the pre-polymerized GA solution was added to the wells of the 96-well plate, sealed with a plastic tape and then incubated overnight at room temperature in the dark. After the incubation, the wells were washed twice with deionized water and once with the $NaHCO_3$ solution. Finally, the plates were dried for 30 min at +37 °C and stored at room temperature in dry and dark conditions before further use.

Glass slides: The slides were incubated overnight with the pre-polymerized GA solution in a plastic box at room temperature (ten slides in 200 ml volume of the solution). The slides were then rinsed with deionized water and dipped in $NaHCO_3$ solution for a few seconds. After drying (30 minutes at +37 °C), the GA-activated glass slides were coated with avidin by immersing the slides overnight in the dark in avidin solution (Catalog number: A003-01, Rockland Immunochemicals Inc., Gilbertsville, PA, USA, 10 µg/ml in the $NaHCO_3$ solution) at room temperature. The slides were then washed three times with PBST (Tween 20 (Fluka, Buchs, Switzerland) in PBS; phosphate buffered saline) and rinsed thoroughly with deionized water. Finally, the slides were dried for 30 minutes at room temperature and then for two hours at +37 °C. The avidin-coated glass slides were stored in plastic boxes at room temperature with silica gel bags and used within 30 days of being coated.

6.2 MULTIWELL BASED PROTEIN MICROARRAYS

The surface activated microtiter plate wells were used for the parallel assays in the serology and the antigen detection (Papers I and II). The antigens and antibodies, used as capture agents, were diluted in $NaHCO_3$ solution, transferred to 384-well plates (polypropylene; Nunc, N.Y., USA) and printed at the bottom of the GA-activated microtiter plate wells using the MicroGrid II microarray printer with MicroSpot 2500 quill pins (BioRobotics, Cambridge, UK). Each pin was estimated to transfer approximately one nanoliter of the capture agent solution per spot onto the surfaces of the wells. The printing was performed at normal room temperature and moisture conditions.

After the spotting, the plates were incubated for at least one hour at room temperature and then washed three times with the PBST. The nonspecific binding sites were blocked

with PBS supplemented with 1-2 % bovine serum albumin (BSA, Rockland Immunochemicals Inc., Gilbertsville, PA, USA) for 15-30 minutes at room temperature followed by three washes with the PBST. The blocked microarrays were then dried and either used in the assays or stored at -70 °C prior to use.

The human serum samples were diluted 1:100 or 1:1000 in sample buffer (BSA in PBS) and 100 µl of each dilution was added to the arrayed wells. After a 15 min incubation at room temperature or at + 37 °C, the wells were washed three times with the PBST. A volume of 100 µl of peroxidase conjugated secondary antibody against human IgG was pipetted onto the microarrays for 15 min and the wells were then washed three times with the PBST. Peroxidase substrate solution was added to the wells and after a few minutes incubation the wells were washed with deionized water and dried for 30 minutes at room temperature before the imaging.

Black-and-white TIFF images of the assayed microarrays were taken by a digital camera in an inverted microscope. Spot intensities and the local background signals on the microarrays were manually measured using ImageJ software (http://rsbweb.nih.gov/ij/).

6.3 GLASS SLIDE BASED PROTEIN MICROARRAYS

The microscope slide based microarrays were used in the analysis of the binding affinity of the transcription factor proteins to the mutated DNA sequences (Paper III and unpublished study). Biotinylated oligonucleotides with controls were diluted in PBS and transferred onto the 384-well polypropylene plate. The dilutions were then printed in an array format onto the avidin-coated glass slides by the microarray printer. Printing was performed at normal room temperature and humidity.

After two days' incubation at room temperature protected from the light and moisture, the arrays were stained with either SYBR Green I (Sigma-Aldrich, St. Louis, MO, USA) (Paper III) or POPO-3 iodide (Thermo Fisher Scientific Inc., Rockford, IL, USA) (unpublished study) dyes and scanned at 488 nm (SYBR Green I) or 546 nm (POPO-3 Iodide) in a ScanArray 5000 (GSI Lumonics, Packard Bioscience, USA) to measure the amount of bound oligonucleotides. After the scanning, the stain was removed before the assays.

