Dissertation zur Erlangung des Doktorgrades der
Fakultät für Chemie und Pharmazie der
Ludwig-Maximilians-Universität München

Development of a protein microarray platform for the characterization of antibodies and quantitative immunoassays

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geboren in Boston, USA

2017

Erklärung

Die vorliegende Dissertation wurde nach §7 der Promotionsordnung in der Fassung vom 28.11.2011 von Herrn Prof. Dr. Lutz Gissmann von der Fakultät für Biologie der Universität Heidelberg betreut und von Herrn Prof. Dr. Gerhard Winter von der Fakultät für Chemie und Pharmazie vertreten.

Eidesstattliche Versicherung

Diese Dissertation wurde wurde eigenständig und ohne unerlaubte Hilfe erarbeitet.

München, den 16.05.2017

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Dissertation eingereicht am: 16.05.2017

Erstgutachter: Prof. Dr. Gerhard Winter

Zweitgutachter: Prof. Dr. Lutz Gissmann, Universität Heidelberg

Tag der mündlichen Prüfung: 14.06.2017

Acknowledgements

The present thesis was prepared under the supervision of Dr. Jörg Hoheisel at the Department of Functional Genome Analysis of the German Cancer Research Center (DKFZ) in Heidelberg, Germany as well as under the supervision of Prof. Dr. Gerhard Winter from the Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics at the Ludwig-Maximilians-University in Munich, Germany.

As the experimental part was mainly carried out in the laboratory of Dr. Jörg Hoheisel in Heidelberg and therefore most of the time was spent here, I would like to express my deepest gratitude to him and his group. From the first encounter on I felt being in good hands, not only because of his own way of providing scientific guidance but also because of the comfortable working environment, which I enjoyed very much. The freedom and confidence provided from his side, gave me the opportunity to be creative and develop own ideas. Without his support, this work would probably be less fragmented but it would have lead to less interesting achievements and it would have had less of an impact on my personal development throughout the last three years.

In the same manner, I want to thank Prof. Dr. Gerhard Winter for his advices and navigation intelligence basically throughout my entire scientific career up until now. Having my own creative mind with many ideas, I do not want to miss his guidance and encouragement to stick to the route. Also, not being present in Munich on a day-to-day basis, I am very grateful to him for allowing me to be part of his group and, even though it was limited, the great time we had together.

Furthermore, I want to express my gratitude to Prof. Dr. Lutz Gissmann for great ideas and discussions during our thesis advisory committee meetings and above all his willingness to attend my defense in Munich.

Special thanks also belong to Dr. Christoph Schröder. Without his input and exchange, it would not have been possible to learn the necessary technical skills this fast. Apart from the scientific support, I am very thankful for the insights into his startup company Scio-

mics. His passion and motivational people management gave me an inspiring idea how a scientific development can be transformed into a commercial business, which might be of great advantage for my own career path.

This work would not have been a success without the help of many cooperation partners such as the group around Prof. Dr. Stefan Dübel and Prof. Dr. Michael Hust from the Institute for Biochemistry, Biotechnology and Bioinformatics at the Technical University of Braunschweig, Germany. At this point I also want to say thank you to Prof. Dr. Thomas M. Gress, PD Dr. rer. nat. Malte Buchholz and PD Dr. Christian Bauer from the Department of Gastroenterology and Endocrinology at the Philipps University, Marburg, Germany for their help with the organization of a clinical trial and the collection of patient samples.

Many thanks also belong to the students I was given the opportunity to work with. Tjaša Berčič, Christoph Harmel and Sandra Schifferdecker it really was good fun working together with you and I wish you all the best for your future.

Last but not least, I want to thank all my labmates in Heidelberg for having such a great time; not only in the lab but also at various other occasions. In this sense, I very much appreciate the scientific and personal support from each of you: Laureen Sander, Patrick Kunz, Eric Dyrcz, Shakhawan Mustafa, Damjana Kastelic, Aseel Marzoq, Smiths Lueong, Adriana Spalwisz, Jessica Schwab, Büsra Turgu, Jeff Munzar, Andrea Bauer, Sandra Widder, Stefanie Kutschmann, Anke Mahler and Marie-Christine Leroy-Schell.

Finally, I want to thank the Helmholtz International Graduate School for Cancer Research at the DKFZ for their financial support.

Contents

Preamble

The goal of the research described in this thesis was to develop a portable diagnostic test for the use at the-point-of-care.

This work was supervised by Prof. Dr. G. Winter from the Department of Pharmacy, Pharmaceutical Technology & Biopharmaceutics of the LMU Munich, yet conducted at the German Cancer Research Center in the group of Dr. Jörg Hoheisel - Division of Functional Genome Analysis.

Identification of biomarkers and development of diagnostic measurement tools is the prerequisite to realize diagnostic concepts. With appropriate technology, it is possible to determine whether a treatment can be started, how much drug has to be administered and if a loss of therapy effectiveness occurs over time. In order to engage patients into the process of disease monitoring, technologies, which can be used with a connected device such as a smartphone from where the generated data can be shared, might become an additional key component of an effective treatment.

The current work applies pharmaceutical technology expertise to design optimized protein-based diagnostic assays with improved usability and make them applicable to established detection systems, which can be widely used with portable medical devices. Adequacy of the systems for therapeutic drug monitoring of biologicals was demonstrated in clinical studies.

1 Antibody characterization using protein microarrays

1.1 Utilisation of antibody microarrays for the selection of specific and informative antibodies from recombinant library binders of unknown quality

The present chapter was published in the journal New biotechnology in September 2016:

Janek Kibat, Thomas Schirrmann, Matthias J Knape*, Saskia Helmsing, Doris Meier, Michael Hust*, Christoph Schröder, Daniela Bertinetti, Gerhard Winter, Khalid Pardes, Mia Funk, Andrea Vala, Nathalia Giese, Friedrich W. Herberg, Stefan Dübel, Jörg Hoheisel‡. Utilisation of antibody microarrays for the selection of specific and informative antibodies from recombinant library binders of unknown quality. *New biotechnology*, 33(5):574?581, 2016.

* These authors contributed equally to this work
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As part of the Affinomics consortium this project was financially supported by the European Commission (EU grant contract no. 241481) with the intention to generate a variety of binder molecules against a multitude of tumor-associated antigens (TAA), which could be of therapeutic and/or diagnostic interest. In this regard, the overall strategy was divided into four steps: 1. Selection of TAA, 2. Preparation of recombinant TAA and scale-up of antigen production 3. Preparation of different binder formats against the produced TAA 4. Quality control of the manufactured binders.

Without the help of our cooperation partners in Copenhagen[1] (supply with antigens) and Braunschweig[2](supply with antibodies), Kassel[3] (surface plasmon resonance experiments) and Heidelberg[4] (clinical samples) this work would not have been possible.

All microarray-related and immunohistochemistry experiments as well as the writing of the manuscript was done by Janek Kibat.

The aim of this work was to describe a quick and convenient way which combines both, the identification of potentially interesting drugable or diagnostic antigen targets as well as the quality characterization of the used antibodies. Other than for small molecules and the corresponding Lipinski's rule of five, a preselection of potentially good or bad candidates is difficult for macromolecules such as antibodies. Therefore, standardized characterization methods and criteria, which determine the suitability of a therapeutic or diagnostic antibody are needed.

1.1.1 Abstract

Many diagnostic and therapeutic concepts require antibodies of high specificity. Recombinant binder libraries and related selection approaches allow the efficient isolation of antibodies against almost every target of interest. Nevertheless, it cannot be guaranteed that selected antibodies perform well and interact specifically enough with analytes unless an elaborate characterisation is performed. Here, we present an approach to shorten this process by combining the selection of suitable antibodies with the identification of informative target molecules by means of antibody microarrays, thereby reducing the effort of antibody characterisation by concentrating on relevant molecules. In a pilot scheme, a library of 456 single-chain variable fragment (scFv) binders to 134 antigens was used. They were arranged in a microarray format and incubated with the protein content of clinical tissue samples isolated from pancreatic ductal adenocarcinoma and healthy pancreas, as well as recurrent and non-recurrent bladder tumours. We observed significant variation in the expression of the E3 ubiquitin-protein ligase (CHFR) as well as the glutamate receptor interacting protein 2 (GRIP2), for example, always with more than one of the scFvs binding to these targets. Only the relevant antibodies were then characterised further on antigen microarrays and by surface plasmon resonance experiments so as to select the

[1]Khalid Pardes, Mia Funk, Andrea Vala
[2]Thomas Schirrmann, Saskia Helmsing, Doris Meier, Michael Hust, Stefan Dübel
[3]Matthias J. Knape, Daniela Bertinetti, Friedrich W. Herberg
[4]Nathalia Giese

most specific and highest affinity antibodies. These binders were in turn used to confirm a microarray result by immunohistochemistry analysis.

1.1.2 Introduction

The demand for highly specific and high-affinity antibodies is continuously and rapidly growing [1]. The advances in genomic sequencing, for example, are uncovering more and more disease-related changes, which frequently need to be elucidated in protein-based assays since they exhibit their functional effect at this molecular level. Companion diagnostics, in which the application of a drug is directly coupled to a patient stratification process, is also of increasing importance. Since most drugs affect proteins, most assays will probably analyse this molecular class, too. Access to antibodies is crucial for such ends. Currently, the antibody field is still dominated by monoclonal antibodies isolated from hybridoma cells [2]. While they represent the gold standard at present, binders that originate from recombinant selection systems are catching up quickly and are expected to become the norm sooner rather than later [3] and [4]. Based on libraries that can match the antibody diversity of the human immune system, they allow the isolation of binders to almost any desired target. They also permit flexibility with respect to the actual application profile. Binders against linear epitopes, for example, may not be suitable for recognition of three-dimensional structures and vice versa. By adaptation of the selection parameters, antibodies from a single library can meet varying requirements. However, similar to antibodies from hybridomas, the production of recombinant binders faces the common problem that individual molecules resulting from the initial selection screens differ tremendously in quality, in particular with respect to specificity and affinity. The majority of generated molecules will eventually fail quality requirements at some stage, making the effort already invested on characterisation useless and void.

In an effort to shorten the usually successively executed processes of antibody production, characterisation, application for antigen identification and selection of binders that exhibit appropriate performance parameters and are of informative value, we propose to combine the last three steps, at least partially, by means of binder microarrays. After an initial selection process - panning from a library by removing the antibodies that do not bind the targeted antigens at all - the remaining antibodies will be arrayed and incubated with protein samples of interest. For lack of any extensive characterisation, a large percentage of the primary antibody hits will be identified immediately as being of inadequate quality.

At the same time, however, molecules will be found that exhibit at least some degree of specificity and yield reproducible results that could be of biological or biomedical relevance. Only antibodies that meet sufficiently both requirements - binding specificity and informative value - will be characterised further in order to determine their quality. Once quality is established, the antibodies can then be used to confirm in other analysis formats the biological finding made in the microarray experiment.

In a pilot experiment, we have investigated a set of 456 recombinant antibodies. They were made within the framework of the Affinomics consortium, the most recent of three European networks for binder production and characterisation [5], and were designed to bind 134 tumour-associated antigens. Instead of selecting the best candidates in a one-by-one analysis of specificity and affinity, they were arranged as an antibody microarray without knowing about their quality. By incubation with clinical protein samples, we could combine the identification of proteins, which exhibit differential expression, with an identification of related antibodies with apparently appropriate performance. These antibodies were then subjected to further characterisation. Molecules that passed the process were subsequently used to validate the original microarray result by immunohistochemistry.

In the analysis, protein extracts from pancreatic and bladder cancer were used as well as appropriate controls. Pancreatic cancer is currently the most lethal cancer entity in the Western world [6]. Most patients die within a year of diagnosis, with mean survival being about five months; mortality is basically identical to incidence. In comparison, bladder cancer is far less lethal and frequently diagnosed relatively early. Low-stage, non muscle-invasive bladder cancer can usually be resected successfully. However, 60% of the tumours recur and then often become invasive [7].

1.1.3 Materials and Methods

1.1.3.1 Antibody microarray production

The antibodies were part of the human single-chain variable fragment (scFv) gene libraries HAL4/7/8 [8] and HAL9/10 [9]. Initial selections against 134 antigens (132 proteins and 2 peptides; Supplementary Tab. 1) of oncological context and poor coverage by commercial monoclonal antibodies were performed by library panning as described [10] leading to a set of 456 scFvs. Concentration dependent binding to the respective target molecules was confirmed by an ELISA using dilution series of the antibodies. The scFvs were then re-cloned so as to link them to a human IgG_1 Fc part as described in detail [8]. The

fusion proteins have superior stability. Experimentally, they behave very similarly to IgG antibodies and can be used with them in immunoassays without changing the underlying protocols. The antibodies were expressed in HEK293 6E cells and purified by affinity chromatography on protein A [11]. Antibody microarrays were prepared as described in detail previously [12]and [13]. In brief, the antibodies were spotted onto epoxysilane-coated slides (25 mm x 75 mm, Nexterion-E; Schott, Jena, Germany) using the contact printer MicroGrid II 610 (Apogent Discoveries, Mittenwald, Germany) and SMP6B pins (Telechem, Sunnyvale, USA) at a humidity of 55 - 65%. The printing buffer was composed of 50 mM sodium bicarbonate, pH 9.0, 100 mM NaCl, 50 mM $MgCl_2$, 0.005% (w/v) sodium azide, 0.25% (w/v) dextran, 0.006% (w/v) Igepal CA-630 (Sigma-Aldrich, St. Louis, USA) and 1 mg/ml of the respective antibody. All antibodies were spotted in quadruplicate on each microarray slide. The spots featured a diameter of about 250 μm. After the actual printing process, the slides were allowed to equilibrate at room temperature and 55-65% humidity overnight. They were then stored in dry and dark conditions at 4°C.

1.1.3.2 Preparation of protein samples

For all tissue samples, written informed consent was obtained from the patients or donors. The analyses performed were approved by the local ethics committee. As a matter of fact, tumour specimens were used, which had been studied before in a different setting. The bladder cancer samples had been isolated from patients with and without tumour recurrence after five years [14]. Also, we used nine tissue samples of patients with pancreatic adenocarcinoma (PDAC) and seven pancreas samples of patients, who had no cancer but had their pancreas removed for other reasons [15; unpublished results]. The protein extracts had been prepared as described in detail previously [13]. Total protein concentration was measured by the BCA assay (Thermo Fisher Scientific, Waltham, USA) and adjusted to 1 mg/ml. The protein was labelled with 0.4 mg/ml of the NHS-ester of the fluorescent dye Dy-549 (Dyomics, Jena, Germany) in 100 mM sodium bicarbonate buffer, pH 8.5, 1% (w/v) Triton-100 on a shaker at 4°C. After 1 h, the reaction was stopped by addition of 10% glycine. Unreacted dye was removed 30 min later and the buffer changed to phosphate-buffered saline (PBS) using Zeba Desalt columns (Thermo Fisher Scientific). While stoichiometric labelling is possible [15], it is not used routinely. Variations in labelling efficiency are normalised during the data analysis process. Subsequently, the Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany) was added as recommended by the manufacturer. All labelled protein samples were stored in aliquots at

−20°C until use. For competitive dual-colour incubation [16], a common reference sample was produced by pooling identical amounts of all protein samples and labelling the pool with the dye Dy-649 (Dyomics). The same reference sample was used throughout the analyses.

1.1.3.3 Antibody microarray analysis

Surface blocking and sample incubation were performed using a Tecan HS 4800 hybridisation system (Tecan, Männedorf, Switzerland). Slides were blocked with the Candor Blocking Solution (Candor Biosciences, Wangen, Germany) for 1 h and incubated with labelled protein for 3 h. Subsequently, the slides were scanned with a Tecan Powerscanner and images were analysed using GenePix 6.0 microarray analysis software (Axon Instruments, Union City, USA). Chipster software version 3.3 (http://chipster.csc.fi/) was applied for loess normalisation and statistical analysis.

1.1.3.4 Antigen microarray analysis

Full-length antigens were produced at the Københavns Universitet NNF Center for Protein Research (UCPH) by expression in E. coli and purified via a polyhistidine tag. To assure their purity, all proteins were tested by SDS-PAGE prior to immobilisation. The list of the 54 antigens used in the analysis is shown in Supplementary Tab. 2. The antigen microarrays were prepared as described for the antibody microarrays. For spotting, however, a glycerol-based buffer (0.4 M $NaHPO_4$, pH 8.6, 10% glycerol, 0.01% (w/v) sarkosyl) was used. Each antigen was spotted in triplicate within each of eight identical sub-arrays placed on one microarray slide. Incubation with antibodies was performed within 24 h after spotting. After an initial blocking with the Candor Blocking Solution, 250 μl 33 nM recombinant antibody was incubated on the arrays for 1 h. After washing, DY-649-P1-labelled protein A/G (Biotrend, Cologne, Germany) was added for detection to a concentration of 20 nM and incubated for 30 min. Experiments were carried out in triplicate.

1.1.3.5 Affinity measurements by surface plasmon resonance (SPR)

A Sierra Sensors SPR-4 instrument (Sierra Sensors, Hamburg, Germany) was used to acquire binding kinetics of selected scFv-Fc:antigen pairs. Antigens were immobilised covalently to a flat amine sensor chip to a level of 350 RU (protein CHFR) or 300 RU (GRIP2). Interaction analysis was performed by injecting several concentrations of the

corresponding scFv-Fc antibody diluted in PBS buffer at a flow rate of 30 μl/min. After each injection the surface was regenerated using 10 mM glycine (pH 1.9) for 30 s. All measurements were performed at 25°C. Non-specific binding, monitored on a blank flow cell, was subtracted from the SPR signals. Rate constants were determined using the SPR-4 analysis software (Sierra Sensors) assuming a 1:1 Langmuir binding model.

1.1.3.6 Immunohistochemistry (IHC)

For immunohistochemistry experiments, frozen tissue sections were produced. They were fixed with −20°C cold acetone for 5 min. Peroxidases were quenched with a 3% (v/v) H_2O_2 solution in methanol. Slides and tissue sections were first blocked with avidin (Dako, Kyoto, Japan) for 10 min, second with biotin (Dako) for 10 min and finally with Power Block (BioGenex, San Ramon, USA) for 1 h. Recombinant antibodies were biotinylated with EZ-Link NHS-PEG4-Biotin according to the manufacturer's protocol, diluted in antibody diluent (Dako S3022) to a concentration of 5-10 μg/ml and incubated on the sections overnight at 4°C. Prior to the incubation with streptavidin peroxidase (Kirkegaard & Perry Laboratories) for 30 min, slides were washed with Tris-buffer supplemented with 0.05% Tween-20. The colour reaction was carried out by incubation for 2 min with liquid DAB+ substrate (Dako) and counterstaining by Mayer's hematoxylin solution (Merck, Darmstadt, Germany). Images were captured using a Zeiss Cell Observer Z1 equipped with an AxioCam (Zeiss, Jena, Germany).

1.1.4 Results

1.1.4.1 Comparative antibody microarray analysis

The binder set used in this pilot study consisted of phage display selected single chain variable fragments (scFvs) isolated from universal phage display libraries, which allow the high-throughput selection of human antibody fragments. After initial selection by panning against 134 proteins or peptides (Supplementary Tab. 1), a set of 456 scFvs was picked from the binding molecules. Each scFv was fused to a human Fc-fragment in order to create scFv-Fc fusion antibodies. This binder format can be used equivalent to human IgGs [17] and [18]. In order to investigate which of the constructs should be studied in more detail, the scFv-Fc antibodies were spotted onto microarrays and incubated with clinical samples (Fig. 1.1 a). Incubation was with fluorescently labelled protein lysates obtained from pancreatic adenocarcinoma (PDAC) and healthy pancreas tissues as well

as resected bladder tumours from patients, who subsequently had either suffered tumour recurrence within five years of tumour resection or had no relapse. All samples had been studied before [14] and were known to exhibit expression differences for proteins other than those represented by the scFv-Fc fusion antibodies used here.

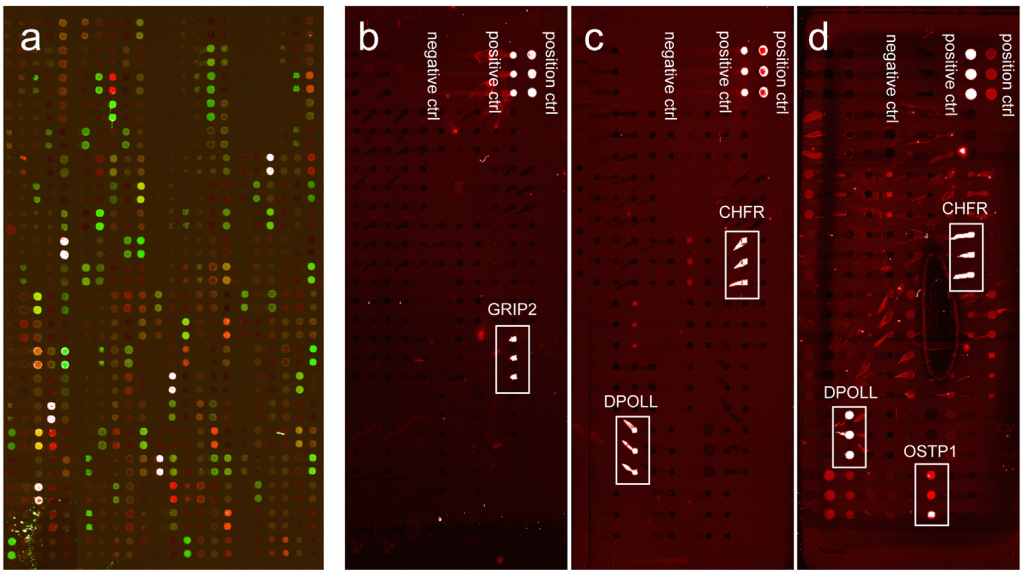

Figure 1.1: Typical results of microarray analyses.
(a) Overlay-image of the scFv-Fc antibody microarray incubated with protein samples from PDAC tissue (green signal) and the common reference (red signal). Depending on the amount of protein binding to each spot in comparison to the common reference, colours vary from green, via yellow and orange to red. (b) - (d) Images are shown of incubations on the antigen microarrays of antibodies DM380-JA70-F6 (b), DM319-JA37-E9 (c) and DM319-JA37-B11 (d). Bound scFv-Fc antibody was detected with fluorescently labelled protein A/G. White spots indicate a signal that is too intense to be presented in colour. Spot streaking is an experimental artefact introduced during drying the arrays with pressurised air.

Sample labelling and incubation as well as data analysis were performed according to well-established procedures [12] and [16]. Volcano plots (Fig. 1.2) present the degree and significance of differential protein expression. In total, antibodies to 31 proteins exhibited a potentially significant up- or down-regulation in PDAC compared to normal tissues, and antibodies to 38 proteins showed such changes when comparing non-recurrent and recurrent bladder cancer tissues. Since 9 proteins were shared by both analyses, an overall total of 60 proteins was found to be potentially differentially expressed in the initial analysis. For the other 84 of the 134 proteins for which antibodies had been isolated, no variation

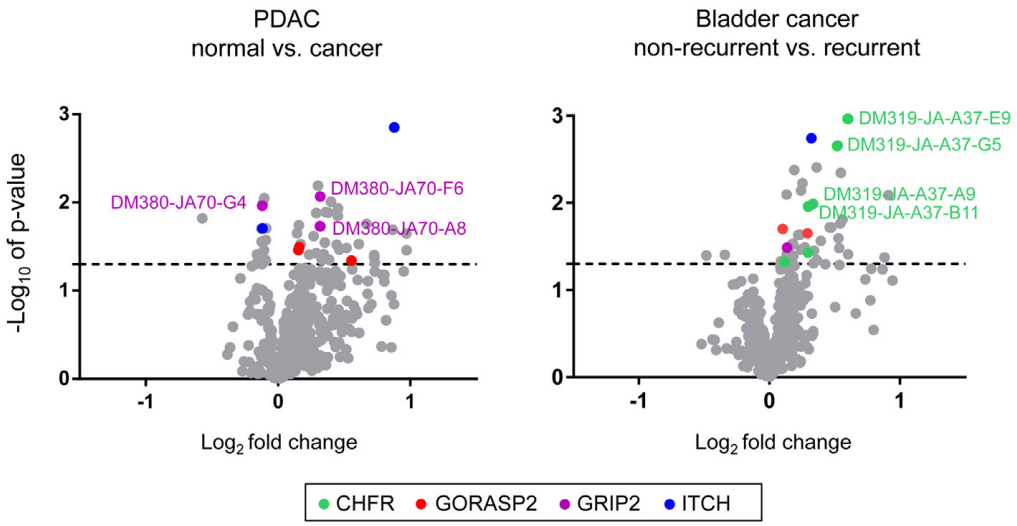

Figure 1.2: Visualisation of the antibody microarray analysis results as volcano plots. (Left panel) Relative variation of protein abundance in lysates of normal pancreatic and PDAC tissues, respectively. A negative $\log_2$ fold change indicates down-regulation, while a positive change indicates overexpression of proteins in PDAC tissue compared to normal tissue. (Right panel) The same is shown for proteins in non-recurrent versus recurrent bladder cancer tissues. Dots of the same colour represent antibodies which were meant to recognize the same antigen; the colour code is given. The names of some relevant antibodies are shown. The dotted horizontal line represents a p-value of 0.05.

was identified. This could be due either to the lack of any such difference in the studied protein samples or the absence of an antibody of sufficient sensitivity or specificity. For the majority of the 60 proteins that yielded varying signals, there was only one antibody that showed this variation, while the other binders that were intended to bind to the same target did not produce such a result. Four proteins - CHFR, ITCH, GORASP2 and GRIP2 - exhibited significant variations on more than one of the antibodies that had been produced against them (Fig. 1.2). The consistent results made it likely that the relevant scFv-Fc antibodies actually bound to the same target and that this target was differentially expressed in the investigated sample cohorts. These antibodies (Table 1.1) were selected for further characterisation.

1.1.4.2 Quality control of selected antibodies

After their initial isolation from the recombinant library, all scFv-Fc antibodies had been used for ELISAs, testing if they recognized the antigen by which they were selected in the

Table 1.1: List of antibodies selected for further characterisation.
'+' indicates the detection of a variation in the abundance of the assumed target protein upon incubation of the antibody microarray with protein extracts isolated from clinical tissue samples.

Antibody name	Antigen specificity (presumed)	PDAC versus normal	Bladder cancer recurrent versus non-recurrent
DM319-JA37-E9	CHFR Uniprot ID: Q96EP1		+
DM319-JA37-G5	CHFR		+
DM319-JA37-A9	CHFR		+
DM319-JA37-B11	CHFR		+
DM319-JA37-H7	CHFR		+
DM319-JA37-D4	CHFR		+
DM379-JA69-F11	GORASP2 Uniprot ID: Q9H8Y8	+	
DM379-JA69-C9	GORASP2		+
DM379-JA69-G7	GORASP2	+	
DM379-JA69-H3	GORASP2	+	
DM379-JA69-E4	GORASP2		+
DM380-JA70-F6	GRIP2 Uniprot ID: Q9C0E4	+	
DM380-JA70-A8	GRIP2	+	
DM380-JA70-G4	GRIP2		
DM380-JA70-B1	GRIP2		+
DM382-JA72-B7	ITCH Uniprot ID: Q96J02	+	+
DM382-JA72-H1	ITCH		

panning process. However, these tests only determined if an antibody was able to bind its target, but did not provide any information about specificity. In order to investigate the degree of cross-reactivity of the candidate antibodies selected by the microarray analysis, they were incubated individually on an antigen array onto which full-length proteins had been spotted (Fig. 1.1 b - d). In our pilot scheme, 54 proteins were presented on the antigen array, including CHFR, ITCH, GORASP2 and GRIP2 (Supplementary Tab. 2). Only the anti-CHFR antibody DM319-JA37-G5 (Fig. 1.3 a) and the anti-GRIP2 antibodies DM380-JA70-F6 and -A8 (Fig. 1.4 a) recognized their target proteins with good specificity. The other candidates were either cross-reactive (anti-CHFR antibodies DM319-JA37-A9, -B11 and-E9) or did not bind to the antigen array at all (anti-GRIP2 antibody DM380-JA70-G4 as well as all anti-ITCH and anti-GROASP2 antibodies). The cross-reactive anti-CHRF antibodies produced very similar binding patterns, also recognizing the proteins DPOLL, OSTP1, OTUB1 and PRDM8, but with varying intensities (Fig. 1.3 a). For confirmation that ITCH and GROASP2 did not interact with their antibodies, the proteins were labelled fluorescently and individually incubated on antibody microarrays. In this inverse system too, there was no binding to the respective antibodies (data not shown), which was surprising as they had exhibited binding in ELISA tests previously.

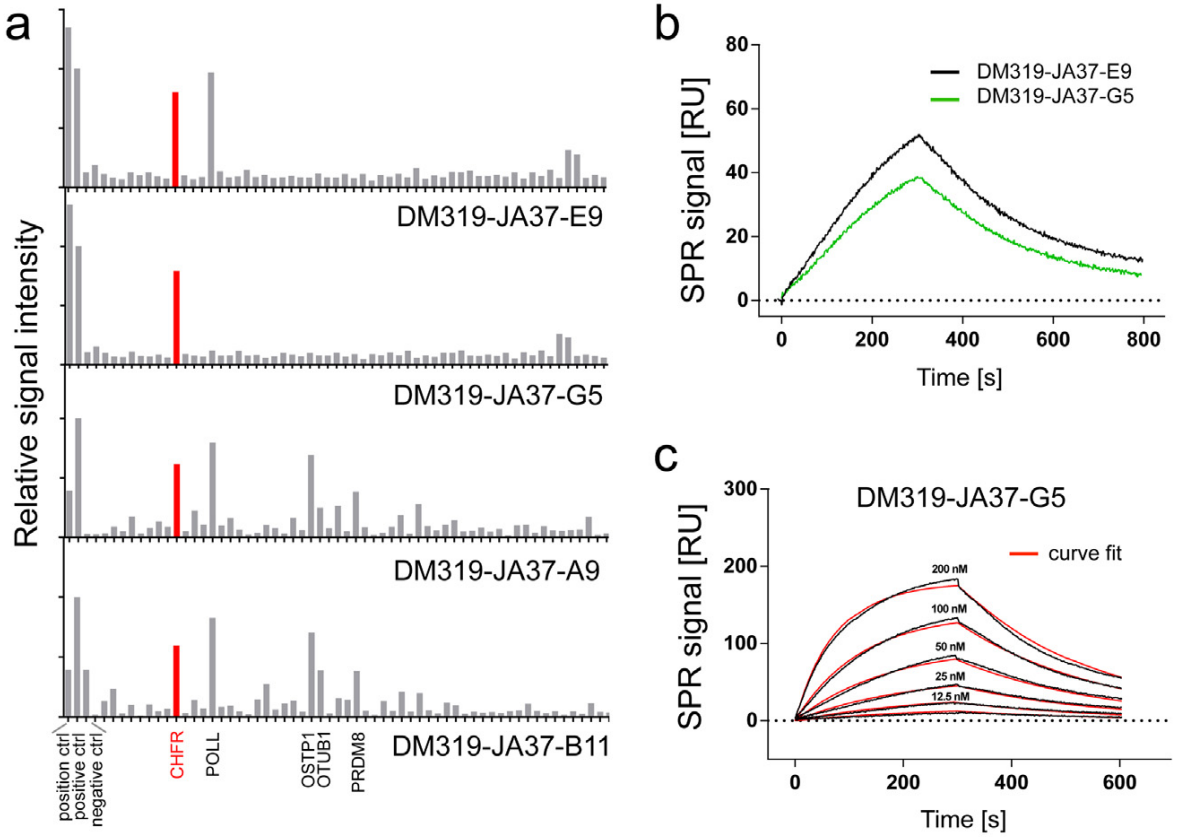

Figure 1.3: Quality control of anti-CHFR antibodies.
(a) Binding intensities on the antigen arrays are shown. Signal intensities were normalised to the value achieved with an immobilised control antibody. The desired target antigen is marked in red. For better visualisation, no error bars are shown. However, the data is available as Supplementary Tab. 3. (b) SPR binding curves of two anti-CHFR scFv-Fc antibodies are shown. (c) SPR binding curves are presented of the best performing antibody (DM319-JA37-G5) at different concentrations; from this, a K_D of 82.2 nM was calculated.

To qualify the array-based results further, the affinities of the anti-CHFR and anti-GRIP2 antibodies were measured quantitatively with SPR. For comparison to the antigen array data, the antigens were immobilised and the antibodies were added in different concentrations. Anti-CHFR antibodies DM319-JA37-G5 (specific) and DM319-JA37-E9 (cross-reactive) showed comparable binding kinetics with moderate dissociation rates (Fig. 1.3 b). Assuming a 1:1 Langmuir binding model, a K_D of 82.2 nM could be calculated for anti-CHFR antibody DM319-JA37-G5 (Fig. 1.3 c). For the anti-GRIP2 antibodies (Fig. 1.4), there was strong binding of the specific binder DM380-JA70-F6, which was also charac-

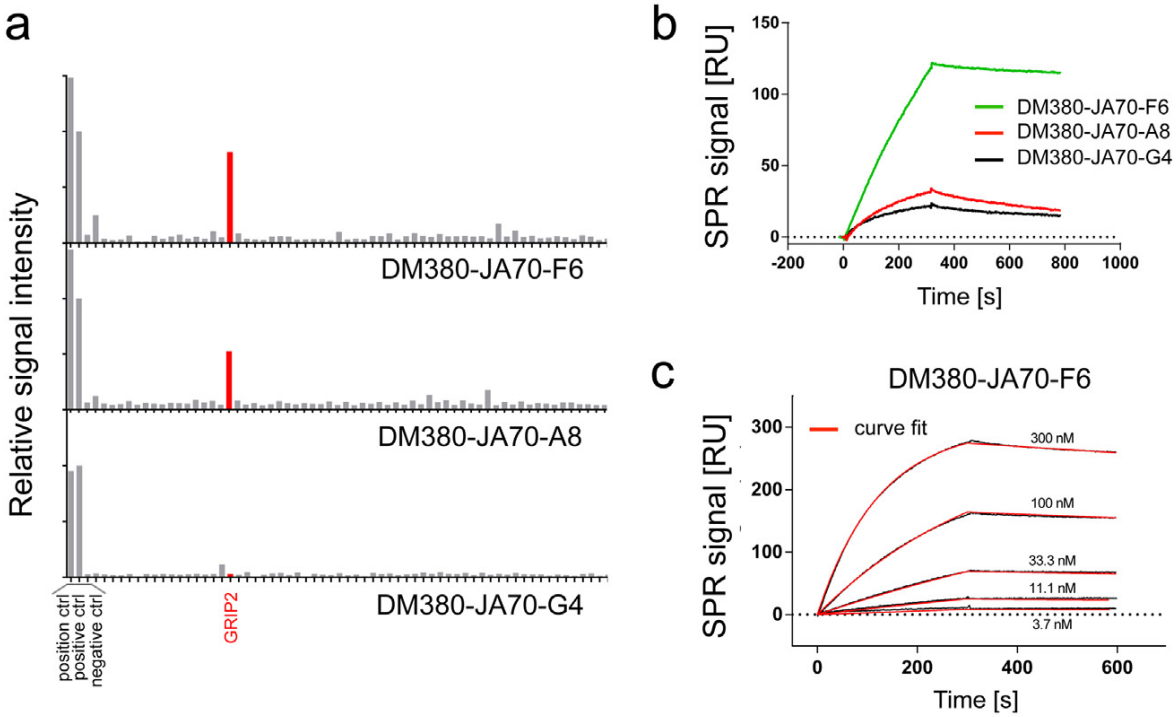

Figure 1.4: Quality control of anti-GRP2 antibodies.
(a) Binding intensities on the antigen array are shown. Signal intensities were normalised to that of an immobilised control antibody. The desired target antigen is marked in red. (b) SPR binding curves of three anti-GRIP2 scFv-Fc antibodies are shown. (c) SPR binding curves are presented of the best performing antibody at different concentrations; from this, a K_D of 6.5 nM was calculated.

terised by its slow dissociation rate, whereas for the antibodies DM380-JA70-A8 (specific) and DM380-JA70-G4 (cross-reacting) binding was significantly weaker. A K_D of 6.5 nM was calculated for DM380-JA70-F6.

1.1.4.3 Confirmation of GRIP2 overexpression by immunohistochemistry

Some of the antibodies that had yielded apparently interesting data in the microarray analysis could be confirmed by the quality control experiments as binders of good specificity and affinity. In turn, we used one of these good performers for validating the differential protein abundance in the tumour samples observed in the microarray analysis. To this end, immunohistochemistry was carried out on frozen tissue sections (Fig. 1.5). Besides confirming the biological variation seen in the microarray assay, the immunohistochemistry assay allowed simultaneous testing of the binder performance. Analysing pancreatic

tissue sections with the specific and high-affinity anti-GRIP2 antibody DM380-JA70-F6 confirmed that GRIP2 was highly abundant in PDAC and chronic pancreatitis compared to normal tissue. As expected, antibody DM380-JA70-G4 produced inconclusive results.

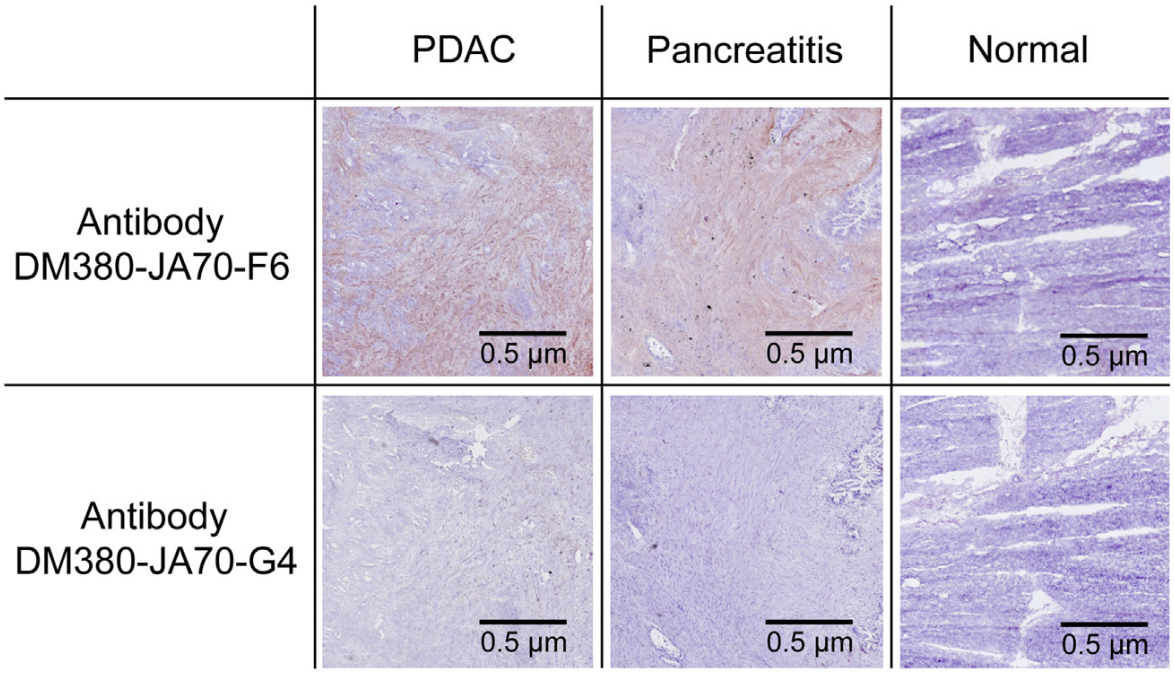

Figure 1.5: Immunohistochemistry analysis of GRIP2 abundance in pancreatic tissues. Tissues isolated from patients with PDAC or chronic pancreatitis and from donors with healthy pancreatic tissue (normal) were stained with two antibodies. The antibody DM380-JA70-F6 clearly showed a higher abundance of GRIP2 in diseased pancreas.

1.1.5 Discussion

Although only performed on a small-scale, these pilot experiments demonstrated the feasibility of screening, by means of antibody microarrays, a preselected antibody library containing binders of unknown quality for the isolation of useful antibodies, without testing the performance parameters of every single candidate beforehand. Simultaneously, utilising representative samples, the process specifically selected antibodies that could be informative for a given application, such as the discrimination of pancreatic tumour from healthy tissue. Actually, there could well be more 'good' antibodies in the set of 456 molecules, useful for analyses other than that of pancreatic and bladder tissues. The strategy is most

suited for a targeted binder selection process. The array format permits flexibility with respect to the number of molecules that could be studied. This number and the degree of selectivity of the microarray assay can be adapted to both the actual needs and the capacity for the subsequent, much more laborious binder characterisation. Similarly, at the other end, the stringency of the pre-selection process, through panning the original binder library against the protein(s) of interest, can be tailored, depending on the capacity of the array analysis.

Our analysis also demonstrated, unsurprisingly, that further characterisation of the selected antibodies is essential; the antibody microarray analysis on its own is clearly insufficient when dealing with molecules of unknown quality. Although there were several antibodies that indicated abundance variations of proteins ITCH and GROASP2, for instance, this result could not be confirmed during the subsequent antibody characterisation. It is likely that the relevant antibodies bind to other proteins exhibiting reproducible abundance variation. Neither when antibodies were applied to the antigen array nor in the reverse experiment, when the individual proteins were incubated on the antibody microarrays, could specific binding be detected. In our pilot scheme, only a small antigen array was used for identification of binding specificity. The use of more comprehensive antigen microarrays would lead to a much better proof of the accurate binding of an antibody to the intended target or the identification of any cross-binding activity. In vitro production of proteins, and in particular an in situ synthesis directly on the microarray surface [18], [19] and [20] permits the production of such complex analysis platforms.

The objective of our study was a demonstration of the utility of the microarray-based selection process. During the course of this, disease related differences in protein abundance were identified and related antibodies of nanomolar affinity were isolated. However, the biological information gained from this is too preliminary to draw any conclusions on the value of GRIP2 or CHFR for diagnosis, for instance. Firstly, the sample number was too small. Secondly, for a comprehensive analysis, further controls would be essential, such as samples from chronically inflamed pancreatic tissue. Nevertheless, even in the very limited setting of the pilot scheme, we succeeded in isolating antibodies that could well be useful for biologically or biomedically motivated studies. Antibody DM380-JA70-F6, for example, is the first monoclonal antibody against GRIP2 according to the Antibodypedia data repository (version 9 of June 2015; http://www.antibodypedia.com) with information on 1 768 896 antibodies.

In conclusion, an efficient strategy was established that combines the identification of protein variations of informative value with the selection of related antibodies from a set of uncharacterised molecules derived from human antibody gene libraries. Only after the application of relevant protein samples to the antibody microarray are binders of potential interest characterised further, so as to isolate appropriately performing antibodies for further utilisation. This limits the task of antibody characterisation - a bottleneck in current antibody production - to antibodies that are informative in a particular biological context of interest.

1.1.6 Acknowledgements

We are grateful to Lukas Selzer, Sandra Schifferdecker and Christoph Harmel for technical assistance. The work was financially supported by the European Commission as part of the Affinomics consortium (EU grant contract no. 241481). The Novo Nordisk Foundation Center for Protein Research is supported financially by the Novo Nordisk Foundation.

1.1.7 Supplementary data

The supplementary data to this article can be downloaded here:

Supplementary Table 1 - List of proteins used for antibody selection.

Supplementary Table 2 - List of proteins that were presented on the antigen array.

Supplementary Table 3 - Relative intensities of binding to protein CHFR as measured on the antigen microarray (Fig. 1.3)

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2 Investigation and optimization of protein microarrays as platform for the absolute quantification of proteins

Apart from the utilization of protein microarrays for the characterization of antibodies and identification of drug or biomarker targets, we aimed to develop a microarray-based diagnostic tool to be used at the point-of-care. The suitability of this platform, to allow quantitative measurements was successfully demonstrated in chapter one. However, the underlying quantification principle here was based on a relative comparison of protein expression levels. In order to be applicable to the measurement of a one ore more individual biomarkers, an absolute quantification strategy had to be developed. In chapter 2.1., the optimization of a quick, specific, robust and sensitive method for the absolute measurement of prostate specific antigen concentrations in blood is described. Furthermore, an approach to investigate the stability of proteins immobilized on the microarray surface was examined in chapter 2.2., as the protein functionality also contributes and determines the assay performance.