The arrays were incubated with BSA in PBS for ten minutes at room temperature to block the non-specific binding sites and then washed with PBST and dried. The transcription factor protein solution was placed on the slides and allowed to react with the arrays for ten minutes at room temperature. The unbound proteins were removed by washing the slides thoroughly with PBST. Transcription factor protein specific antibody was diluted in PBS and the solution was incubated with the arrays. After ten minutes of incubation at room temperature, the slides were rinsed with PBST. Fluorescence labeled secondary antibody was diluted in PBS and the solution was added onto the arrays. Slides were incubated for ten minutes at room temperature followed by washing first with the PBST and then with deionized water before being dried. The assayed arrays were scanned in a ScanArray 5000. The fluorescence intensities of the spots and the background were analyzed with Spotfinder software (http://www.tm4.org/spotfinder.html).

7 Results and Discussion

The main results of the protein microarray fabrication will be presented and discussed in this chapter. The focus of this research project was to evaluate a new surface activation method using different protein microarray applications. More detailed results of the assays including tables and analyses, and their biological sense can be found in the original published articles mentioned in parenthesis.

7.1 MICROARRAY PLATFORMS

The conventional glass slides (Paper III and unpublished study) and 96-well polystyrene plates (Papers I and II) were chosen for use in these protein microarrays because of their low cost and the compatibility with standard laboratory equipment (Mujawar et al. 2012, North et al. 2010, Kwon et al. 2008). The surfaces of the solid supports were chemically activated using pre-polymerized glutaraldehyde. The feasibility of the new activation method and the functionality of the surface were field tested with two different kinds of applications. In the first example, various kinds of capture agents (antibodies, proteins, peptides and whole viruses) printed onto the activated surface were used to perform a simultaneous human serum antibody and antigen detection which could be applied for both diagnostic and research purposes (Papers I and II). In the second application, the influence of DNA mutations in the binding affinity of the transcription factor proteins to their target DNA sequences was studied using DNA sequences with mutated nucleotide sequences (oligonucleotides) printed onto the activated surface via the biotin-avidin technique (Paper III and unpublished study).

The oligonucleotide based microarray screening studies were performed on glass slides since a large number of the DNA variants were required in order to evaluate the affinity binding profile of the transcription factor proteins (Paper III and unpublished study). Because of the larger printing area of the slides, when compared with the limited area of the microtiter plate wells, it proved possible to screen all of the oligonucleotides both simultaneously and in a parallel manner with only a relatively small quantity of the protein.

Thus, the transcription factor protein assays were used to screen a large number of different oligonucleotides immobilized onto solid support which allowed quantification of the binding affinity of the one or two transcription factor proteins. In contrast, the infection diagnostic based microarrays were focused on the screening of panels of the human serum samples using relatively small arrays of the immobilized capture proteins.

In the serological assays, 96-well plates were used (Papers I and II) based on their capacity to screen several human samples with identical arrays at the same time with the same assay, which cannot be achieved with the more traditional slide-based assays with their restricted arrays. The multiwell plates are also compatible with the currently employed automated methods used for the EIA and this minimizes the possibilities for mistakes and decreases the time required for completion of the assay. In addition, the plates have significantly larger sample volumes compared with the slides, enabling sample dilution and thus reducing the non-specific binding of the other biomolecules present in the sera.

7.2 SURFACE ACTIVATION

The conventional glass slides (Paper III and unpublished study) and 96-well polystyrene plates (Papers I and II) were chemically activated using the pre-polymerized glutaraldehyde solution. Glutaraldehyde is the most widely used crosslinking for protein-protein and the protein-other molecule conjugations and it is also known as a fixative reagent suitable for immobilizing proteins, cells and tissues onto the glass slides prior to microscopic analyses (Wine et al. 2007, Migneault et al. 2004, Margel et al. 1979). In the protein microarrays, glutaraldehyde can be used to undertake the chemical activation of hydrogels (Son et al. 2011, Derwinska et al. 2008, Wei et al. 2004) and particles (Liu et al. 2011). Although glutaraldehyde is a widely used reagent in protein microarray and other technologies, there are no reports investigating the feasibility of using pre-polymerized glutaraldehyde for the direct chemical activation of the solid supports for protein arrays.

In the surface activation procedure, glutaraldehyde was used as the starting monomer for the polymerization (Figure 3). The polymerization of the glutaraldehyde was performed in a carbonate buffer at pH 9.5 (1 % (v/v) glutaraldehyde in 10 mM $NaHCO_3$). Under these alkaline conditions, glutaraldehyde undergoes polymerization, generating a mixture of variable sized polymeric chains which express the active aldehyde groups in the individual units and each end of the polymer molecules (Figure 3). All types of solid support materials are capable of undertaking the direct activation of the formed glutaraldehyde polymers leading to the generation of a functionalized surface since the polymeric glutaraldehyde forms a grid onto the surface of the solid support containing several reactive aldehyde groups available for binding the proteins (Figure 4) (Migneault et al. 2004).