2.1 Optimization of a microarray-based method for the quantification of biomarkers in blood

2.1.1 Abstract

The overall goal of this project was to develop a portable biomarker detection system, which provides quick results with sufficient sensitivity and is potentially operable in combination with a smartphone. For this purpose, the prostate specific antigen (PSA) as one of the most established and low abundant protein biomarkers was chosen as model protein. In this work, different parameters influencing the quantification performance of a

microarray-based competitive PSA quantification strategy were systematically characterized. After the optimization of the major components - detection antibody, presentation of antigen and incubation time - a method was established, which allows the quantification of PSA in spiked-in whole blood samples in the range of 1 - 100,000 ng/ml within 15 minutes. In this proof-of-concept work, we could further demonstrate a functional multiplexed quantification of PSA and tumor necrosis factor alpha (TNF-α) with the option to include additional protein candidates.

2.1.2 Introduction

In medicine, biomarkers are defined as measurable indicators of a disease state. Usually, the parameter to be measured is the concentration of a specific molecule, which, in the ideal case, exhibits a low value at the healthy state and can be understood as a sign of disease development once it exceeds a certain threshold. The monitoring of a biomarker can be very helpful for any kind of disease as it provides the opportunity to potentially intervene at a very early disease stage [1]. Biomarkers are not only useful for the detection of certain diseases but can also provide therapy supporting information e.g. when it comes to the question which therapy to select or when to switch to another treatment due to an acquired resistance. These approaches, combining diagnostic as well as therapeutic concepts, are described with the term precision medicine.

The molecules of interest are often DNA fragments, especially in mutation driven diseases such as cancer. Taking into account that the translation of DNA into a functional protein is dependent on many factors and can lead to different protein modifications, each exhibiting a different function, it is of advantage to investigate the proteome and use specific proteins as biomarker [2, 3]. A drawback of this approach is because, from an analytical and technical point of view, many proteins exhibit a rather low abundance and cannot be amplified as it is possible with DNA. Commonly and typically used LC-MS methods feature a sufficient sensitivity, yet, because of the biological specimen matrix, these methods are laborious in terms of method development as well as sample preparation and limited regarding throughput. Therefore, sensitive and robust methods for the multiplexed detection of proteins are needed.

In order to develop a protein biomarker quantification method, which could be used as a diagnostic tool, immunoassay strategies seem to offer advantages with respect to their sensitivity, high specificity and their read-out practicability in a non-laboratory environ-

ment [4]. A convenient format, especially if a multiplexed analysis is needed, are microarrays [5]. Amongst the variety of available microarray platforms - planar, bead- or microtiter-based - the focus was set on planar microarrays in this work.

The immobilization of small protein spots on a functionalized surface allows us to print a huge amount of different proteins on a rather small area. In this miniaturized fashion, a relatively small amount of sample material is needed, compared to a microtiter plate. By this the simultaneous measurement of several parameters of interest in one sample can be enabled. The protein microarray platform in the form of an antibody microarray is typically used for quantitative proteomics such as the identification of biomarkers which had been described in the previous chapter. The quantification strategy used here is a relative one, which is based on the comparison of protein levels in samples from healthy and diseased patients. However, for the measurement of an individual biomarker with a diagnostic tool, an absolute quantification is needed to distinguish if a critical, disease indicating concentration is exceeded. Microarray-based methods, which allowed the detection of single molecules are described in literature and highlight the suitable sensitivity of this application [6].

Due to the mentioned advantages of a handy platform and because the read-out of microarrays can be accomplished with a simple fluorescence detector, we focused on the development of a portable diagnostic test, which could be employed at the point-of-care or even by the end user (physician or patient). The development of portable microarray read-out systems is an emerging field and several systems have already been described in literature [7–9]. Consequently, it is more of importance to select and develop a suitable immunoassay detection format as the test itself can be easily transferred onto present systems. In this work, the prostate-specific antigen (PSA), a well-established biomarker for prostate cancer, was selected as model protein to develop and test a microarray-based immunoassay in the first place. PSA is primarily present in the blood of men at a concentration of 0 - 4 ng/ml in healthy individuals. If the threshold of 4 ng/ml is exceeded, a biopsy is indicated to check if a carcinoma can be localized, which is the case in about 30 % [10]. As a matter of fact, our test had to feature a quantifiable range of at least 1 - 10 ng/ml.

Based on findings of our group, a good understanding of general parameters, which influence the performance of protein microarrays, had been acquired over the time [11,12]. This includes factors such as spot morphology, spot uniformity, choice of surface functionalization and protein attachment methods, but also protein concentration and suitable blocking

reagents. Nevertheless, as the aim of this project was to develop an absolute quantification strategy for a limited amount of protein candidates, a refinement for the particular use was needed. Apart from a conventional ELISA-like sandwich assay, we decided to choose a competitive quantification strategy (see figure 2.1), because it exhibits the advantage of an adjustable quantification range [13].

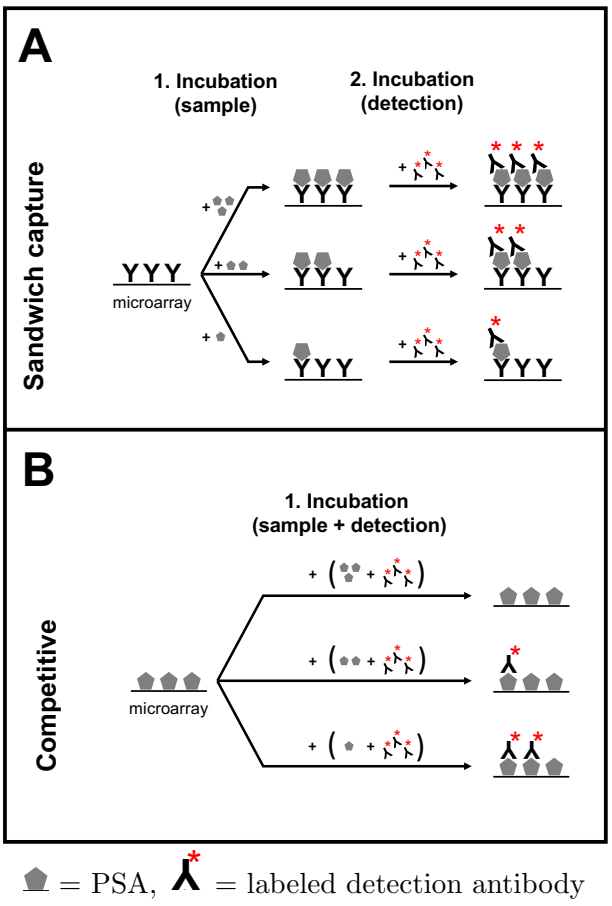


Figure 2.1: A - Direct quantification in sandwich capture format
A microarray with anti-PSA antibodies is incubated with the PSA sample solution (illustrated: high, middle and low concentration). After the first incubation a labeled anti-PSA detection antibody is incubated in a second step.
B - Competitive quantification
The PSA sample solution (illustrated: high, middle and low concentration) is mixed with a defined amount of labeled anti-PSA detection antibody. This mixture is incubated on a microarray with PSA. Depending on the amount of PSA more or less unbound labeled anti-PSA detection antibody will be generated and able to bind to the immobilized PSA on the microarray surface.

The theoretical background of a competitive quantification can be explained by the following equations:

If a competitive quantification strategy is employed, a labeled detection antibody is added to the sample solution containing the antigen of interest. The amount of detection antibody (Y) as well as antigen (A) determines the amount of the forming immune complex YA according to the law of mass action.

$$[Y] + [A] \rightarrow [YA] \tag{2.1}$$

$$K = \frac{[YA]}{[Y] \cdot [A]} \tag{2.2}$$

In this system, the component with the lower concentration will limit the amount of immune complexes, so we can transform the equation as a function of the amount of the formed immune complex:

$$[Y] = [Y_0] - [YA] \tag{2.3}$$

$$[A] = [A_0] - [YA] \tag{2.4}$$

if [YA] = x

$$K = \frac{x}{([Y_0] - x) \cdot ([A_0] - x)} \tag{2.5}$$

$$\frac{x}{[A_0] - x} = K[Y_0] - Kx \tag{2.6}$$

In other words:

$$\frac{antigen_{bound}}{antigen_{unbound}} = K[Y_0] - Kx \tag{2.7}$$

Equation 2.7 indicates that the equilibrium of bound and unbound antigen is dependent on the affinity constant K and the initially added amount of the detection antibody $[Y_0]$. If this solution is now incubated on a microarray surface with immobilized antigen, the unbound amount of detection antibody will also bind to the immobilized antigen.

2.1.3 Materials and Methods

Table 2.1: Materials and chemicals

Name	Description	Supplier
DY-649P1	NHS-ester, MW: 1066.10 kD	Dyomics
NHS-PEG5k-Biotin	bifunctional polyethylene glycol MW: 5 kD	Nanocs
UDP-$GalN_3$	Click-iT Enzymatic Labeling System Kit	Thermo Fisher
QD655	quantum dots - SiteClick Antibody Labeling Kit	Life Technologies
Triton X-100		Sigma-Aldrich
Tween® 20	Polysorbate 20	Sigma-Aldrich
$NaHCO_3$		Sigma-Aldrich
DMSO	Dimethylsulfoxide	Sigma-Aldrich
Nexterion E	2D epoxysilanized glass slide	Schott
3D Epoxy	3D epoxysilanized glass slide	PolyAn
2D Alkyne	2D alkyne functionalized glass slide	PolyAn
3D Alkyne	3D alkyne functionalized glass slide	PolyAn
3D Malemide	3D malemide functionalized glass slide	PolyAn
Neutravidin	neutravidin-coated 2D epoxysilanized glass slide	PolyAn
Streptavidin	streptavidin-coated 2D epoxysilanized glass slide	PolyAn
Coating buffer	Coating buffer 10x	Candor
Dextrane-based spotting buffer (2x)	0.2 M $NaHCO_3$ (pH 8.5), 1 % (w/V) dextran, 0.012 % (w/V) Igepal® CA-630, 100 mM NaCl, 10 mM $MgCl_2$	
Glycerol-based spotting buffer (2x)	0.4 M $NaHPO_4$ (pH 8.5), 10 % (w/V) glycerol, 0.01 % (w/V) sarkosyl	
PSA	Prostate specific antigen (UniprotID: P07288)	Abcam
CHYH2	monoclonal mouse anti-human PSA IgG1	Anogen
TNF-α	Tumor necrosis factor alpha (UniprotID: P01375)	Biomol
Inflximab	anti-TNF-α (UniprotID: DB00065)	MSD

2.1.3.1 Protein modifications

Antibody labeling with DY-649-P1
The detection antibody was labeled using the NHS-ester of the Cy5-like fluorescent dye DY-649P1. For this purpose, the antibody concentration was adjusted to 1 mg/ml in a 0.1 M NaHCO3 buffer with a pH of 9.0 supplemented with 0.01 % (w/v) Triton X-100. The dye was dissolved in dry DMSO at 1 mg/ml and added to the protein solution with a molar ratio of 4 - 32:1 (dye : protein) to reach labeling efficiencies of 1, 2 and 4 dye molecules per protein. After overnight incubation at 4°C, the buffer of labeled protein was exchanged to PBS using Amicon ultra-0.5 centrifugal filter units (Merck Millipore) with a MWCO of 3 kDa.

Antibody labeling with QD655
Antibodies were labeled with QD655 according to the manufacturer's protocol provided with the SiteClick Antibody Labeling Kit.

PSA PEGylation
The PEGylation of PSA was carried out as already described for the antibody labeling. Instead of a fluorescent dye, NHS-PEG4-N3 or NHS-PEG5k-Biotin was used. The PSA-PEG-Biotin conjugate could be purified and analyzed using size exclusion chromatography (ÄKTAexplorer, GE Healthcare, mobile phase: PBS, column: Superdex 200 10/300 GL, detection: absorption at 280 nm).

2.1.3.2 Protein microarray production

For the preparation of protein microarrays glass slides with different functionalities were used (see above). Proteins were adjusted to the needed concentration, mixed with twofold spotting (see table 2.1) and spotted using a MicroGrid II 610 (Biorobotics, USA) contact printer. Prior to sample incubation, slides were blocked for 1 h with the Candor Blocking Solution (Candor, Wangen im Allgäu, Germany) and washed with PBS supplemented with 0.05 % (v/v) Tween 20 (Sigma Aldrich) using a Tecan HS 4800 hybridization system (Tecan Group Ltd., Männedorf, Switzerland).

2.1.3.3 Sample preparation, microarray incubation, read-out and data analysis

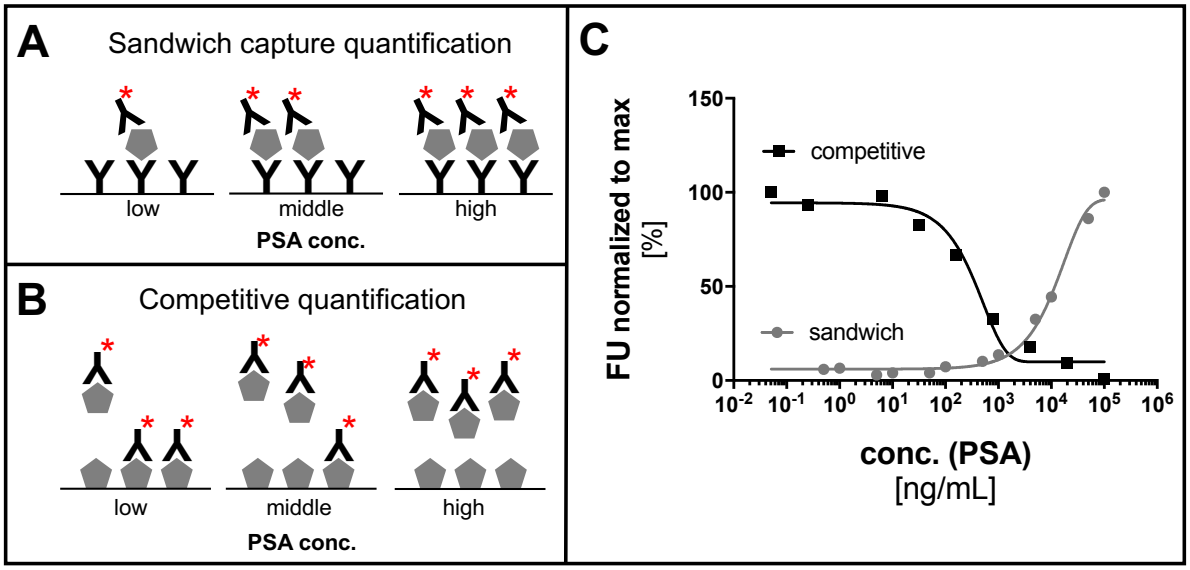
Serial dilution series of PSA were mixed in PBS, blocking solution or spiked into whole blood from female donors. The sample solutions were diluted 1:1 with blocking solution containing the detection antibody (CHYH2-DY649P1). The blocked slides were sealed with ProPlate® 16-well incubation chambers (Grace Bio Labs). Each well was filled with 70 μL of sample solution and incubated for up to 30 minutes at room temperature. After incubation, each well was washed separately with PBST (PBS supplemented with 0.05 % v/v Tween 20) and ddH20. The dried slides were than scanned using a Tecan PowerScanner (Tecan Group Ltd., Männedorf, Switzerland) and images were analyzed using the GenePix 6.0 microarray analysis software (Axon Instruments, Inc., Union City, CA). The median spot intensities without background correction were used for the analysis. At least five spots were averaged per detection area (intraarray). For precise quantifications, the intraarray averaged values were then again averaged with the values of three separate incubations (interarray).

2.1.4 Results and discussion

Instead of a sandwich capture immunoassay format, which requires two to three incubation steps with several detection antibodies, a competitive strategy was chosen as only one detection antibody and just one incubation step was needed. Furthermore, this approach offered the opportunity to individually adjust the desired quantification range of each investigated protein. The two detection principles are opposed in figure 2.2.

In contrast to the sandwich format, the competitive quantification strategy yielded the highest signal intensities at low PSA concentrations and the lowest intensities at high PSA concentrations - illustrated in figure 2.2 A and B as well as in the resulting quantification curves in 2.2 C. The actual quantification range in the competitive setup was defined, but also limited, by the dynamic range between the zero and the infinite concentration response asymptote, forming an upper and a lower limit of detection.

Comparing the lower limit of detection (graphical interpretation: inflexion point from the zero-response asymptote towards increasing or decreasing signal intensities) of both formats, the competitive strategy showed to be ten times more sensitive than the sandwich capture format. Yet, for a suitable quantification of PSA, further optimization was needed.



= PSA, = labeled detection antibody, FU = fluorescent units

Figure 2.2: **A** - Direct quantification in sandwich capture format.
B - Competitive quantification.
C - Resulting quantification curve in sandwich vs. competitive format (four parameter logistic curve fit).

In the following sections, we describe the investigation of each assay component such as the detection antibody, the antigen as well as the surface to evaluate its effect on the assay performance in terms of incubation times, signal intensities, detection limits and multiplexing characteristics.

2.1.4.1 Detection antibody

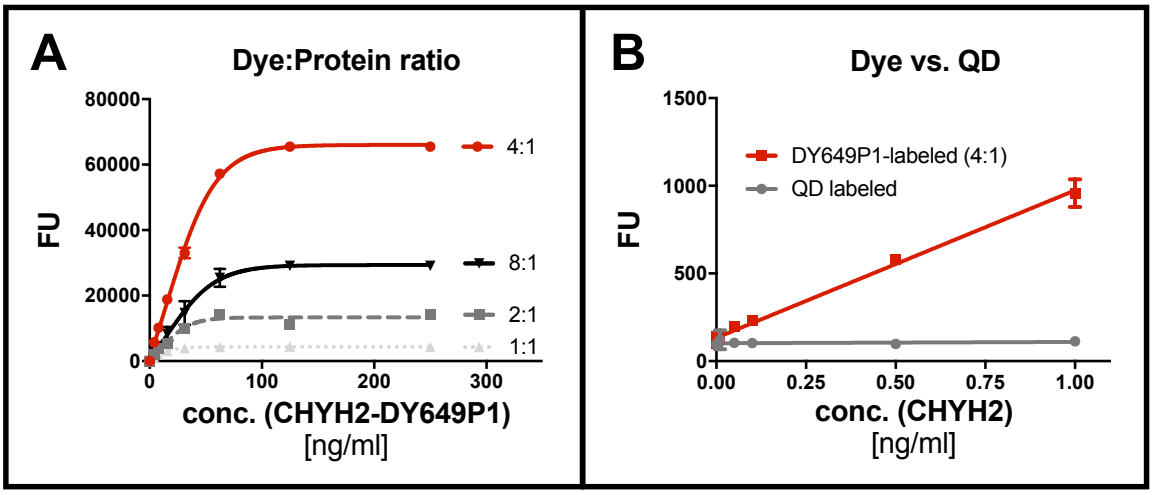
In the chosen format of a competitive quantification, the detection antibody represents the signal-giving component. Several parameters such as labeling ratio, incubation time and detection antibody concentration were optimized to enhance the signal intensity and thereby tune the limit of detection.

Selection of antibody with highest affinity
As described in the theoretical part earlier, the affinity of the used detection antibody as well as its concentration, had to be considered when optimizing the competitive quantification method. Amongst several detection antibodies, the mouse anti-human PSA antibody with the clone number CHYH2 exhibited the highest affinity with a K_D of 8.2 nM and was therefore chosen for all subsequent experiments.

Labeling of detection antibody
A directly labeled detection antibody was preferred rather than the utilization of a secondary antibody to keep the number of incubation steps as low as possible. As the fluorescent properties of the labeled antibody influence the overall assay sensitivity, it was tested which labeling strategy provided the highest signal intensities. As depicted in figure 2.3 A, a fluorescent dye was conjugated to the detection antibody in different dye:protein ratios. The gained fluorescent intensities of the dye labeled antibody were then compared with a quantum dot (QD) labeling as seen in figure 2.3 B. Quantum dots are known to exhibit a fluorescent yield well above the ones of dyes and therefore might be an alternative option to increase signal intensities.

An increased dye:protein ratio resulted in higher signal intensity (figure 2.3 A). An overlabeling, which lead to a reduced affinity and binding, was, however, observed at a dye:protein ratio of 8:1. Consequently, a dye:protein ratio of 4:1 was found to be the most suitable strategy because the highest signal intensities were achieved here. Moreover, concentration changes resulted in a more pronounced signal intensity difference and smaller concentration changes could better be detected.

A site-click quantum dot labeling (quantum dot was conjugated to glycosylation of antibody) did not show any superior fluorescent properties in comparison with a dye labeling. An explanation for this might be the increased size of a quantum dot (26 nm in diameter). During the incubation process, the quantum dot-coupled antibody might behave



CHYH2 = anti-PSA detection antibody, DY649P1 = Cy5-like fluorescent dye, QD = quantum dots, FU = fluorescent units

Figure 2.3: Labeling of detection antibody using the Cy5-like fluorescent dye DY-649P1 (A) or fluorescent quantum dots (B).
Experimental setup: Different concentrations of labeled detection antibody were incubated on 2D epoxysilanized microarrays with immobilized PSA.

more like a rolling nanoparticle with a size of approximately 36 nm, rather than a free moving molecule. In this regard, a reduced diffusion rate due to sterical hindrances and mass transportation limitations might prevent the antibody from binding. Similar observations are also described and discussed in literature [14].

Other signal increasing methods such as rolling circle amplification or reporter enzymes for the generation of fluorescent dyes would not have met the requirements of a fast and reproducible one-step solution for the desired application.

Incubation time

Investigating the incubation time as the most time-consuming step, a compromise between an adequate signal intensity and a short duration had to be made. The gained results, represented in figure 2.4, indicate that the steady-state antibody binding capacity was attained after one hour. In contrast to the quickly rising signal intensity, only a moderate background noise increase was observed. As the signal-to-noise ratio (S/N) did not further improve at incubation times beyond fifteen minutes, this incubation time was chosen for our setup. As discussed by Kusnezow et al. [15], the optimal design of a microarray assay to compensate kinetic limitations would span an incubation time of several hours.

Yet, it is also mentioned that prolonged incubation times lead to a pronounced dissociation. Consequently, a longer incubation time would not necessarily result in a better assay performance as dissociation processes can be dependent on additional and probably more uncontrollable parameters. This also applies for washing steps, which in our setup were reduced to a minimum of two PBST washing steps followed by three rinsing steps with water at the end of each incubation.

Moreover, choosing a time point not in the plateau level but in the dynamic range, required an exact incubation time control to enable a precise quantification.