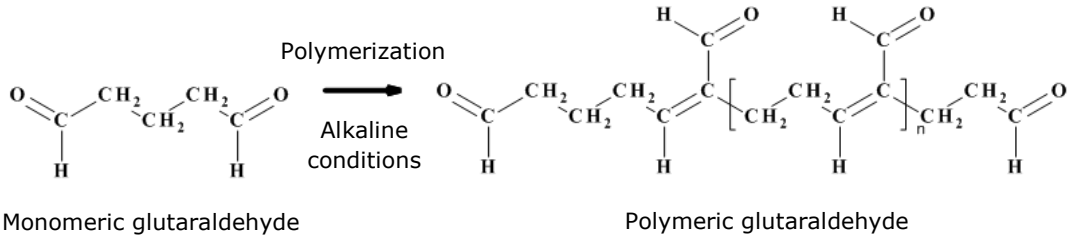

Figure 3. The glutaraldehyde undergoes polymerization in the alkaline solutions, generating a mixture of variable sized polymeric chains which express the active aldehyde groups in the individual units and at each end of the polymer molecules (Wine et al. 2007, Migneault et al. 2004).

The aldehydes react readily with primary amines present in the proteins (Figure 4) forming Schiff's bases after a nucleophilic attack by the ε-amino groups of the lysine residues in the protein. Most proteins contain lysine residues which are located on the surface of the protein. The lysine residues are not generally involved in a catalytic site, which means that they can react with the aldehyde without altering the protein's conformation or losing its biological activity. Proteins can attach to the aldehyde activated surfaces in a variety orientation since proteins typically display many lysines all over on their surfaces (Migneault et al. 2004). The reaction of the aldehyde with the protein occurs rapidly (within minutes to hours). The aldehyde groups can form covalent bonds with the many functional groups present on the protein's surface, but reactions with the protein's primary amines tend to predominate (Wine et al. 2007, Migneault et al. 2004).

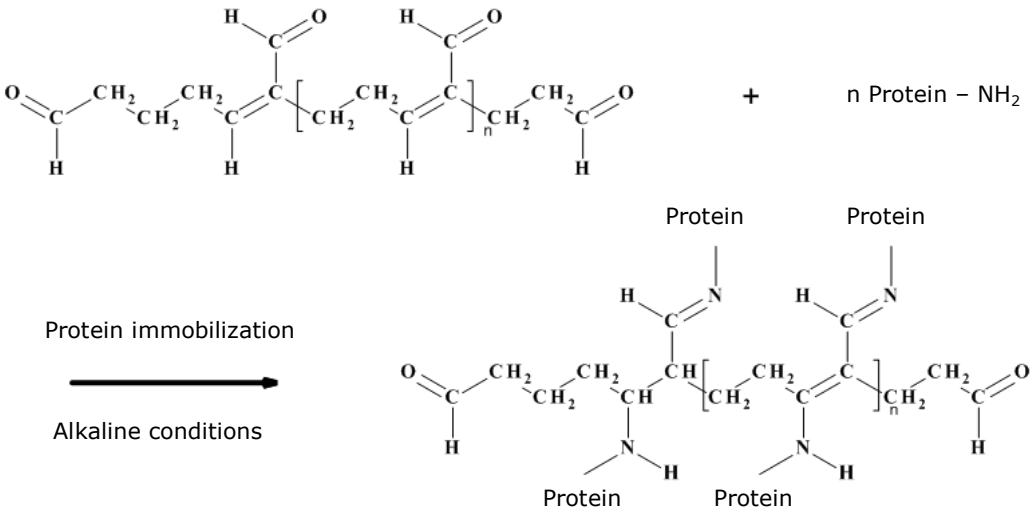

Figure 4. Protein immobilization via aldehyde groups of polymeric glutaraldehyde (Wine et al. 2007, Migneault et al. 2004).