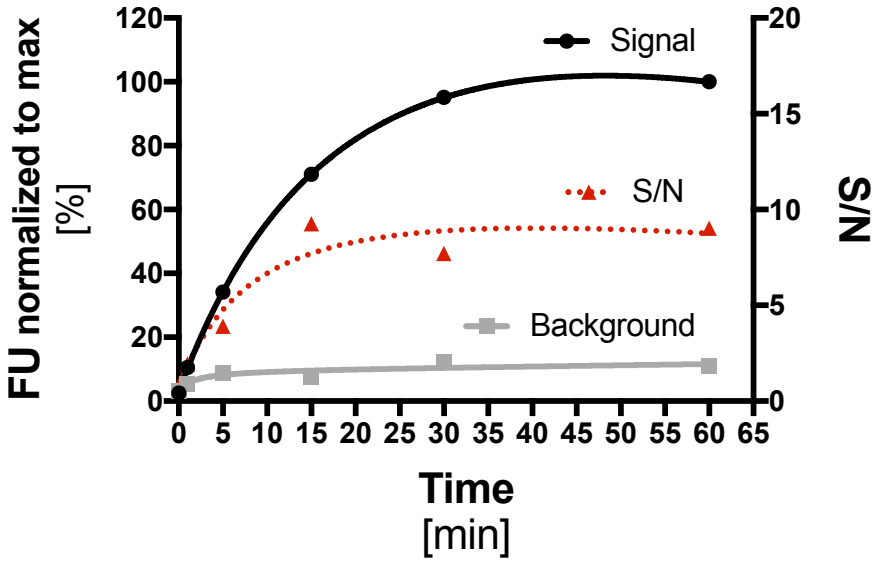


Figure 2.4: Influence of incubation time on signal intensity
Experimental setup: Labeled detection antibody in a concentration of 3 ng/ml was incubated for different amounts of time on a 2D epoxysilanized microarray with immobilized PSA.

Concentration of detection antibody

According to the equation transformation made in the introduction, the concentration of the detection antibody turned out to be the most influential parameter regarding the quantification range. In theory, an equilibrium between the detection antibody in solution, the antigen in solution and the immobilized antigen has to be reached. As demonstrated in figure 2.5, the dynamic range of the sigmoidal competitive quantification curve could be adjusted by the concentration of detection antibody. Hereby, the inflexion point is de-

termined by the antibody affinity and defined as the point at which the ratio of antigen bound and antigen unbound in solution equals 1:1. Given a target concentration of PSA of 0.035 - 0.35 nM (1 -10 ng/ml) in solution, the most suitable concentration of the detection antibody was at 0.02 nM (3 ng/ml). Lower concentrations would have been thinkable too, but taking into account that lower concentrations of detection antibody would also result in a decreased absolute fluorescent signal, a compromise had to be made here.

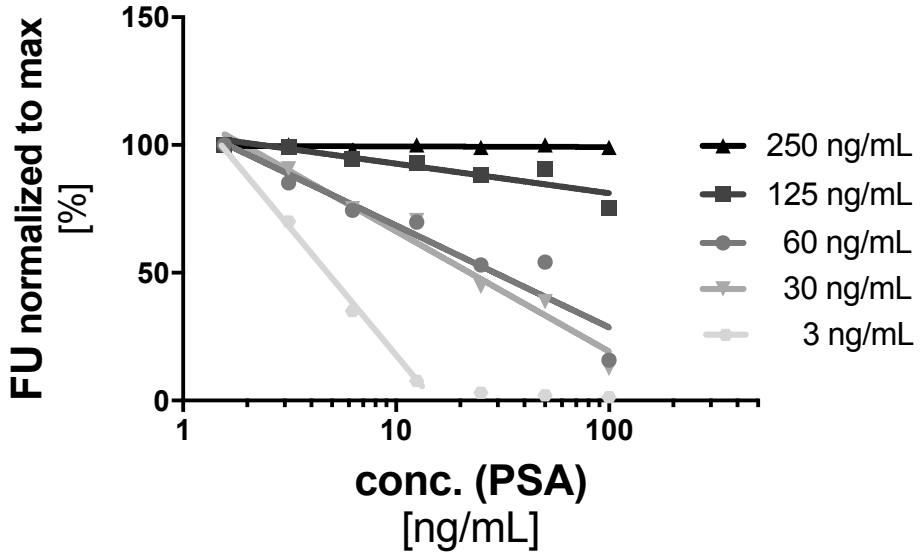


Figure 2.5: Effect on the dynamic range using different detection antibody concentrations

Experimental setup: Each sample of a PSA serial dilution series was incubated with a defined concentration of labeled detection antibody. Sample mixture with detection antibody was then given on a 2D epoxysilanized microarray with immobilized PSA. After washing, fluorescence intensity of each PSA spot was measured and analyzed. A semilog curve fit was performed for data points within the dynamic range.

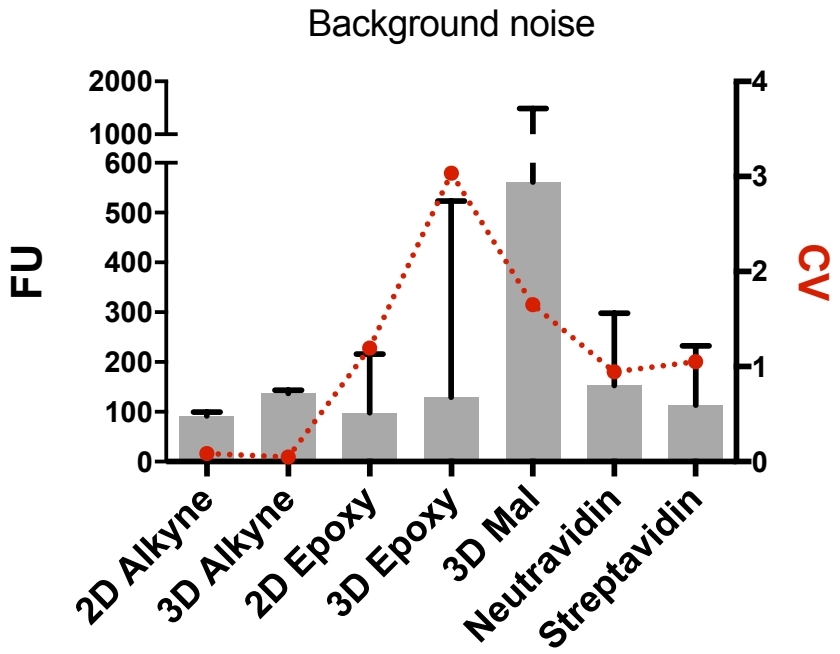
2.1.4.2 Antigen presentation

Apart from the detection antibody, the presentation of the antigen on the microarray surface was another crucial parameter as the antibody is only able to bind a target structure which remains recognizable. The concentration of the antigen on the surface was no factor influencing the quantification performance because, under the assumption that the antigen is covalently bound to the surface forming a saturated monolayer, the immobilized antigen functions as a capturing sensor and does not significantly influence the equilibrium in solution.

Thinking of a sensitive and 3D-shaped protein, it would be possible that the antigen's recognition site could be altered or masked by the immobilization process or on-chip degradation stress. For this reason, we investigated different effects on the antigen presentation such as immobilization strategy, spotting buffer, matrix effects and protein stability. These parameters were studied less to increase the signal intensity but rather to ensure a consistent test performance.

Slide chemistry
The read-out of fluorescent spots on a microarray surface using a fluorescent scanner results in an image. As a matter of fact, the actual signal measurement is strongly related to the field of digital image analysis. According to the Rose criterion a signal-to-noise ratio of at least 5 is needed to be able to distinguish image features at 100% certainty [16]. This emphasizes that not only the signal intensity itself, but also the background noise had to be considered [17].

To evaluate which surface functionalization would be the most suitable, the background signal intensities of different slide formats was compared. According to figure 2.6, the highest background noise was measured for 3D-malemide slides. 2D-epoxy, 3D-epoxy, neutravidin and streptavidin slides exhibited a lower background signal, yet a pronounced coefficient of variation. Alkyne slides featured the lowest background intensities and because of this the most appropriate properties. For a proper comparison, signal intensity and signal-to-noise ratios would be needed in addition. As the attachment of PSA on each surface demanded for its own immobilization strategy, an independent comparison was not feasible at this point.



CV = coefficient of variance, FU = fluorescent units

Figure 2.6: Fluorescence background intensities of different slide coatings
Background measured with buffer blank

Immobilization strategy

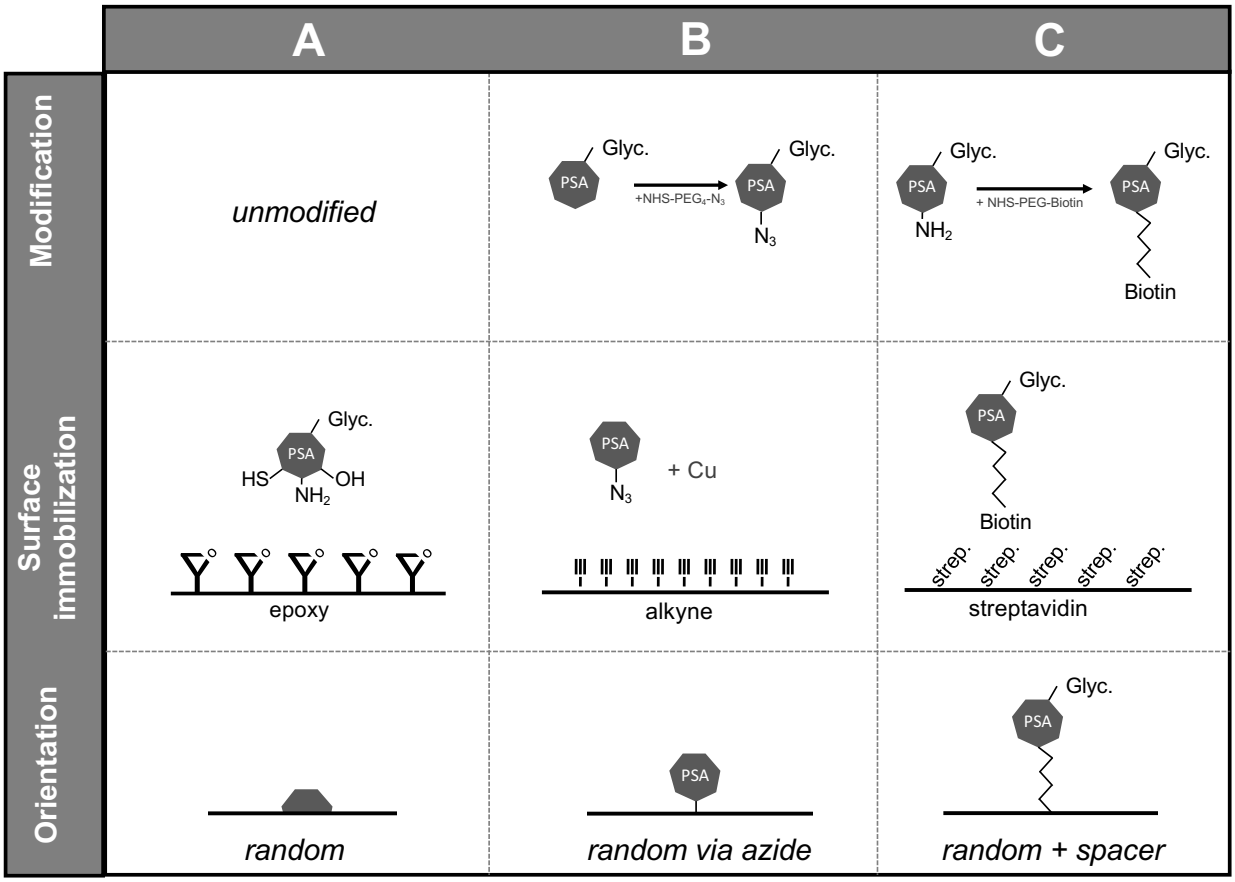
Based on the results of our background intensity level test, three different immobilization strategies, needed for each particular surface functionalization, were investigated. As illustrated in figure 2.7, a random immobilization on 2D epoxysilan slides (A), a surface coupling on a 2D alkyne slide with an azide modified PSA (B) as well as the connection of the protein to a 2D streptavidin surface via a spacer (C) were compared.

Immobilization on epoxysilanized slides

An immobilization on epoxysilanized slides does not require any protein modification as the protein is covalently linked to the surface via free and deprotonated amino functions, but also to some extent via other nucleophilic thiol and hydroxyl groups.

Click immobilization

For the utilization of alkyne slides, an azide moiety had to be coupled to PSA prior to immobilization. This was achieved by the coupling of NHS-PEG4-N_3 to free amine groups of the PSA. To click the azide-modified PSA onto an alkyne functionalized surface required



Glyc. = glycosylation, N_3 = azide moiety

Figure 2.7: Immobilization strategies
A - Immobilization of unmodified PSA on 2D-epoxysilanzied slide.
B - Azide modification and following 'click' immobilization on alkyne surface.
C - PSA PEGylation via NHS chemistry and attachment to streptavidin-coated surface.

copper as catalyst. Copper is known to potentially denature proteins, so it had to be tested if PSA would remain recognizable when employed. Surface staining of attached proteins using SyproRuby revealed that PSA could be immobilized via the explained route also indicating that the modification was successful (data not shown). However, the gained results shown in figure 2.8 A pointed out that PSA degraded under the influence of copper, as it could not be recognized by the detection antibody anymore. This effect was independent of the copper concentration. An immobilization was observed if no cooper was added, but it can be assumed that this was mainly due to an unspecific interaction - as shown in figure 2.8 B, PSA could be attached to the 2D alkyne surface without any modification, too.

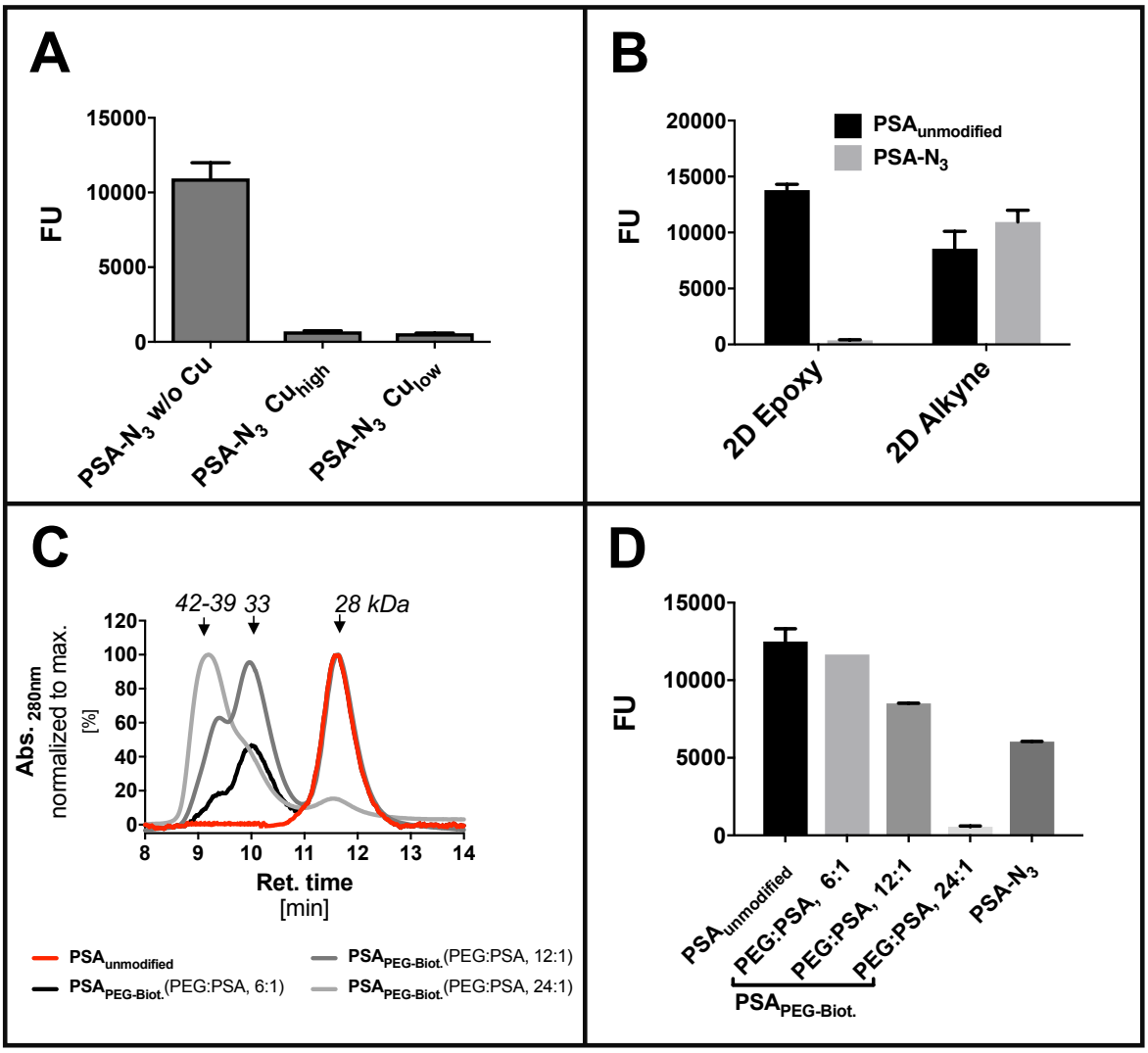


Figure 2.8: Immobilization strategies data
A - Immobilization yield of azide-functionalized PSA on 2D-alkyne surface. Copper concentration: high = 100 μM, low = 100 nM.
B - Immobilization yield of unmodified and azide-functionalized PSA on different surfaces. PSA-N_3 did not bind on the epoxy surface because free amine residues were blocked by the modification with NHS-PEG_4-N_3.
C - SEC chromatogram of PEGylated PSA. **D** - Immobilization yield of functionalized PSA on streptavidin surface

Immobilization with spacer

Apart from an oriented immobilization, it was further investigated whether the detection could be increased if PSA was linked to the surface via a single spacer. In this way, the protein would be able to float in the sample solution being more accessible for antibodies.

The immobilization via a spacer demanded for a conjugation with a bifunctional PEG featuring an amine reactive N-Hydroxysuccinimide (NHS) end for the linkage with PSA as well as a biotin end to connect it to a streptavidin-coated surface. In this regard, the PEGylation degree had to be adjusted to ensure a mono-PEGylation as it was assumed that, due to sterical hindrance, a di-PEGylation would block the antibody from binding.

The PEGylated PSA could be detected and purified employing size exclusion chromatography (SEC), as seen in figure 2.8 C. In additional MALDI experiments (data not shown), we could further confirm that PSA was preferably mono-PEGylated ($\approx$ 33 kDa) if a molar PEG:PSA ratio of 6:1 was used for conjugation. An increased amount of di-PEGylated PSA ($\approx$ 39 kDa) was detected at a molar PEG:PSA ratio of 12:1 and di- as well as tri-PEGylated PSA was generated with a molar ratio of 24:1.

Based on the results represented in figure 2.8 D, the detectability of PSA could not be increased if anchored to the surface via a 5 kDa PEG spacer. Moreover, the highest recognition was achieved by unmodified PSA, which unspecifically attached to the streptavidin surface. An explanation might be that the PEG spacer is not rigid enough to hold the protein in an upright position. In this case, PSA might lay down covered by other PEG chains comparable with rolled lumber. To confirm this assumption, additional experiments using time-of-flight secondary ion mass spectrometry (Tof-SIMS) or atomic force microscopy would have to be carried out [18, 19].

Table 2.2: Summarized comparison of different immobilization strategies.

Immobilization Strategy	**Background**	**Modification required**	**Modification yield**	**Detectability**	$\sum$ [+]
2D Alkyne	+++	y	++	o	5
3D Alkyne	++	y	++	o	4
2D Epoxy	++	n	+++	+++	8
3D Epoxy	o	n	+++	++	5
3D Malemide	o	n	+++	++	4
Neutravidin	+	y	+	+	2
Streptavidin	+	y	+	+	2

Comparing the acquired information on background intensity, modification performance and achieved detectability for each immobilization strategy, as done in table 2.2, we could conclude that strategies, which did not require a PSA modification step, exhibited a better performance. This could also be considered as time and material saving advantage, when thinking of a multiplexed analysis with many protein candidates. The 2D epoxy surface

was finally chosen as the best protein detectability could be achieved with unmodified PSA here and as it provided acceptable background properties.

2.1.4.3 Spotting buffer

The choice of an appropriate spotting buffer depended on the chosen immobilization and slide chemistry. To this extend, the immobilization of unmodified PSA on a 2D epoxysilanized surface via lysine-related free amine residues demanded for a slightly basic pH (amount of lysine residues in PSA = 9). Comparing different pH values, the highest immobilization could be confirmed for a pH of 8.5 (figure 2.9).

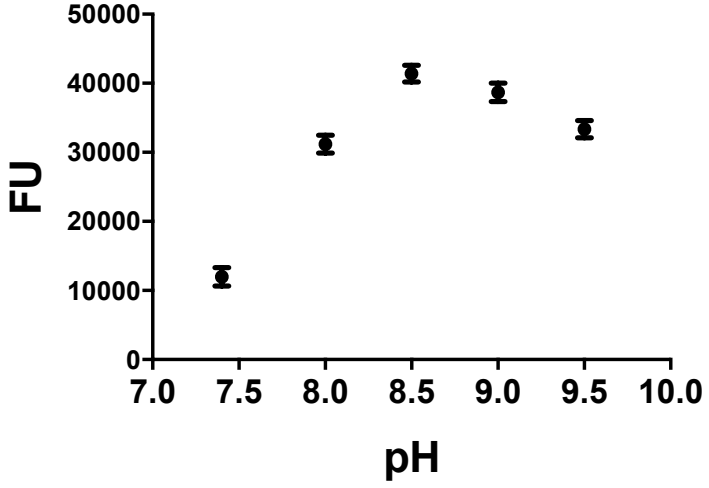


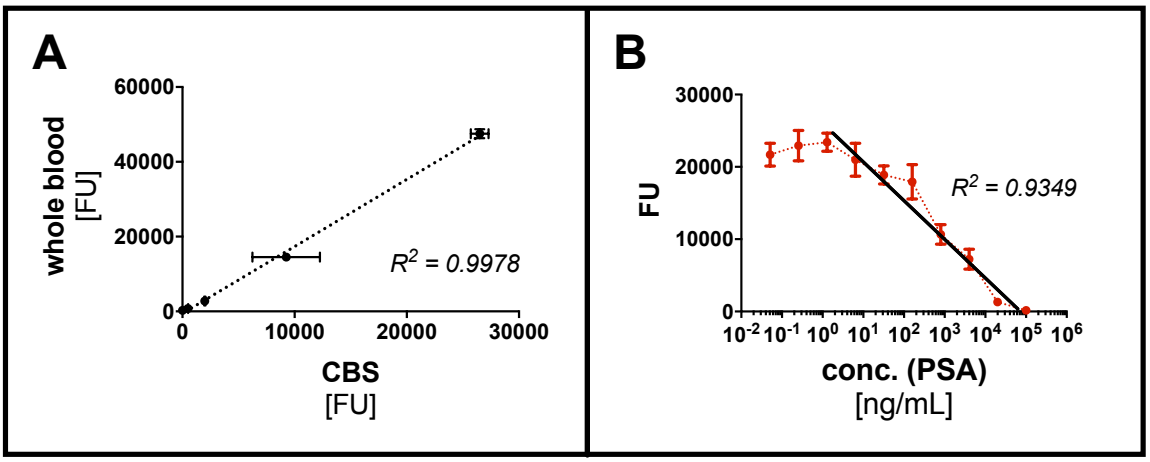
Figure 2.9: Influence of pH on PSA immobilization

Testing three different spotting buffer types - a dextran-based, a glycerol-based and a commercially available (unknown formulation) - for the immobilization of PSA, no differences could be observed regarding the spot morphology and the signal intensity upon detection with a detection antibody (data not shown). Additional properties to evaluate the performance characteristics of the available spotting buffers would include the capacity to preserve the on-chip protein stability. To investigate the on-chip protein stability, new methods had to be developed, which will be described in the following chapter.

2.1.4.4 Matrix effects

Apart from the antigen presentation on the surface, it was further tested if the quantification performance was altered in different incubation scenarios. In the desired setup, a

biomarker quantification would be possible in whole blood to minimize the amount of time and avoid a sample preprocessing. In this regard, PSA was spiked into whole blood from female donors as well as into blocking solution. The results, represented in figure 2.10 A, showed that blood as sample matrix did not alter the quantification performance.



CBS = candor blocking solution (based on casein)

Figure 2.10: **A** - Correlation of PSA quantification in CBS vs. in whole blood;
B - Final quantification performance in whole blood.
(Black line = Semilog fit of dynamic range)

In summary, the best quantification results were obtained using unmodified PSA for the immobilization on a 2D epoxysilanized surface, a detection antibody labeled with 4 fluorophores in a concentration of 3 ng/ml and an incubation time of 15 minutes on the chip.

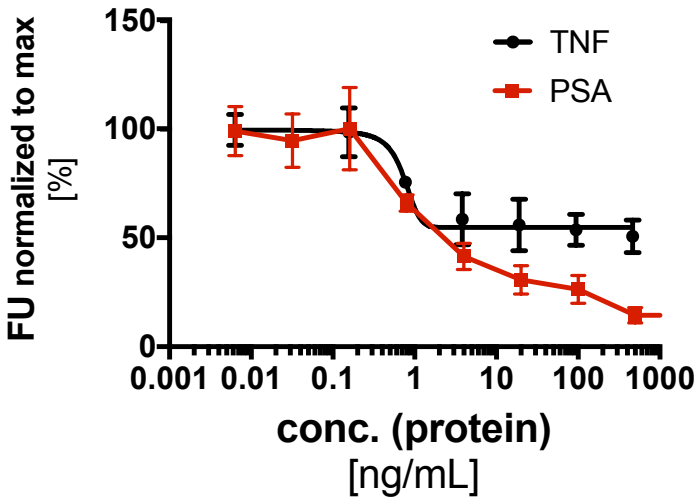
Based on these findings, a calibration curve with a dynamic quantification range of 1 ng/ml - 10 μg/ml was generated as presented in figure 2.10 B. As initially mentioned, physiological PSA concentrations range from 0 - 4 ng/ml in healthy men. A PSA concentration between 4 - 10 ng/ml indicate a confirmatory biopsy and concentrations above 10 ng/mL are considered critical. With the calibration curve at hand, it is possible to accurately determine whether the PSA concentration is above or below 10 ng/ml. However, for a precise PSA quantification in the desired concentration range of 1 and 10 ng/ml, the quantification performance seemed to be not robust enough as it exhibited a pronounced standard deviation of signal intensities. This might be due to the fact that the limit of detection was adjusted to a concentration of 1 ng/ml. In such a competitive setup, the limit of detection is very much dependent on the equilibrium adjustment between the target protein in the sample solution, the detection antibody as well as the immobilized target protein on the

microarray (see equations 2.1 to 2.7). In addition, other parameters such as a potential multicomplex formation, which consists of several detection antibodies cross-bound to one analyte molecule (rather than one antibody bound to one analyte molecule), might result in an increased amount of surface bound detection antibodies. Known as 'hook effect', this phenomenon might influence the transition phase, especially at low analyte concentrations, and lead to the pronounced signal intensity variation [20]. For this reason, further investigations with regard towards reproducibility to control this effect would be needed in the next steps [21]. This would also include the inclusion of a suitable calibration standard either as an internal standard in the sample, a technical reference on the chip or a combination of both [22].

2.1.4.5 Multiplexed Quantification

The previously mentioned advantage of the microarray platform is related to the option of multiplexing. By the immobilization of many different proteins on a small area, the parallel testing of multiple analytes in one sample can be enabled, which again can improve the diagnostic accuracy. In this regard, we were interested in developing a multiplexed assay for the simultaneous quantification of PSA and the tumor necrosis factor-alpha (TNF-α). TNF-α exhibits elevated concentrations in patients with rheumatic diseases. Peak concentrations can reach 100 pg/ml or higher but usually concentrations are in the lower pg/ml range [23]. However, the combinational investigation of these two parameters might be very valuable in cancer diagnostics as also discussed by others [24–26] Despite this, a quantification method for TNF-α was of interest for further tests as described in the following chapters.

The multiplexed detection of PSA and TNF-α, as represented in figure 2.11, shows that a simultaneous and comparable quantification of both proteins was possible. Regarding the quantification performance of PSA, it was possible to achieve a dynamic quantification range from 0.2 - 500 ng/ml. This trend is similar to the previously described calibration curve (figure 2.10 B), yet no protein concentrations above 500 ng/ml were used in this experiment and the dynamic range exhibited a logarithmic rather than a linear decreasing character. The logarithmic decrease also did not allow a proper four parameter logistic curve fitting. Therefore, a clear correlation between the single and multiplexed quantification performance was not possible here. Even though an altered quantification behavior in the dynamic range is not to be expected, it would still be possible to generate a separate PSA quantification calibration curve for the multiplexed setup with TNF-α.



Curve fitting for TNF: four parameter logistic curve

Figure 2.11: Multiplexed quantification of PSA and TNF-α
Experimental setup: A dilution series in CBS containing PSA and TNF-α was prepared in a way that the sample with highest PSA concentration exhibited the lowest TNF-α and vice versa. To each sample a solution with labeled anti-PSA and anti-TNF-α detection antibody was added and the mixture was incubated on a microarray surface where PSA as well as TNF-α had been immobilized.

A less broad quantification range from 0.1 to 4 ng/mL was observed in the case of TNF-α. A potential reason for the narrow dynamic quantification range might be due to the fact that infliximab, as the used detection antibody, featured a very high affinity with a K_D of 4.2 pM [27]. In this regard, the transitional equilibrium phase might be less pronounced, especially at the investigated detection antibody concentration. Also, an additional refinement of parameters as performed for PSA and described in this work, might improve this assay.

Nevertheless, with this proof-of-concept experiment we could demonstrate that a parallel quantification of two proteins in a competitive fashion is possible. In the next steps, other proteins of interest could be included in such an assay. Interferences in array performance are not be expected as long as the detection antibodies specifically recognize their targets and do not feature any cross-reactivity, neither among each other nor with blood components.

2.1.5 Conclusion

Based on the gained results, it could be pointed out that it is possible to quantify PSA in the desired range of 1 - 10 ng/ml on a microarray platform employing a competitive quantification strategy. This could be enabled by the optimization of several parameters such as the detection antibody concentration, its labeling and the on-chip incubation time. Whereas PSA concentrations above 10 ng/ml can be measured accurately, a precise quantification of concentrations around and below 1 ng/ml still remains difficult to control. In this regard, a sensitivity increase by an optimized antigen presentation could not be mediated. Nevertheless, the fact that just a small area is required to perform multiple measurements within 15 minutes emphasizes the potential to apply this assay in a point-of-care diagnostic test; portable read-out systems have already been developed and can easily be used for this purpose. Concerning a valid quantification quality and a potential shelf life of a protein microarray-based assay, the protein stability states a parameter, which needs to be studied in further experiments as described in the next chapter.

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2.2 Investigating the stability of proteins immobilized on microarrays using thioflavin T

2.2.1 Abstract

Preserving the stability of a protein is of outmost importance not only in the development of a pharmaceutical formulation but also in the development of a immunoassay-related diagnostic test to guarantee a valid test performance. Apart from a pharmaceutical formulation, proteins are primarily attached to a solid support, e.g. microtiter plates, membranes, beads or microarrays, in an immunoassay setup. The amount of surface bound protein is usually in the lower nanogram range and therefore sensitive and preferably easy methods are needed to investigate its stability. In the present work, it was investigated whether thioflavin T, an extrinsic fluorescent dye, which is 'activated' by the the incorporation into denatured proteins, could be used as quantitative probe to detect and quantify the degree of stability loss of proteins, immobilized on a microarray surface. In this regard, four different protein candidates, representing different protein classes, were immobilized on a microarray and stored at different conditions. To monitor the degree of functional loss, the capability to be bound by detection proteins as well as the thioflavin T-associated fluorescence were measured. The correlation of data points, which were generated by these two measurement methods, revealed a connection between an increasing loss of protein functionality and an increasing thioflavin T-associated fluorescence. The suitability to use thioflavin T as a quantitative probe for the evaluation of the protein stability depended on the secondary structure of the investigated protein. The developed method offers a convenient and feasible method to determine the degree of protein denaturation on a microarray surface and could be used for the optimization of storage conditions such as the selection and optimization of storage buffers.

2.2.2 Introduction

Microarrays offer a versatile platform to perform thousands of individual binding assays simultaneously. They can be used as DNA but also protein arrays. Arising from DNA chips, several preparation technologies have been adopted for the preparation of protein microarrays. Other than the straight-forward immobilization of linear DNA strands, protein microarrays are quite demanding with regard to their preparation as each protein represents a different molecule featuring its own diversity of suitable and less suitable im-

mobilization sites [1]. Apart from the immobilization itself, proteins are per se rather instable macromolecules in comparison to DNA. Especially during the preparation process, influences of humidity, evaporation, dehydration, temperature, pH, stirring, light and interaction forces with surfaces will stress the protein stability.

In general, investigation of protein stability did not deserved the same attention in diagnostics as in the formulation development of the pharmaceutical industry where tremendous efforts are made to stabilize sensitive therapeutic proteins [2, 3]. With an expanding interest in protein microarrays as diagnostic application, efforts should also be made here to better control the quality of such assays. The preserved intactness of the protein in a diagnostic assay will not only determine the shelf life but more importantly ensure the test performance, especially in the case of quantitative assays.

Based on advances in pharmaceutics many protein stability analysis methods are known, yet the biggest challenge, regarding protein microarrays, is to identify and develop simple methods which are sensitive enough to analyze the few picogram of protein mass which are printed to the microarray surface.

In this regard, it had to be further clarified which stability indicating parameters are important in the particular case to select the most appropriate technology. The function of a protein microarray is to present proteins which can interact with distinct binding partners. By way of example, the interaction between an antibody and its antigen is very dependent and based on a specific structural sequence, consequently, the intactness of the protein shape determines its stability in this context. To investigate the shape of a protein, spectroscopic methods such as Fourier transform infrared spectroscopy (FTIR), circular dichroism spectroscopy (CD), nuclear magnetic resonance spectroscopy (NMR) and X-ray diffraction but also H/D-exchange measurements are typically employed. However, for the analysis of proteins immobilized on a microarray surface these methods are not feasible as the protein can only be analyzed in solution (CD, NMR, H/D-exchange) or are not sensitive enough for the given amount of protein (FTIR). To this extent, more advanced technologies like X-ray photoelectron spectroscopy (XPS), sum frequency generation (SFG) as well as time of flight secondary ion mass spectrometry (ToF-SIMS), which can detect the protein conformation at the interface of protein and surface would be in favor, yet laborious.

The tertiary structure and conformation of a protein is furthermore dependent on the underlying chemical stability. Therefore, methods which allow to monitor chemical changes (denaturation, oxidation, deamidation) can be used to predict conformational changes

too [4]. An alternative and very inexpensive approach to characterize proteins is the investigation of fluorescence-based parameters such as the intrinsic tryptophan fluorescence or the interaction of extrinsic fluorescent dyes with denatured proteins. These dyes e.g. are used to visualize the formation of amyloid fibrils related to neurodegenerative diseases such as Alzheimer's disease [5, 6]. A list of suitable and available dyes featuring different properties has been summarized by Hawe et al. [7].

The aim of this work is to investigate the functional stability of proteins immobilized on a microarray surface by means of detectability using a corresponding binding partner and to establish a method which allows to monitor the chemical and the related conformational stability of proteins using an extrinsic fluorescent dye. For this purpose, a correlation between loss of antibody/antigen recognition and increase in denaturation-based thioflavin T (ThioT) fluorescence had been elaborated. With the developed tool at hand, it will be possible to improve the evaluation of immobilization procedures because the basis of valuation could then be complemented by the stability factor.

2.2.3 Materials and Methods

Table 2.3: Utilized proteins

	Protein	Class	MW [kDa]	Disulfide bonds	Glyco. sites	Uniprot ID/ Accession no.
Immobilization	PSA	enzyme	28.7	5	1	P07288
	TNF-α	cytokine	25.6	1	1	P01375
	Infliximab	IgG1	149.1	16	2	DB00065
	DM380 JA70-F6 (anti-GRIP2)	scFv-Fc (fusion protein)	110	12	2	n.a.
Detection	anti-PSA (clone: CHYH2)	IgG1 (mouse anti-human)	150			n.a.
	Infliximab	IgG1	149.1			DB00065
	TNF-α	cytokine	25.6			P01375
	GRIP2	Glutamate receptor -interacting protein 2	112.5			Q9C0E4

The following chemicals were obtained from Sigma-Aldrich: Thioflavin T (CAS# 2390-

54-7), Glycerol 86 % (w/V, CAS# 56-81-5), Sarkosyl (CAS# 137-16-6), Igepal® CA-630 (CAS# 9002-93-1), Tween® 20 (CAS# 9005-64-5), dextran (enzym. synthesis, CAS# 9004-54-0) as well as all salts $NaHPO_4$, NaCl, $MgCl_2$, $NaHCO_3$.

Detection proteins were labeled with the Cy5-like fluorescent dye DY-649-P1 (Dyomics GmbH, Jena, Germany) via NHS-chemistry in a molar ratio of 12:1 (dye:protein).

2.2.3.1 Protein microarray preparation and storage

For the preparation of protein microarrays epoxysilanized glass slides (Nexterion E, Schott, Jena, Germany) were used. The investigated proteins are listed in table 1. Each protein solution was adjusted to a concentration of 1 mg/ml, mixed with twofold spotting buffer (glycerol-based: 0.4 M $NaHPO_4$, pH 8.6, 10% glycerol, 0.01% sarkosyl; dextran-based: 0.2 M $NaHCO_3$, 1 % (w/V) dextran, 100 mM NaCl, 10 mM $MgCl_2$, 0.012 % (w/V) Igepal CA-630) and spotted using a MicroGrid II 610 (Apogent Discoveries, Mittenwald, Germany) contact printer equipped with SMP6B pins (Arrayit). Thus, droplets with a volume of 0.6 nl featuring spots of around 220 μm in diameter were generated. In each incubation block (0.7 x 0.7 cm) 15 spots of one protein in one spotting buffer with a spot-to-spot distance of 1.5 mm were printed (4 proteins x 2 spotting buffers = 8 incubation blocks, each with 15 spots). Prior to sample incubation, slides were blocked for 1 h with the Candor Blocking Solution (Candor, Wangen im Allgäu, Germany) and washed with PBST (PBS supplemented with 0.5 % (v/v) Tween 20 using a Tecan HS 4800 hybridization system (Tecan Group Ltd., Männedorf, Switzerland). The prepared slides were then packed in air-proof bags with drying silica gel pearls (blue, Sigma Aldrich) and stored at -20, 4, 25 and 60 °C for 1, 7, 30 and 90 days.

2.2.3.2 Incubation and analysis of samples - Antibody/antigen recognition

The blocked slides were sealed with ProPlate® 16-well incubation chambers (Grace Bio Labs). Each well was filled with 70 μl of detection solution (corresponding detection proteins labeled with DY-649-P1 dissolved in the blocking solution at a concentration of 100 ng/ml) and incubated for 15 min at room temperature. After incubation, each well was washed separately with PBST as well as ddH_2O for three times and dried with pressurized air. The dried slides were than scanned using a Tecan PowerScanner (Tecan Group Ltd., Männedorf, Switzerland) and images were analyzed using the GenePix 6.0 microarray analysis software (Axon Instruments, Inc., Union City, CA).The median spot

intensities without background correction were used for further data analysis.

2.2.3.3 Incubation and analysis of samples - Thioflavin T fluorescence

Similar to the procedure described above, a fresh solution of Thioflavin T (0.5 mM in PBS) was produced and 70 μl of this solution were added to each well. After an incubation of 30 minutes protected from light, microarray slides were rinsed with ddH_2O for three times. The resulting fluorescence was measured using a SensoSpot Fluorescence Microarray Analyzer (Sensovation, AG, Radolfzell, Germany).

2.2.4 Results and discussion

Protein microarrays, as an alternative platform for immunoassays, rely on the formation of antigen-antibody complexes. In the ideal case, the immobilization as well as the storage of proteins on microarray surfaces should not alter the protein in its ability to form immune complexes. However, since the protein interacts with the microarray surface and is exposed to external denaturation stresses such as humidity and temperature changes, it is likely that the protein undergoes conformational transformations. To assess conditions, which prevent or accelerate a protein's denaturation when immobilized on a microarray surface, it was of prior importance to develop suitable methods which allowed an investigation of the on-chip protein stability.

In this regard, two methods were employed and compared:
1. Antigen/antibody detectability - To investigate to which extent a protein loses functionality on the microarray surface, it was measured and compared how much of the corresponding binding partner could still be bound after storage. Simulating a controlled microarray experiment, this method was used as our reference method. It allowed us to quantify the loss of function. In this context, the loss of function should be understood as a general loss of protein detectability preferably due to conformational alterations.
2. Thioflavin T fluorescence - The extrinsic fluorescent dye thioflavin T is known to incorporate into the hydrophobic sites of denatured proteins - preferably β-sheet-rich amyloid fibrils [8,9] - and it is widely used already in protein formulation analytics to detect conformational changes at rather early stages. Despite this, it had never been tested whether this approach is sensitive enough to monitor conformational changes on microarray surfaces.

We hypothesized that the degree of function loss of an immobilized protein correlates with an increasing thioflavin T fluorescence, in a way that thioflavin T might be used as a

quantitative probe.

As stability characteristics may vary from protein to protein, four candidates, representing different protein classes, were chosen and investigated in this study. Prostate specific antigen (PSA), tumor necrosis factor alpha (TNF-α), infliximab and a scFv-Fc fusion protein (properties listed in table 2.3) were spotted on epoxysilanized microarray slides using a glycerol- as well as a dextran-based spotting buffer. The prepared slides were then stored at -20, 4, 25 and 60 °C for 1, 7, 30 and 90 days.

2.2.4.1 Antibody/antigen detectability

To illustrate the loss of protein functionality after immobilization on a microarray surface and the resulting loss of detectability by the corresponding binding partner an exemplary data set is shown in figure 2.12. The data points represent the relative amounts of how much antigen could be bound by the immobilized antibody-like scFv-Fc fusion protein after 1, 7, 30 and 90 days of storage at a storage temperature of 4 °C.

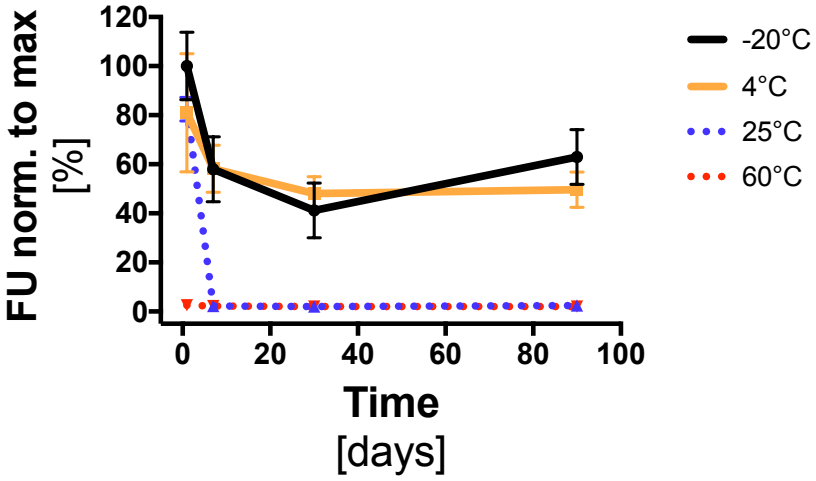


Figure 2.12: Loss of protein recognition over time
The monoclonal IgG1 antibody infliximab was immobilized on a microarray surface (glycerol-based spotting buffer), stored at different temperatures and detected with DY-649-P1-labeled TNF-α after different time points.

Over a time interval of 90 days, a protein recognition decrease of 37.1 % was observed if the protein microarray was stored at -20 °C. At a storage temperature of 4 °C the degradation rate and degree was comparable. If stored at 20 and 60 °C hardly any recognition could be measured after one day of storage (a time point t_0, which would have been immediately

after printing, was not feasible as at least 24 h were needed for the protein to immobilize). The fact that the protein detectability could not be preserved neither at -20 °C nor at 4 °C implied the sensitivity of this particular protein.