The shelf-life of the aldehyde activated or the avidin coated surfaces, or the printed arrays on top of them, was not determined. The surfaces were used for printing within one month after their activation and the printed arrays were usually tested no more than one week after their printing. The coated glass slides were kept in a plastic box with silica gel bags at room temperature to protect them from light and moisture (Paper III and unpublished study). The coated polystyrene 96-well plates were kept dark at room temperature sealed with adhesive tape (Papers I and II).

7.3 IMMOBILIZATION OF CAPTURE AGENTS

The array printing was automatically performed onto the activated glass slides according to the standard printing protocol using the MicroGrid II microarray printer (Paper III and unpublished study). The printing onto the wells of the microtiter plates was achieved with the microarray printing robot controlled by custom developed microplate arraying software (Papers I and II). The immobilization of the printed capture agents (Papers I and II) and the coating of the avidin layer (Paper III and unpublished study) onto GA-activated surfaces were performed under alkaline conditions using the carbonate buffer (10 mM $NaHCO_3$, pH 9.5) which enhance the protein binding during the immobilization (Mujawar et al. 2012, Olle et al. 2005, Seong 2002). PBS was used as the printing buffer in the arraying of the biotinylated oligonucleotides, (Paper III and unpublished study).

After the printing, the array quality was visually confirmed by checking the spots under a microscope. The visual checking was based on the detection of salt crystal spots formed during the drying of the printing buffer. The unsuccessful arrays were not used further. The studies performed to measure the binding affinity of the proteins to the oligonucleotides require that one determines the amount of the immobilized DNA. Thus the arrays of the immobilized oligonucleotides were also screened with fluorescence staining (Paper III and unpublished study) so that it would be possible to normalize the assay results. In the assays on the arrayed wells (Papers I and II), normalization was not necessary since the assays were mainly utilized to detect the presence, absence or concentration of the specific antigens and antibodies in the serum samples. The assay

validity was performed using the internal and external standards as well as by applying the reference methods.

The aldehyde activated surfaces achieved the immobilization of the proteins, whole viruses, peptides and oligonucleotides. Intact viruses display proteins on their surface which can undergo covalent bonding with the surface aldehydes (Paper I). The peptides and the oligonucleotides used in the assays, have only one or a few binding sites based on their small molecular weights, thus the direct immobilization via the aldehyde groups would lead to loss of their probe binding capacity. For this reason, the biotin-avidin technique was used achieve the immobilization of the small weight capture agents (Papers I and III and unpublished study). The biotinylated peptides were printed onto the aldehyde activated surface with the conjugated avidin (Paper I) and the biotin conjugated oligonucleotides were printed onto the avidin coated aldehyde surface (Paper III and unpublished study).

7.4 MICROARRAY ASSAYS

The non-specific binding sites of the arrayed surfaces were blocked with the BSA containing PBS buffer before the assays. This treatment quenches the unreacted aldehydes on the surfaces and forms a molecular layer which reduces the non-specific binding of the other molecules present in the assay.

The antigen-antibody assay molecule configurations used in the infection diagnostic based studies (Papers I and II) have general applications in protein microarray experiments (Natesan and Ulrich 2010). The detection of the captured probes was also performed via the general method, by using the enzyme labeled antibodies as a form of secondary detection (Figure 5). The enzyme precipitation was used as the assay method since it allows for array imaging with a conventional microscope camera which detects the formation of the visible spots (Figure 5).

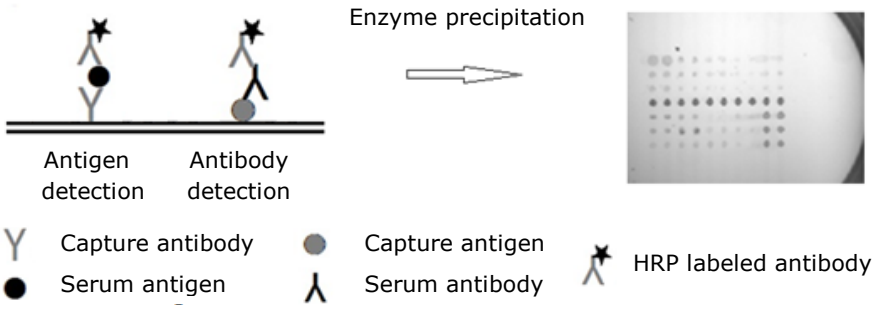

Figure 5. The molecule configurations used in the serological protein microarray analysis (Papers I and II) and a photograph of one array after the assays. The detection was performed by enzyme precipitation and photographed by a conventional microscope camera.