2.2.4.2 Thioflavin T fluorescence

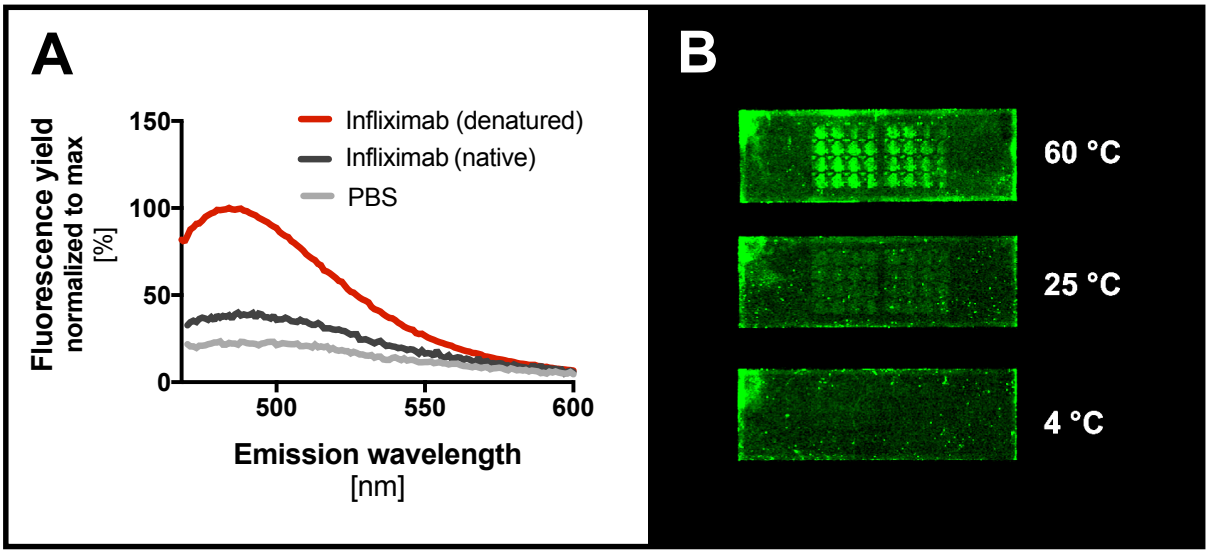


Figure 2.13: Thioflavin T as fluorescent denaturation probe
A - Thioflavin T fluorescence in solution (excitation: 440 nm), Infliximab was diluted in PBS at a concentration of 1 mg/ml. The denatured specimen was analyzed after a storage over three days at 60 °C. Measured with Tecan infinite M200 plate reader.
B - Fluorescence scan of antibody microarray with 660 entities stored at different temperatures for three days. Measured with SensoSpot Fluorescence Microarray Analyzer (Sensovation).

The reason why thioflavin T was chosen as indicative dye was mainly due to its fluorescent properties (excitation: 440 nm, emission max.: 475 - 495). Typically, microarray scanners feature a green (580 ± 21 nm) and a red (675 ± 18 nm) filter setting for the measurement of Cy3 and Cy5-like dyes. The SensoSpot Fluorescence Microarray Analyzer (Sensovation) was equipped with an additional blue filter setting (500 ± 15 nm) allowing to quantify the amount of thioflavin T fluorescence on the microarray surface. Illustrated in figure 2.13 A and B, an increase in degradation-dependent fluorescence could be observed in solution using a Tecan infinite M200 fluorescence plate reader as well as on the protein microarray surface using the SensoSpot Fluorescence Microarray Analyzer.

2.2.4.3 Analysis of on-chip protein stability

In the following sections, the gained findings are summarized by opposing the results from the two employed methods, separately for each protein and each storage temperature.

PSA

The observed results represented in figure 2.14 indicate that temperature and storage stress did not have a pronounced effect on the recognition of PSA by the used detection antibody. Moreover, instead of a function loss, an increasing recognition trend independent of the storage temperature seemed to be the case. Also, the choice of the used spotting buffer did not show a major difference in recognition performance.

Regarding the measured thioflavin T fluorescence, an elevating signal intensity was seen if PSA was stored in the glycerol-based spotting buffer. This effect was more pronounced the higher the selected storage temperature. If PSA was spotted with the dextran-based buffer hardly any increase in thioflavin T fluorescence could be achieved.

TNF-α

Similar to the previously specified function loss properties of PSA, TNF-α exhibited an increasing or constant recognition behavior at storage temperatures of -20 and 4°C respectively. This effect was independent and comparable amongst the two employed spotting buffer systems. However, a tremendous loss of detectability was observed if TNF-α was stored in the glycerol spotting buffer at higher temperatures. At 25 °C a loss of 74.7 % within seven days was recorded, whereas hardly any protein remained detectable from day one on if stored at 60°C.

In contrast to PSA, a trend, exhibiting an increasing thioflavin T fluorescence with decreasing protein recognition was monitored for TNF-α. This was particularly the case at storage temperatures above 4°C. Nevertheless, it has to be put into question whether the signal intensity jump of the specimens at 60°C day 7, stating the overall maximum, could also be considered as measurement error or outlier as this effect was also seen in the following analysis. A possible explanation might also be that after a one month storage at 60°C only minor protein residues, which would allow thioflavin T incorporation, are present.

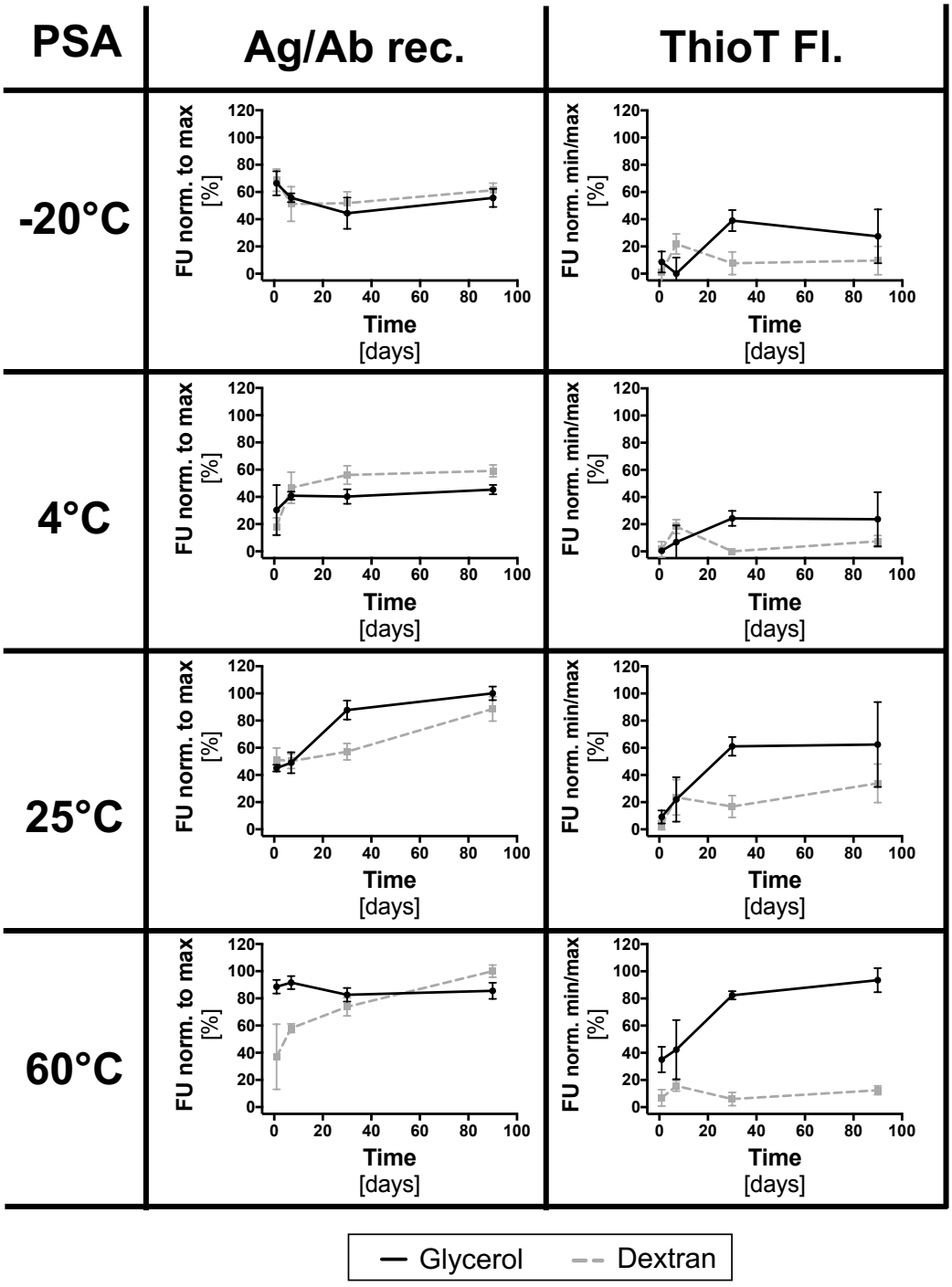
Infliximab

Other than PSA and TNF-α, infliximab is a therapeutic anti-TNF-α monoclonal IgG1-type antibody used for the treatment of rheumatic patients. Investigating its on-chip stability, revealed that the antibody structure was more likely to lose its function. Even at storage temperatures of -20 and 4°C a loss of up to 50.5 % was measured. It was further noticed that the utilization of the dextran-based spotting buffer seemed to provide a slight stability preserving effect.

ScFv-Fc fusion protein

As previously mentioned, the antibody-like scFv-Fc fusion protein exhibited the most sensitive stability characteristics amongst all investigates proteins as a strong loss of function could already be noticed if stored in a glycerol environment at 4°C for seven days. However, with a closer look at the measured binding capacity at a storage temperature of 25°C, the employment of the dextran-based spotting buffer was able to better preserve the protein's stability.

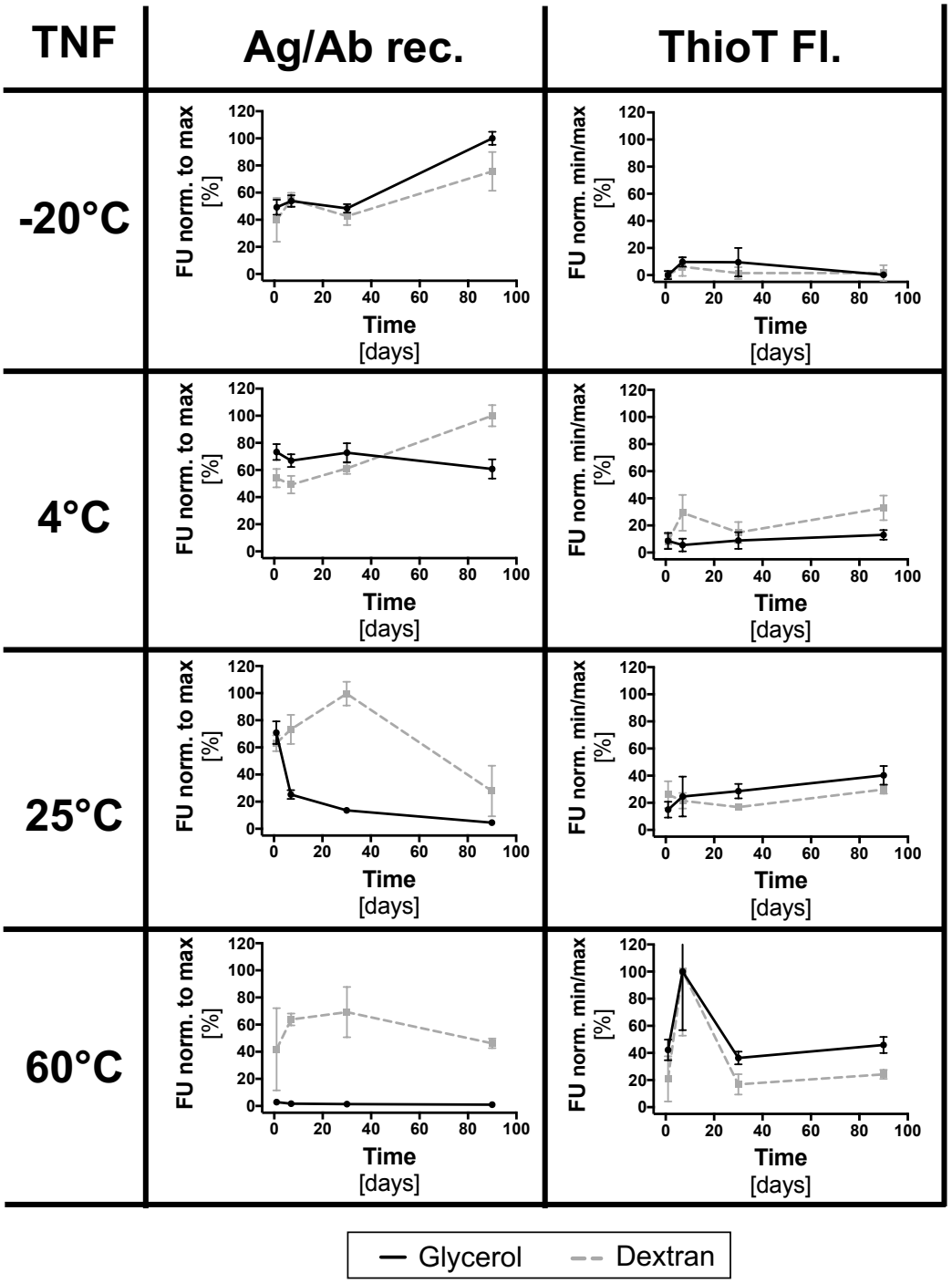
Similar to the observations made in the case of infliximab, a higher degree of denaturation lead to an, in average, higher thioflavin T intensity.



(Ag/Ab rec. = antigen/antibody recognition, ThioT Fl. = Thioflavin T fluorescence)

Figure 2.14: PSA

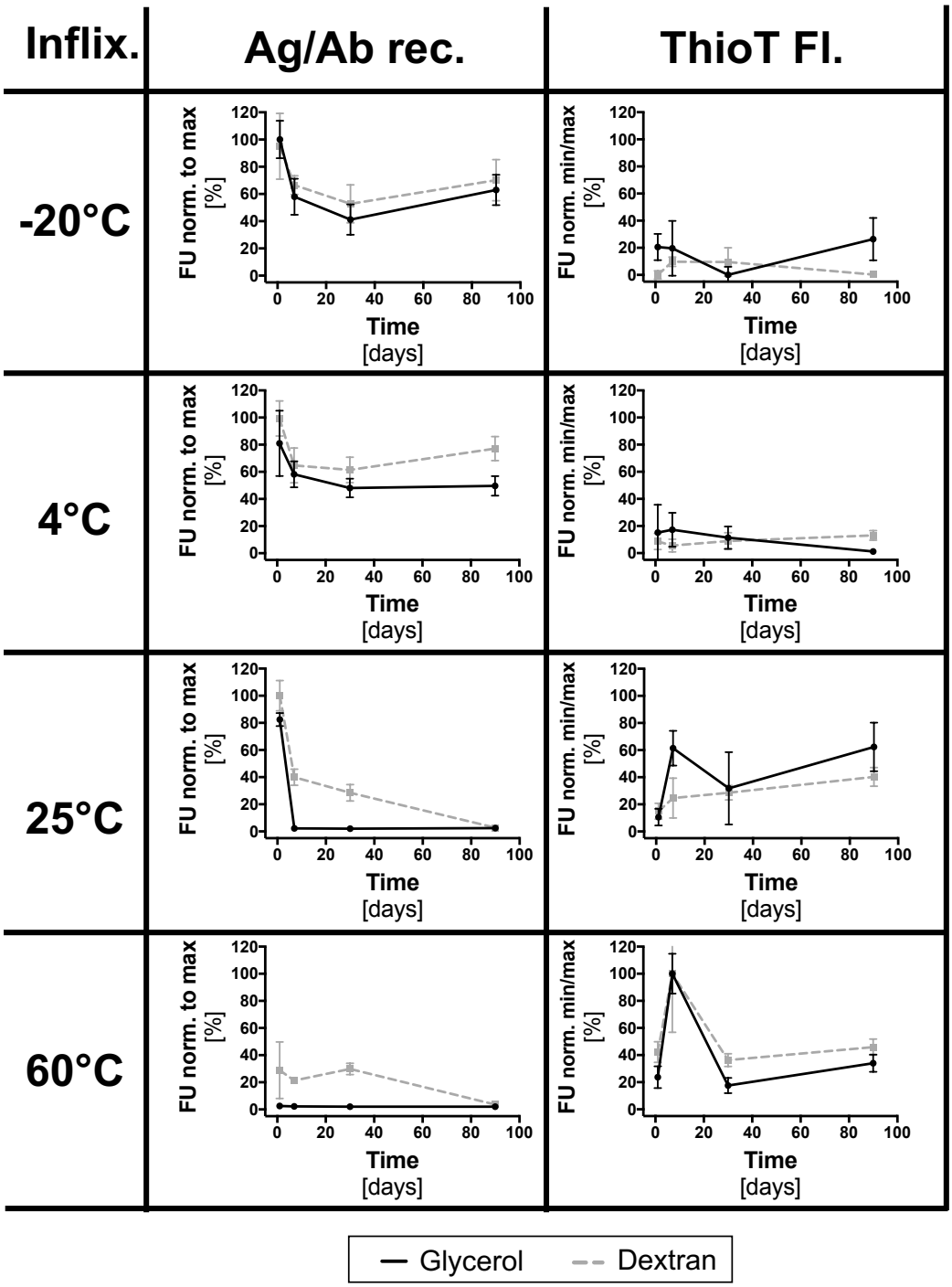
Degree of protein function loss vs. degree of thioflavin T incorporation and resulting fluorescence due to storage and temperature stress. Values were normalized to the maximal fluorescent intensity of all measured values.



(Ag/Ab rec. = antigen/antibody recognition, ThioT Fl. = Thioflavin T fluorescence)

Figure 2.15: TNF-α

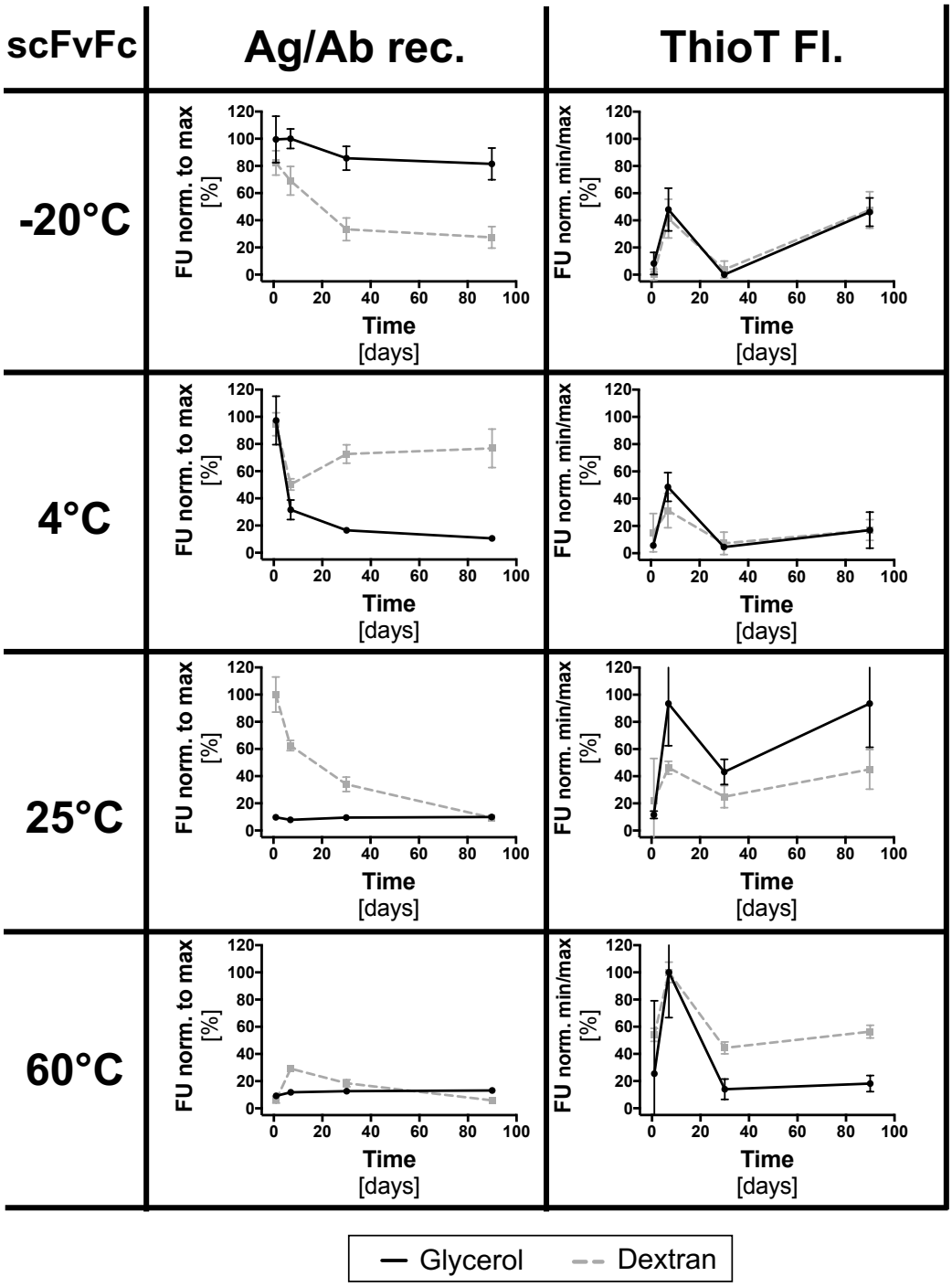
Degree of protein function loss vs. degree of thioflavin T incorporation and resulting fluorescence due to storage and temperature stress. Values were normalized to the maximal fluorescent intensity of all measured values.



(Ag/Ab rec. = antigen/antibody recognition, ThioT Fl. = Thioflavin T fluorescence)

Figure 2.16: Infliximab

Degree of protein function loss vs. degree of thioflavin T incorporation and resulting fluorescence due to storage and temperature stress. Values were normalized to the maximal fluorescent intensity of all measured values.



(Ag/Ab rec. = antigen/antibody recognition, ThioT Fl. = Thioflavin T fluorescence)

Figure 2.17: ScFv-Fc fusion protein
Degree of protein function loss vs. degree of thioflavin T incorporation and resulting fluorescence due to storage and temperature stress. Values were normalized to the maximal fluorescent intensity of all measured values.