In the affinity assays (Paper III and unpublished study), the DNA oligonucleotides were immobilized via the biotin-avidin technique (Figure 6). After the normalization signal was detected (Figure 6a), the DNA-stain was removed. The transcription factor proteins were allowed to react with the oligonucleotide arrays, and the bound proteins were detected by the double-antibody method with transcription factor protein specific antibodies and fluorescence labeled secondary antibodies (Figure 6b). The assayed arrays were imagined by the fluorescence scanner (Figure 6).

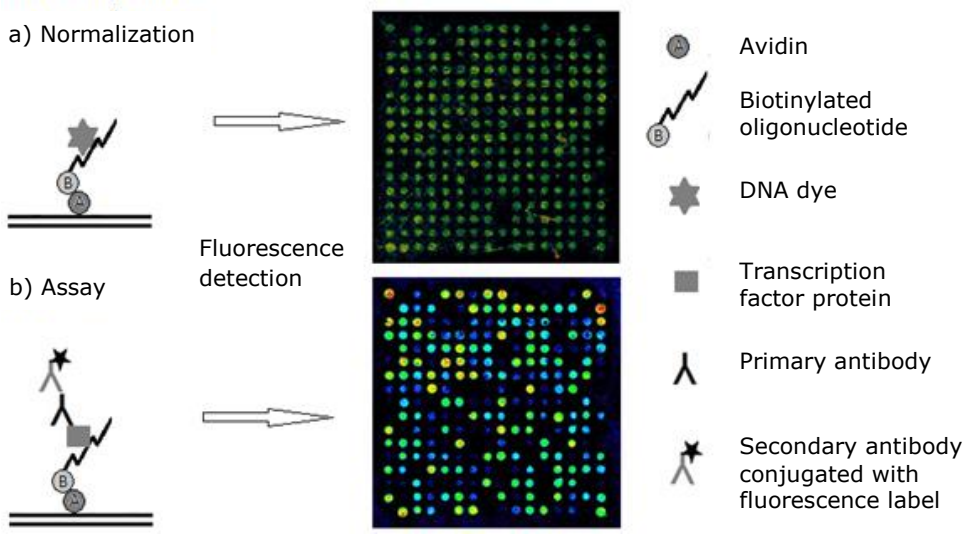

Figure 6. Transcription factor protein affinity assays with the protein microarray. The assay (b) was performed after the normalization signal (a) had been detected. The figures of performed array tests were taken by a fluorescence scanner (Paper III and unpublished study).

7.5 FUNCTIONALITY OF ASSAYS

Reference methods (described in the original publications) were used to evaluate functionality of the developed protein microarray assays.

The serological microarray assays were performed to evaluate whether this multiplexed method could be utilized for the simultaneous detection of antibodies against CNS pathogens (central nervous system, Paper II) and for a virus-specific antibody detection method combined with a quantitative C-reactive protein (CRP) assay (Paper I).

The arrays of selected antigens and antibodies were evaluated using serum sample panels which were tested with the existing reference assays. The enzyme immunoassays and immunoblots were used as reference methods for the antibody assays (Papers I and II), and commercial methods (rapid test and analyzer) for the CRP assays (Paper I). The sensitivity and specificity values of the developed microarrays were estimated in comparison with the reference assays.

The CRP quantification (Paper I) showed an excellent correlation to the reference methods. The measured CRP concentrations showed a linear correlation (R^2=0,960) in the range of 0-150 mg/ml (n=22, Figure 7) with both commercial tests. The antigen detection showed the high sensitivity, the lowest concentration which could be determined using purified CRP antigen was 0,024 µg/ml (data not shown).

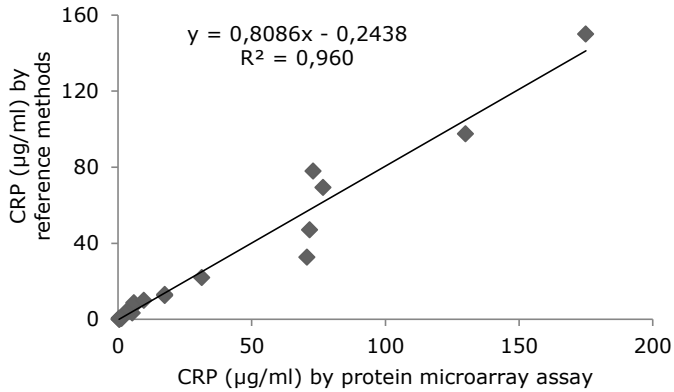

Figure 7. Measurements of serum CRP concentrations by protein microarray assays and reference methods were showed high linear correlation (R^2=0,960) in range of 0-150 mg/ml (n=22) (Paper I).

In the parallel microarray assays of the serum CRP and viral antibodies (Paper I), the human immunodeficiency virus type 1 (HIV-1) and Puumala virus (PUUV) assays revealed identical results to the reference methods and the antibody screening with Sindbis virus (SINV) and adenovirus yielded individual negative and positive discordant samples (overall concordance 96% for both viruses with the reference tests). More detailed results are shown in Table 1.

In the second experiments with the serological protein microarrays (Paper II), the microarray sensitivity for the screening of antibodies against herpes simplex virus 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus (VZV), adenovirus and *Mycoplasma pneumoniae* ranged from 77% to 100%, with the specificity ranging from 74% to 97% (Table 1). In the evaluation of the antigens from the species of *Borrelia burgdorferi* sensu lato, the sensitivities and specificities were varied (Table 1) and the analytical sensitivity of the microarray was found to depend on the borrelial IgG concentration of the serum sample.

Table 1. Microarray performance of two applications for different pathogens in comparison to reference methods (Papers I and II).

	Reference method	Protein microarray assay positive (n)	negative (n)	Total (n)	Sensitivity %	Specificity %
Screening of viral antibodies combined with CRP quantification (Paper I)						
HIV-1	positive	1	0	1	100	100
	negative	0	22	22		
PUUV	positive	10	0	10	100	100
	negative	0	13	13		
SINV	positive	3	1	4	75	100
	negative	0	19	19		
Adenovirus	positive	16	1	17	94	100
	negative	0	6	6		
Screening of microbial antibodies in parallel with antigen evaluation (Paper II)						
HSV-1	positive	31	3	34	91	89
	negative	5	42	47		
HSV-2	positive	22	3	25	88	74
	negative	15	42	57		
VZV	positive	59	5	64	92	92
	negative	1	12	13		
Adenovirus	positive	58	0	58	100	84
	negative	3	16	19		
M. pneumoniae	positive	33	10	43	77	96
	negative	1	26	27		
B. burgdorferi sensu lato (six different borrelial antigens)					63-96	40-89

DNA-protein binding microarrays were performed to measure the binding affinity of the transcription factor (TF) proteins: NRF2 (nuclear factor, erythroid 2 like 2, encoded by *NFE2L2* gene) heterodimerized with small MAF proteins (Paper III) and two TCF7L2 isoforms (transcription factor 7-like 2, with and without E-tail) (unpublished study) on a variety of representative DNA sequences. The assays were used to study how single nucleotide polymorphisms (SNPs) in transcription factor binding sites could alter the binding properties of the TF and thus affect the gene regulatory potential of the TF (Reddy et al. 2012).

The binding affinities of each tested DNA sequence, processed bioinformatically by a collaborator, were ultimately expressed as the measured binding relative to reference sequence binding. The binding profile of the singly varied sequence set was compared to the binding motifs derived from chromatin immunoprecipitation coupled with deep sequencing (ChIP-seq) data. There was a high correlation between the protein microarray assay and the reference method (Figure 8).

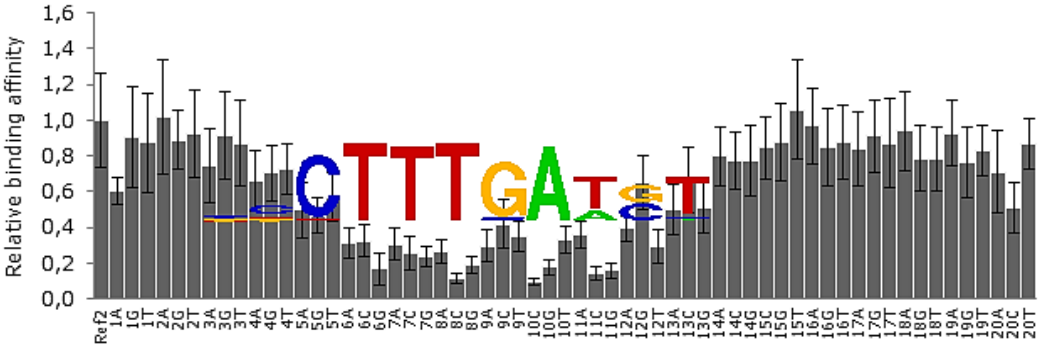

Figure 8. The relative binding affinity profile of singly mutated oligonucleotide sequences based on preliminary data analysis (unpublished results). The binding affinities of reference (Ref2) and variant sequences for the TCF7L2 isoform with an E-tail are expressed as relative to reference sequence (Ref2) binding (mean±SD). The overlaid sequence logo displays the ChIP-seq derived TCF7L2 binding motif.