In summary, PSA and TNF-α could still be well recognized after a 90-days storage if kept at typically temperature conditions of -20 and 4°C. PSA did not lose its function even at elevated storage temperatures of 25 and 60°C. A reason for this might be due to the fact that the used PSA detection antibody was directed against a linear peptide epitope. In this case, it is less of relevance whether a protein exhibits an intact structure or not. For the recognition of the more temperature sensitive TNF-α, infliximab was used as detection antibody. The underlying immune complex formation here seemed to be more dependent on an intact three-dimensional structure of TNF-α, which is well in accordance with findings on the binding geometry of infliximab and TNF [10].

In contrast, an on-going degradation was observed in the case of the infliximab antibody as well as the scFv-Fc fusion protein. It is arguable whether this loss of functionality was only dependent on storage effects (humidity, spotting matrix, temperature) or also on immobilization effects. As the storage conditions at -20 and 4°C represented a protein stability maintaining environment, we hypothesized that the functional loss was a more immobilization-dependent process. An increased molecular weight of the antibody and the scFv-Fc fusion protein offers a higher amount of potential interaction sites between protein and surface such as nucleophilic lysine, thiol and hydroxyl groups. Consequently, and because the epoxysilanized surface had not been blocked prior to storage, it was assumed that the proteins continuously bind to the reactive surface and by this are stretched out on the solid support. Technically, it would have been easy to reduce or deactivate the functional epoxy residues by blocking as emphasized by others [11]. Apparently, the visual appearance of the spotting pattern is then washed away and a visual inspection, which is needed for handling and orientation purposes, cannot be carried out anymore.

Drawing a comparison to other surface bound protein techniques such as Western Blot or ELISA, it seemed to be less problematic to present an antigen in a way that it can be recognized by an antibody rather than immobilizing an antibody and maintaining its active function of binding antigens. Even though an antigen might lose its native three-dimensional structure and stretches out on the surface, the corresponding detection antibody might still be able to bind to its epitope. This might be the case especially, if it is an antibody which is known to be suitable for applications such as Western blot or IHC. In contrast, it should be considered that even if an antigen remained detectable, this does not automatically imply that the protein did not degrade. This fact could be of importance if an antigen microarray is used to detect antibodies, which only recognize their target in its native three-dimensional structure as previously discussed in chapter I.

Looking at the measured thioflavin T fluorescence values, without a correlative comparison with the antigen/antibody recognition results at this point, increasing fluorescence values were achieved the longer proteins were stored and the higher the storage temperature was set. This was observed for all investigated protein candidates and is in good accordance with the findings in figure 2.13 B. In addition, it is striking that the thioflavin T fluorescence intensity jumped from day one to day seven in the case of TNF-α, infliximab and the scFv-Fc fusion protein, especially if kept at 25 or 60°C. An explanation for this might be that the proteins denature within seven days and in the following twenty-three days then reach a state beyond denaturation, which is determined by a more hydrophilic character due to oxidation procedures hindering the thioflavin T to incorporate.

A detailed correlation between function loss and thioflavin T fluorescence is discussed in the next section.

2.2.4.4 Correlation of antigen/antibody recognition vs. thioflavin T fluorescence

Correlating the gained thioflavin T fluorescence with the achieved signal intensities of the detection assay allowed us to evaluate the hypothesis if a thioflavin T-associated fluorescence is quantitatively correlated with a loss of detectability. For the correlation displayed in figure 2.18, the raw signal intensities of the detection assay are opposed with the gained raw thioflavin T fluorescence values. In more detail, all values in regard of spotting buffer, storage time and storage temperature were used for the analysis of each protein. However, data points after a 7-day storage at 60°C were, based on Grubbs testing and graphical interpretation, considered as outliers and excluded from the correlation analysis.

Testing on a confidence interval of 95 %, a significant correlation could be pointed out in the case of PSA (p-value = 0.0005), TNF-α (p-value = 0.0008) and infliximab (p-value $<$ 0.0001); no correlation was observed for the scFv-Fc fusion protein (p-value = 0.2366). Whereas data on TNF-α and infliximab confirmed a negative correlation between detectability and thioflavin T fluorescence ($r_{\text{TNF-}\alpha}$= -0.53 , $r_{\text{infliximab}}$= -0.67) as initially expected, PSA exhibited a positive correlation (r_{PSA}= 0.58).

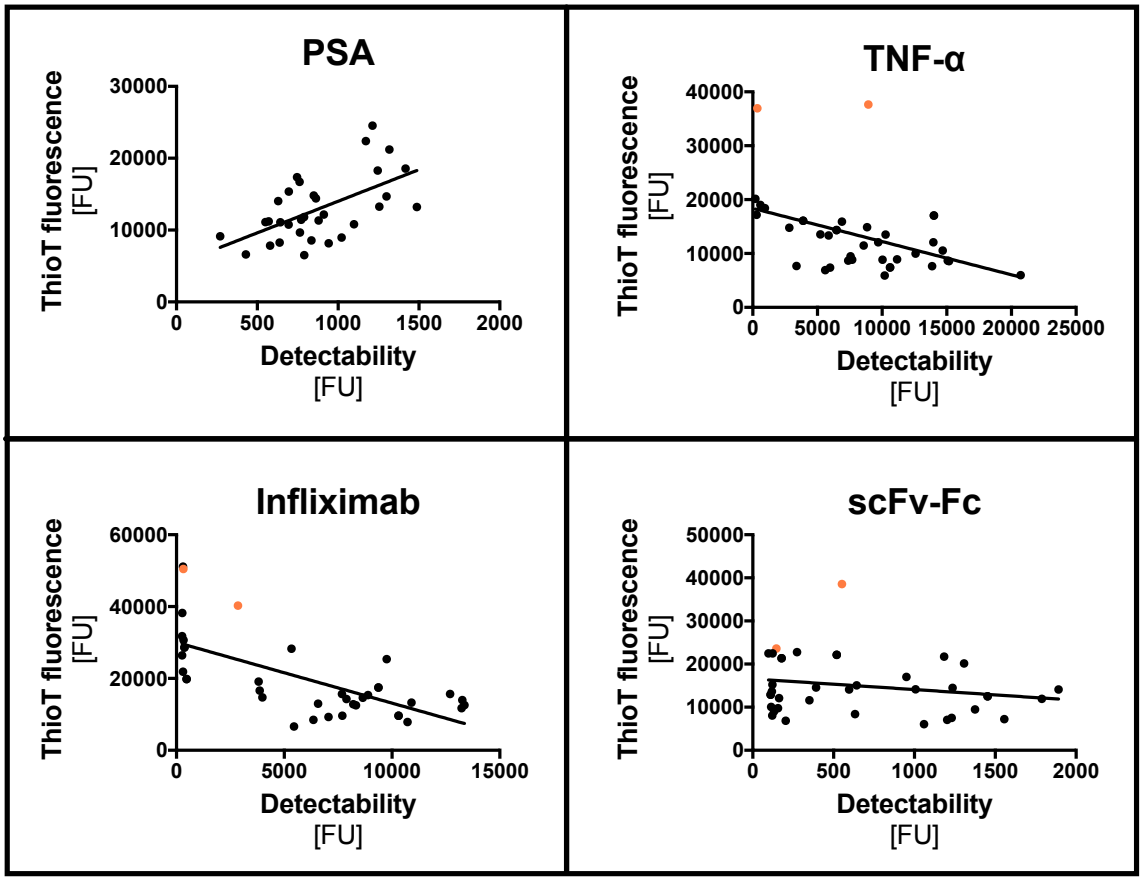


Figure 2.18: Correlation of antigen/antibody recognition and thioflavin T fluorescence

Correlation of antigen/antibody recognition and thioflavin T fluorescence with all measured data points over time and temperatures. Data points in orange were considered as outlier (graphical interpretation + Grubbs test) and not included into the correlation analysis.
P-values:
PSA = 0.0005, TNF-α = 0.0008, Infliximab < 0.0001, scFv-Fc = 0.2366.

With a closer look at the molecular properties of the investigated proteins and discussing the incorporation mechanism of thioflavin T on this level, it might be able to generate a better understanding why the correlation might be more significant in the case of infliximab rather than for TNF-α, PSA or the scFv-Fc fusion protein.

The underlying principle of thioflavin T incorporation implies a dynamic and potentially irreversible change in the secondary protein structure. Consequently, a good correlation indicates that those thioflavin T-sensitive secondary structure alterations are linked to and

determine the binding affinity of the detection antibody.

The monoclonal IgG1 antibody infliximab is composed of several β-sheet structures within its heavy and light chains which makes thioflavin T a suitable dye to assess the conformational as well as colloidal stability as indicated by Tim Menzen [12].

As reported by Narhi et al., unfolded TNF-α contains only β-sheet structures, which partially transform into irreversible α-helical aggregates through thermodynamically induced denaturation processes [13]. These structural changes seem to create a hydrophobic and therefore thioflavin T friendly environment. Taking the secondary structure-based binding geometry of the TNF/infliximab complex into account [10], these findings also lead to the conclusion that a correlation between thioflavin t-associated fluorescence, protein stability as well as detectability is possible which is in good accordance with our results.

Other than that, an increased thioflavin T-associated fluorescence of PSA seemed to display a more non-denatured state. However, since PSA remained detectable even after a 90-day-storage at 60°C, it had to be concluded that the recognition site of the detection antibody is independent of the stability of the protein, which can be confirmed as the used detection antibody is also suitable to detect linearized PSA on nitrocellulose membrane in Western blot experiments.

Based on the gained results for infliximab, similar findings were expected for the IgG1 antibody-like scFv-Fc fusion protein. According to Jaeger et al. the single chain fragment variables (scFv), consisting only of the variable regions of the immunoglobulin light and heavy chain were connected to the IgG Fc moiety by a soluble and flexible oligopeptide forming the scFv-Fc fusion protein [14]. The interpretation of our data, represented in figure 2.12 already revealed that the scFv-Fc fusion protein was much more temperature-sensitive than infliximab which might be due to a less condensed structure. As a consequence, the amount of thioflavin T, which is able to incorporate into denatured scFv-Fc protein, is limited.

In summary, we concluded that each investigated protein exhibited its own specific denaturation characteristic. Furthermore, the ability to monitor this denaturation process using thioflavin t is very dependent on the structure of the protein itself but also on the used detection protein. As already discussed by others, an underlying β-sheet assembly seems to be a basic requirement to employ this assay. Despite this, our results showed that thioflavin T in general is a simple, quick and cost-saving method which can be used to monitor the degree of denaturation once the specific denaturation characteristics of the

protein are known.

2.2.4.5 Comparison of two different spotting buffers

The employed methods and obtained results were also used to compare two different spotting buffers: a glycerol-based as well as a dextran-based system. In the previous chapter, the importance of the spotting buffer was already emphasized - the composition of different excipients determines parameters such as spot size, spot morphology, immobilization efficiency, evaporation but also protein stability. With regards towards their spotting performance, the investigated spotting buffer exhibited good results. However, less was known about their capacity to preserve the protein stability on the microarray surface.

For the comparison of the stability preserving performance of the two buffers, the antigen/antibody recognition as well as the thioflavin T fluorescence could be used. However, due to a non-Arrhenius behavior (involvement of too many influential parameters) it was not possible to calculate and compare time- and temperature-independent kinetic constants. As a matter of fact, the area under the curve (AUC) was calculated for each protein separately for each spotting buffer and temperature. The values were added over time and normalized to the overall sum. By this, it was possible to compare which spotting buffer provided the higher antigen/antibody recognition or thioflavin T fluorescence at different storage temperatures as seen in figure 2.19.

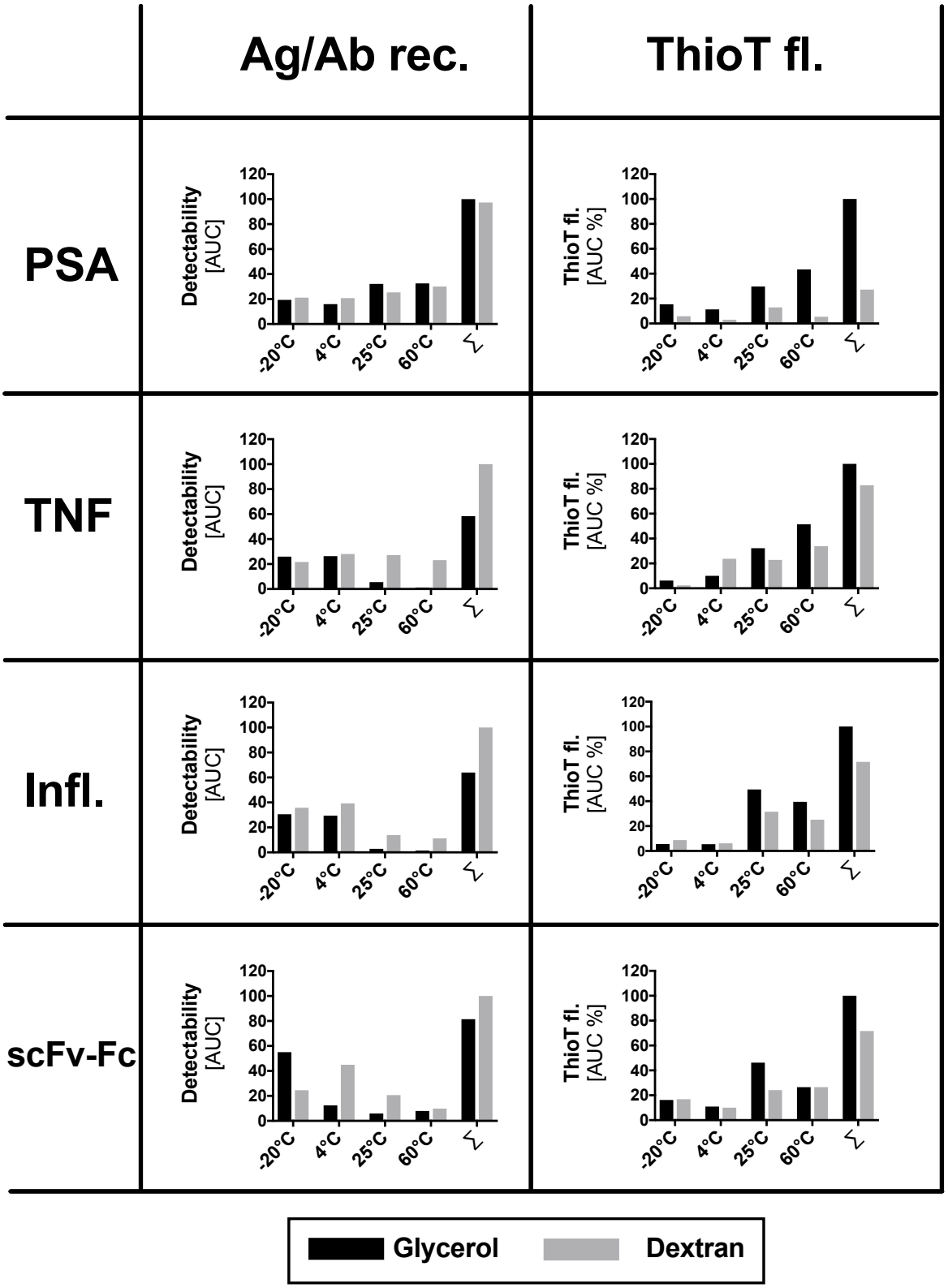


Figure 2.19: Comparison of spotting buffer performance
By means of normalized AUC values.

Based on these findings, it was possible to determine that, except for PSA, the dextran-based spotting buffer featured the overall superior protein preserving properties. For TNF-α, infliximab and the scFv-Fc fusion protein a higher detectability and a lower thioflavin T fluorescence, also in a storage temperature resolved manner, was calculated. Regarding the evaluation of PSA, it has to be keept in mind that, in contrast to the other candidates, a higher thioflavin T-associated fluorescence indicated a better recognizable protein.

Nevertheless, concerning practicability aspects such as spot morphology, glycerol-based spotting buffer systems are reported to provide beneficial properties especially with regard towards microarray printing [15, 16]. Dextran-based systems, which are commonly used in surface plasmon resonance measurements, are well known for their stabilizing capacity. Nevertheless, it should be mentioned that these buffers are less used for spotting rather than for coatings [17–19].

2.2.5 Conclusion

In the present work, it was demonstrated that thioflavin T is an appropriate probe to detect protein denaturation process not only in solution but also for proteins immobilized on microarray surfaces. A significant negative correlation between protein detectability and thioflavin T fluorescence could be pointed out for two of the four investigated proteins. Regarding its potential to serve as a quantitative protein function loss indicator, our correlation analysis showed that it depends on the protein to be investigated as well as the utilized detection protein, in particular the underlying secondary protein structure (e.g. β-sheet rich regions). It would have been of further interest to compare the gained results with results achieved using alternative techniques. Unfortunately, no useable results could be generated neither with time-of-flight secondary ion mass spectrometry nor with sum frequency generation. This emphasizes the importance of the developed method at hand, which allowed us to investigate and characterize the on-chip protein stability in a feasible fashion. In this regard, it was finally shown that a dextran-based spotting buffer features an improved stabilization performance in comparison to a glycerol-based matrix.

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3 Assay development for the immunological quantification of therapeutic antibodies and anti-drug antibodies

The evidence obtained by the investigations described in the previous chapters - antibody characterization and optimization of quantitative immunoassay - were used to design an applicable immunoassay for the analysis of clinical samples. In this regard, the detection of anti-drug antibodies was addressed.

In chapter 3.1. a microarray-based strategy is described, which can be implemented into existing portable microarray read out systems, whereas in chapter 3.2. a smartphone attachable blood glucose meter was used as read-out system.

All experiments as well as the drafting of the manuscripts was done by Janek Kibat.

Chapter 3.2. was submitted to PLOS ONE in April 2017.

Janek Kibat, Christian Bauer, Malte Buchholz, Thomas Gress, Christoph Schröder, Gerhard Winter and Jörg D. Hoheisel‡, Quantification in plasma of the protein drugs infliximab and adalimumab as well as the patients' corresponding immune response using a smartphone-attachable blood glucose meter. *PLOS ONE*.

‡ Corresponding author

3.1 Simultaneous quantification of Infliximab, adalimumab and corresponding anti-drug antibodies in plasma using protein microarrays.

3.1.1 Abstract

From a pharmacological point of view, a therapeutic drug monitoring of biological drugs such as proteins is of essential importance, not only to control the drug level and its effectiveness but also to investigate a potential abundance of anti-drug antibodies (ADAs). By means of humanized protein engineering, formulation design and administration schedules, the occurrence of ADAs might be minimized. Nevertheless, as therapeutic proteins can be understood as a foreign body, an immunogenicity potential remains. This risk is especially elevated in rheumatic patients which are under permanent treatment. In this work, we developed and optimized a microarray-based drug monitoring test for adalimumab and infliximab, which combines several individual assays such as drug type and drug concentration determination as well as IgG- and IgM-ADA quantification. The test performance allows a drug quantification within a range of 0.01 to 4 μg/ml (competitive strategy) and an ADA quantification starting from 10 to 10,000 ng/ml (bridging format) with a free drug tolerance of 1 μg/ml. The assay was further employed for the analysis of clinical patient samples to test its applicability and performance.

3.1.2 Introduction

The administration of highly specific therapeutic proteins is part of the state-of-the-art treatment options for many diseases such as cancer, hemophilia, anemia, diabetes as well as rheumatic and infectious diseases. However, the formation of anti-drug antibodies (ADA) is a potential risk, limiting the applicability of biological drugs especially if given over a longer period of time [1]. The risk can be reduced if the active protein is well designed and exhibits a low immunogenicity or if the patient is additionally treated with immunosuppressive drugs [2, 3]. Aside from the protein engineering and the therapeutical strategy itself, the inclusion of a diagnostic strategy is needed to detect and to handle this complication adequately. In the ideal case, the patient is under continuous monitoring which includes both, a therapeutic drug monitoring as well as an evaluation of ADA concentrations [4–6]. By this, the drug plasma concentration could be precisely adjusted to avoid overdosing

(which could again trigger the formation of ADAs) and alternative therapy concepts could be selected before a full drug resistance would have regarded by the patient [5, 7–9]. Apart from an increased therapy effectiveness, an appropriate therapy monitoring would also reduce the amount of needless injections e.g. when it comes to overdosing due to an unexpectedly low elimination or in the treatment of drug-resistant patients and would therefore also reduce costs [10, 11].

The detection of ADAs in plasma samples from patients, especially from those who are currently under treatment, is a challenging task. This is due to the fact that the majority of ADAs will be bound to the present drug and thereby are hardly accessible in an immunological assay without immune complex dissociation. Furthermore, the data interpretation is not always clear in a way that it might be difficult to assess whether the amount of detected ADAs represents a clinical relevant immune response. In this context, it is not just one specific ADA rather than a patient-individual collection of different antibody isotypes against different epitopes. Amongst all ADAs, preferably the neutralizing candidates, which are directed against the functional epitope of the drug, are the ones limiting the pharmacological function most.

By now, many techniques and data analysis tools have been published, describing how to circumvent or control influencing factors and how to approach a valid ADA quantification [12–14]. The experimental setup can be quite laborious, especially if an isotyping is pursued. For a patient-individual and continuous monitoring, also known as personalized medicine, a reliable test is needed, which can be carried out in a clinical routine setting. The requirements for such a clinical use would comprise a short test duration, basic laboratory equipment as well as the possibility to implement it into an automated process.

With respect to the mentioned aspects and being aware of interfering parameters, a protein microarray-based method was developed, which combines the quantification of drug levels, the determination of related ADA concentrations as well as the differentiation between an IgM or IgG predominant immune reaction in one assay. As protein microarrays offer a suitable platform for more than just one drug, we designed a proof-of-concept test for the two most widely used anti-rheumatic drugs - adalimumab and infliximab [15, 16]. By multiplexed combinations it was possible to answer the following questions when analyzing patient samples: Is drug present in the sample? If yes, which drug is present and how high is its concentration? Are ADAs present? If yes, against which drug and in which concentration? Which ADA isotypes can be detected?

After testing the assay performance in terms of limit of detection, drug tolerance level and correlation with ELISA results using surrogate ADAs, clinical patient samples could be analyzed. The obtained samples from rheumatic Crohn's disease (MC) and ulcerative colitis (UC) patients under adalimumab and infliximab treatment were used to test the assay applicability and to evaluate clinically relevant ADA concentrations.