8 Conclusions

Various surface modification methods, in addition to those described here in more detail, have been developed to enhance the binding affinity of proteins onto the solid support in the microarrays. The quality of the surface is critical for determining the uniformity and the coupling efficiency of the immobilized molecules and it will often determine the overall success of a microarray experiment.

Each different type of platform has its own advantages and disadvantages resulting from the inherent characteristics of the surface. For example, hydrogels have a 3-D structure which increases their loading capacity; it also provides an aqueous environment with near-physiological conditions for immobilized proteins (Kim et al. 2008, Rubina et al. 2008, Derwinska et al. 2007). Although this semi-liquid environment allows the proteins to maintain their structure and biological activity (Feyzkhanova et al. 2014) the complex 3-D surface structure tends to decrease diffusion rates and thus slows down the assay kinetics, and as a result, molecular recognition events may require a prolonged incubation time. The diffusion of the printed proteins onto the hydrogel layers will enlarge the size of the protein spots in continuous gel surface and this reduces the assay's sensitivity (Li et al. 2011, Kusnezow and Hoheisel 2003).

The aldehyde activation places the functionalized monolayer surface onto the planar supports which may be as effective as the 3-D surfaces due to their larger surface area than can be attained with 2-D matrices. However, in comparison with 2-D supports, most of 3-D surfaces are more difficult to fabricate; they may require several reagents and their production typically involves multiple step surface chemistry in order to obtain an activated surface suitable for protein immobilization. The method evaluated here for the surface activation with the pre-polymerized glutaraldehyde is straightforward and simple and has low risk of failing to coat the surface material.

The aldehyde surface activation method proved to be suitable for the immobilization of many different kinds of proteins and it did not require any pre-treatment. Many reagents, in addition to glutaraldehyde, could be used for the pre-activation of the proteins but the treatment and the subsequent purification steps might lead to a loss of the proteins and furthermore these techniques may be expensive or involve complicated manufacturing procedures. In addition, the proteins should to be activated directly prior to spotting since they might not remain stable in this state for a prolonged time (Moschallski et al. 2010, Kusnezow and Hoheisel 2003).

The novel aldehyde surface described here proved to be suitable not only for immobilizing proteins, but by exploiting avidin-biotin chemistry, it was also possible to immobilize small molecular weight molecules, such peptides and oligonucleotides. The evaluated glutaraldehyde polymer surface maintained the properties of the proteins in a stabilized form and thus the proteins and other capture molecules remained in an active conformation capable of binding the probe molecules. Even if the activities of the attached proteins may be partially lost due to the non-oriented type of covalent binding when being immobilized onto the surface, there should still be enough activity remaining to bind the molecules of interest.

9 Future Perspectives

The protein microarray is a versatile tool for screening, identifying and analyzing of numerous molecules or samples both simultaneously and in parallel. It could be easily automated for analysis of large sample series needed for example in hospital laboratories. Lower reagent consumption and shorter analysis time of protein microarrays compared to the traditional EIA method might allow large financial savings and faster results.

The devised straightforward and low-cost surface fabrication method involving glutaraldehyde polymer activation can be readily exploited in novel protein microarray applications. Although high quality slides with the pre-activated surfaces are commercially available, many researchers still wish to fabricate their own surfaces and arrays, and thus there is a clear need for new coating methods with well-defined protocols. The glutaraldehyde polymer surface coating evaluated here is a straightforward, easy and inexpensive method for diverse applications in protein microarray based research.

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Protein microarray is a versatile tool for screening, identifying and analyzing of numerous molecules both simultaneously and in parallel requiring only tiny amounts of sample and reagent volumes. This thesis represents evaluation of new simple and low-cost method for molecule immobilization, which is a major issue for successful microarray fabrication. This new glutaraldehyde polymer based surface activation method can be readily exploited in a wide variety of protein microarray applications.

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