3.1.3 Materials and Methods

3.1.3.1 Protein microarray preparation

For the preparation of protein microarrays epoxysilanized glass slides (Nexterion E, Schott, Jena, Germany) were used. Adalimumab (Humira®; Abbvie Ltd.), Infliximab (Remicarde®, MSD Sharp & Dohme GmbH), HCA203, HCA213, (AbD Serotec - Bio-Rad Laboratories Inc.), TNF-α (ATGen Ltd) as well as human IgM (myeloma, whole molecule, Thermo Fisher Scientific) were adjusted to the needed concentration, mixed with twofold spotting buffer (0.4 M $NaHPO_4$, pH 8.6, 10% glycerol, 0.01% sarkosyl) and spotted using a Micro-Grid II 610 (Apogent Discoveries, Mittenwald, Germany) contact printer. Prior to sample incubation, slides were blocked for 1 h with the Candor Blocking Solution (Candor, Wangen im Allgäu, Germany) and washed with 1 M glycine buffer (pH 2.6) supplemented with 1.0 % (v/v) Tween 20 (Sigma Aldrich) using a Tecan HS 4800 hybridization system (Tecan Group Ltd., Männedorf, Switzerland).

3.1.3.2 Incubation and analysis of samples

Serial dilution series of adalimumab, infliximab, anti-adalimumab (HCA203) or anti-infliximab (HCA213) were spiked in the pooled plasma of 5 healthy individuals. These sample solutions as well as the patient plasma samples were diluted 1:1 in blocking solution. The blocked slides were sealed with ProPlate® 16-well incubation chambers (Grace Bio Labs). Each well was filled with 70 μL of sample solution and incubated for 1 hour at room temperature. Prior to the incubation with 70 μL of detection solution, each well was washed with PBST (PBS supplemented with 0.05 % v/v Tween 20) for three times. The detection solution consisted of adalimumab and infliximab labeled with DY-649-P1 (Dyomics GmbH, Jena, Germany) each at a concentration of 0.05 μg/ml dissolved in the blocking solution. Each well was washed separately with PBST and ddH_2O after 1 hour incubation. The dried slides were than scanned using a Tecan PowerScanner (Tecan Group Ltd., Männedorf, Switzerland) and images were analyzed using the GenePix 6.0 microarray

analysis software (Axon Instruments, Inc., Union City, CA).

3.1.3.3 Drug tolerance

Plasma samples were spiked with HCA203 and HCA213 at a constant concentration of 250 ng/ml and different drug concentrations form 1 mg/ml to 10 ng/ml. Samples were then further processed as described above.

3.1.3.4 ELISA

ELISA experiments were used as reference method for the quantification of drug and anti-drug antibody levels. 50 μL of a solution containing 5 μg/ml TNF-α, adalimumab or infliximab were filled in each well of a 96-well microtiterplate (Maxisorb; Nunc, Roskilde, Denmark) and incubated overnight at 4°C. The following day, the protein solution in the wells was discarded and wells were blocked with 150 μL of 5 % (w/v) skim milk for 3 hours at 4°C. The blocked wells were incubated with 40 μL of sample solution. As detection antibody biotinylated adalimumab and infliximab (EZ-Link NHS-PEG_4-Biotin, Thermo Fisher Scientific) were used. After an incubation time of 1 hour and three washing steps with PBST, 20 μL of a 1 μg/ml Streptavidin-HRP (BioLegend) solution were added to each well for 30 minutes. The final read-out was performed by adding TMB substrate (Thermo Fisher Scientific) and using a plate reader (Infinite M200, Tecan) to measure the absorbance at 450 nm.

3.1.3.5 Plasma samples

Plasma samples of 10 inflammatory bowel disease patients under the treatment of adalimumab (n=4) and infliximab (n=6) were collected by the Department of Gastroenterology, Marburg, Germany. Analysis of the collected samples was approved by the ethics committee of the University Hospital of Marburg and Gießen and carried out according to standardized laboratory work procedures for infectious materials.

Table 3.1: Clinical information on plasma samples

Patient ID	Sex	Age [y]	Disease	Drug treatment	Therapy duration [m]
P1	m	26	UC	Infliximab	7
P2	m	20	MC	Infliximab	3
P3	f	54	CI	Adalimumab	1
P4	f	32	MC	Adalimumab	3
P5	m	22	UC	Adalimumab	9
P6	m	39	MC	Infliximab	8
P7	m	30	MC	Infliximab	8
P8	m	57	UC	Infliximab	13
P9	m	29	CC	Infliximab	86
P10	f	44	UC	Adalimumab + MTX	25

MC= Crohn's disease, UC = ulcerative colitis CI = Colitis indeterminata, CC = Crohn's colitis,
MTX = Methotrexate

3.1.4 Results and discussion

3.1.4.1 Assay and array design

The assay was designed to characterize a patient sample by the simultaneous investigation of three parameters: (i) the type and concentration of free drug, (ii) the presence and amount of ADAs against different drugs as well as (iii) an estimation of the amount of IgM within the quantity of all ADA isotypes. The quantification of free drug was necessary for this test as the detection of ADAs provided valid results only if the drug level was below a certain concentration - also known as drug tolerance level. Despite the quantification of ADAs itself, the quantification of anti-drug specific IgMs was further included in order to distinguish between an IgM-dominated early-stage or an IgG-related late-stage immune reaction.

As illustrated in figure 3.1 A and B, TNF-α, adalimumab, infliximab, HCA203 (anti-adalimumab), HCA213 (anti-infliximab) and a serial dilution series of human IgM were spotted on the microarray surface. After the surface had been blocked, the sample material, consisting of drug antibodies (adalimumab or infliximab) and ADAs (against adalimumab, infliximab or both), was incubated. Depending on the individual composition of each sample, adalimumab and infliximab antibodies bound to TNF-α as well as the

corresponding surrogate anti-drug antibodies (HCA203 and HCA213). ADAs bound to either adalimumab or infliximab. In the next step, the sample solution was replaced by a detection solution which consisted of labeled adalimumab, infliximab and anti-human IgM.

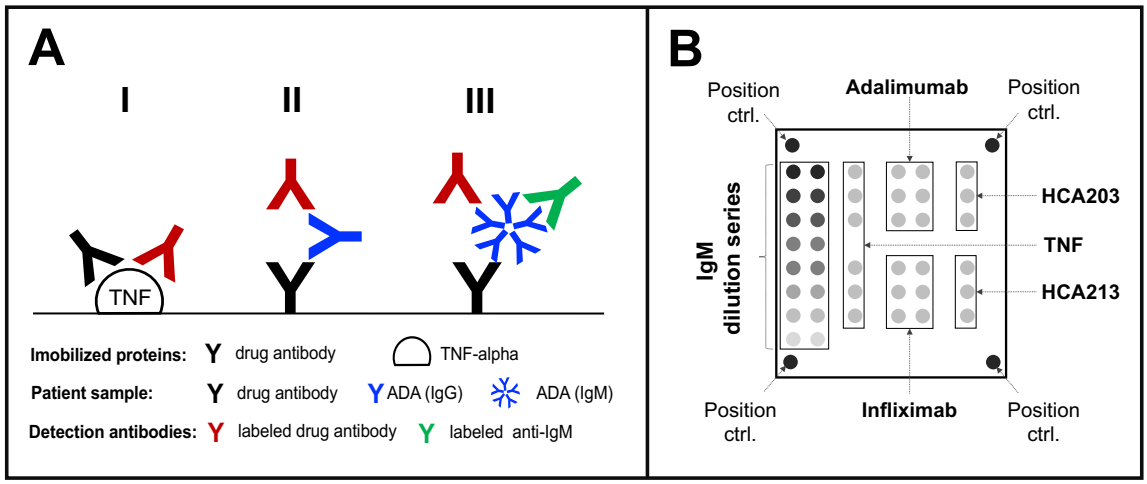


Figure 3.1: Immunodetection principles and spotting pattern
The three detection principles represented in A consist of a competitive drug quantification (I), an ADA bridging assay (II) and the detection of IgM ADAs (III). In B the spotting pattern is pictured, which includes a human IgM dilution series for the estimation of IgM levels.

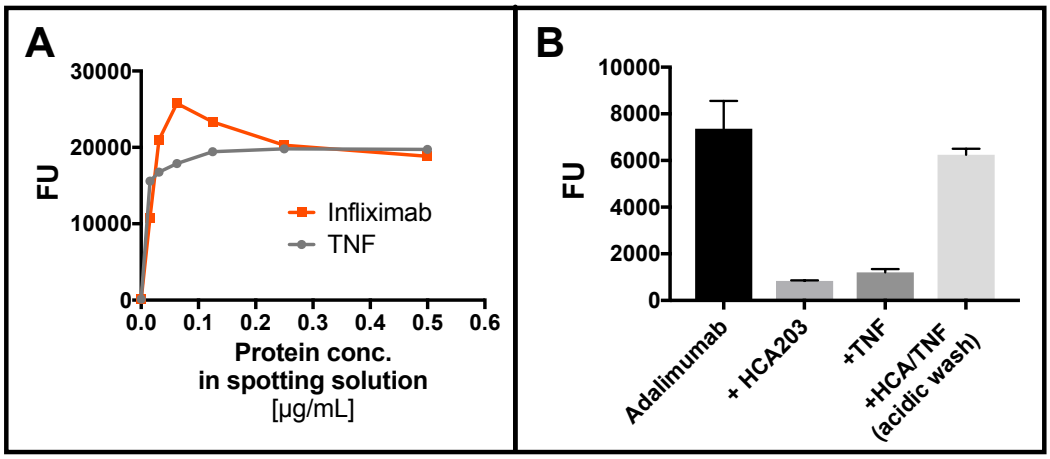
The type and concentration of free drug could be determined, considering HCA203 as binding target for adalimumab, HCA213 as target for infliximab and TNF-α as target for both. The free drug in the patient sample competed with the fluorescently labeled drug in the detection solution against the mentioned binding sites, allowing a competitive quantification.

The amount of ADAs was determined using a bridging format, in which the ADAs in the sample functioned as a bridge between immobilized and fluorescently labeled drug. Utilizing the same detection antibody in the competitive quantification strategy as well as in the bridging format, the calculation of drug and ADA concentrations could be combined in one step.

By the addition of a labeled anti-human IgM detection antibody, which bound to the IgM-ADAs forming the bridge as well as the human IgM dilution series, it was further possible to correlate and calculate the amount of anti-drug specific IgMs.

3.1.4.2 Microarray preparation

Spotting several binding pairs (TNF-α and infliximab/adalimumab, adalimumab and HCA203, infliximab and HCA213) on one microarray test field demanded for an optimization of our preparation process so that TNF-α could not interfere with adalimumab and infliximab or vice versa. As depicted in figure 3.2 A, a saturation of the surface with TNF-α was reached at a concentration of 0.25 μg/ml. Consequently, the amount of interfering protein, which did not immobilize on the surface, would have been increased if spotting solutions with a protein concentration above 0.25 μg/ml had been used.



FU = fluorescent units

Figure 3.2: **A** - Solutions with different infliximab and TNF-α concentrations were spotted on epoxysilanized glass slides. The immobilized proteins were detected using fluorescently labeled TNF-α or infliximab.
B - Achieved signal intensities of adalimumab spots after incubation with fluorescently labeled TNF-α in different test fields. On the 'adalimumab' test field only adalimumab was immobilized. On the '+HCA203' test field adalimumab and HCA203 were immobilized. On the '+TNF' test field adalimumab and TNF-α were immobilized. On the '+HCA203/TNF' test field adalimumab, HCA203 and TNF-α were immobilized, but an additional acidic washing step was performed.

In contrast to the expected concentration dependent immobilization of TNF-α, a hook effect was observed for the immobilization of infliximab. An explanation for this phenomenon might be described in a model: At the point of complete saturation a monolayer of antibodies covers the slide surface. Using a protein concentration which is above the monolayer saturation level, may lead to the buildup of additional antibody layers. Antibodies in the upper layers will preferably bind levitating antigens during incubation. Still,

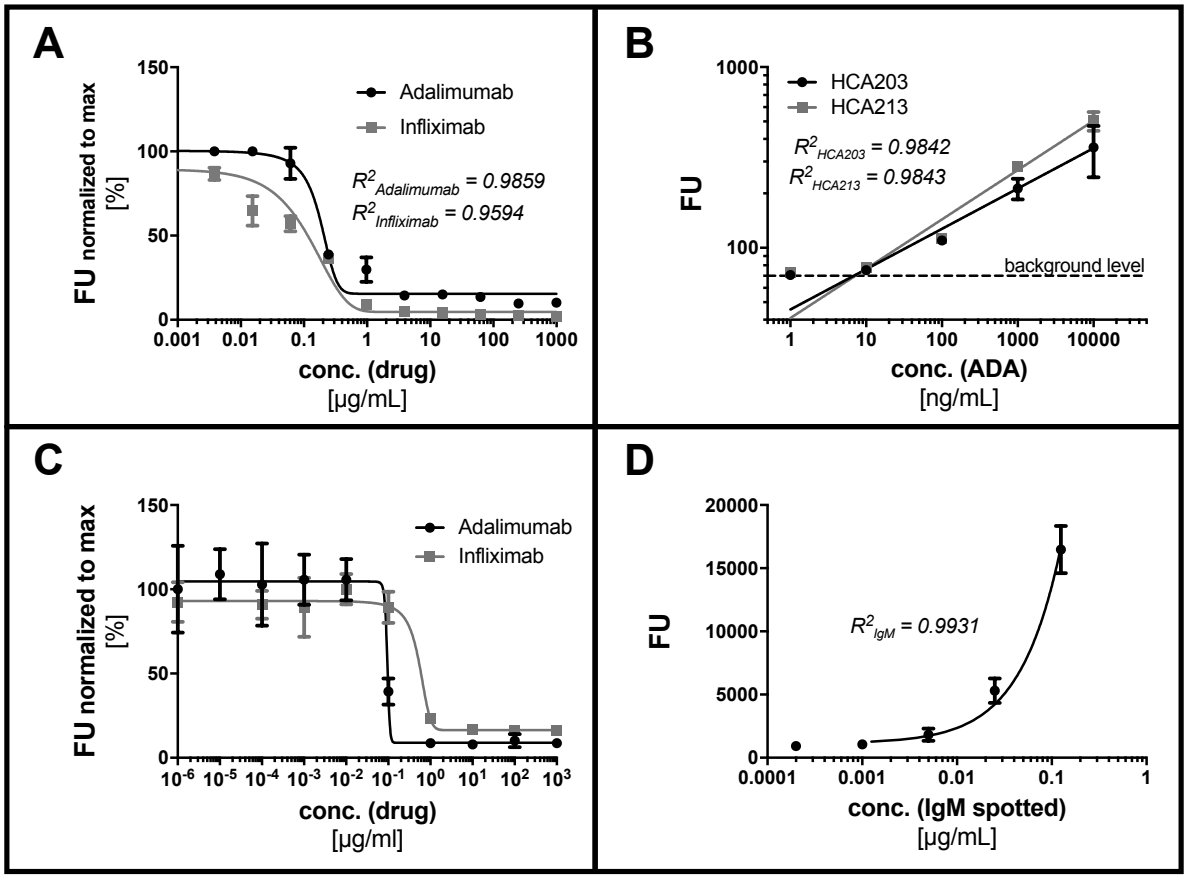
the formed immune complexes with antibodies in the upper layers tend to be rinsed in the final washing step, paradoxically leading to a reduced signal intensity. The likeliness of layer formation might be dependent on the interaction forces between proteins of the same kind such as aggregation behavior.

Even though proteins were spotted in concentrations at the saturation level, the interference of different binding partners could not be avoided as illustrated in figure 3.2 B. If either HCA203 or TNF-α was spotted next to adalimumab and no acidic washing step was included prior to the incubation with labeled TNF-α, the majority of adalimumab recognition sites was occupied leading to a signal intensity decrease of 88.6 or 83.6 %. Including a final acidic washing step using 1 M glycine buffer (pH 2.6) supplemented with 1 % (v/v) Tween 20 into the microarray preparation workflow, the formation of interfering immune complexes could be avoided or dissolved.

3.1.4.3 Assay performance

Detection as well as quantification of adalimumab and infliximab concentrations
Plasma concentrations of adalimumab and infliximab were determined using a competitive quantification strategy: the free drug antibody in the plasma sample competed against the fluorescently labeled drug antibody for the available binding sites either on the TNF-α or the corresponding surrogate anti-drug antibody (HCA203 and HCA213). As the used drugs bound to TNF-α with a higher affinity (K_d(adalimumab) = 8.6 pM, K_d(infliximab) = 4.2 pM - [17]) than against HCA203 and HCA213 (K_d(HCA203) = 0.15 nM, K_d(HCA213) = 1.8 nM), TNF-α was in favor for the calculation of drug concentrations. However, HCA203 and HCA213 were needed to differentiate which of the two TNF-binding drugs was present in the sample.
Figure 3.3 A reveals that it was possible to quantify adalimumab in a concentration range from 0.01 to 4 μg/ml and infliximab in a concentration range from 0.004 to 4 μg/ml. Depending on the amount of labeled detection antibody, the dynamic quantification range could also be shifted towards either higher or lower concentrations.

ADA quantification with bridging format
The entity of all ADAs in a patient sample is an individual collection of antibodies, which differ in isoform, epitope specificity as well as affinity. For this reason, standardized and well characterized surrogate anti-drug antibodies - HCA203 and HCA213 - were employed to determine a calibration curve for the quantification of ADA levels. As seen in figure 3.3



FU = fluorescent units

Figure 3.3: Assay performance

A - Competitive quantification of adalimumab and infliximab (four parameter logistic curve fit)
B - Quantification of HCA203 and HCA213 in bridging format (log-log fit)
C - Investigation of drug tolerance level at an ADA concentration of 250 ng/ml (four parameter logistic curve fit)
D - Quantification of human IgM via the reference dilution series (linear fit)

B, HCA203 and HCA213 concentrations starting at 10 ng/ml could be quantified, which complies the FDA industry guideline 'Assay Development and Validation for Immunogenicity Testing of Therapeutic Protein Products' recommending a test sensitivity of at least 250 - 500 ng/ml.

Investigation of drug tolerance

Quantifying ADAs in patient samples using the chosen bridging format provides the most valid results if the concentration of free drug is below 0.01 μg/ml. Represented in figure

3.3 C, the signal intensities for a constant surrogate ADA level at 250 ng/ml decreased with increasing concentrations of the corresponding drug. At a concentration of 1 μg/ml and higher, a detection of ADAs is no longer possible. Therefore, the calculation of ADA levels in patient samples with a free drug concentration above this threshold had to be interpreted with care - ADAs could be present, yet not detectable in such a sample. For this reason, it was of importance to collect samples before a new dosage of adalimumab or infliximab had been administered to the patient.

In order to circumvent ADA quantification interferences with higher drug levels, an acidification strategy, to dissolve and reassemble drug and ADA immunocomplexes, was tested. Neither a sample acidification with a 0.2 M glycine buffer (pH 2.6) nor with 0.15 M acetic acid and a following neutralization using Tris or $NaHCO_3$ (pH 9.0) buffers resulted in about 80 % lower signal intensities compared to the unacidified samples (data not shown). As the pH adjustment from around 3 to 7 - 8 was monitored, an insufficient neutralization capacity of the used buffers could be denied. By the addition of 30 μl neutralizing buffer to the 70 μl of incubated sample solution, the volume was increased by 42 %. This volume increase leads to a reduced concentration of analytes, which might be the most plausible explanation for the decreased ADA signal intensities. Still, the reduced concentration was not expected to result in a signal intensity reduction of 80 % and should have also been partly compensated by the amount of ADAs, which had been made accessible by this. An optimization in this regard would be necessary. However, up until now no acidification step was included into the method stream.

Estimation of IgM level
The IgM calibration curve in figure 3.3 D was derived from the spotted serial dilution series and used as quantification reference. As a precise calibration with target bound IgM antibody could not be carried out, it could only provide an estimation of the actual IgM concentration

3.1.4.4 Analysis of clinical samples

Samples of ten patients under a current adalimumab or infliximab treatment were analyzed (see table 3.1). In contrast, the plasma samples of five healthy individuals were pooled and used as negative control.

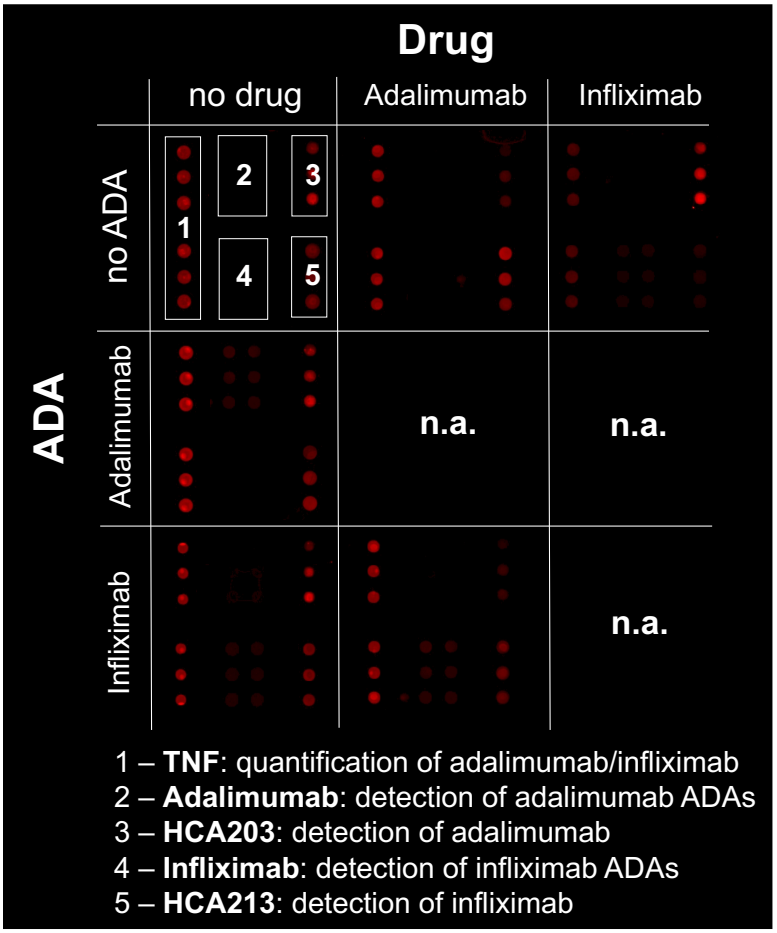


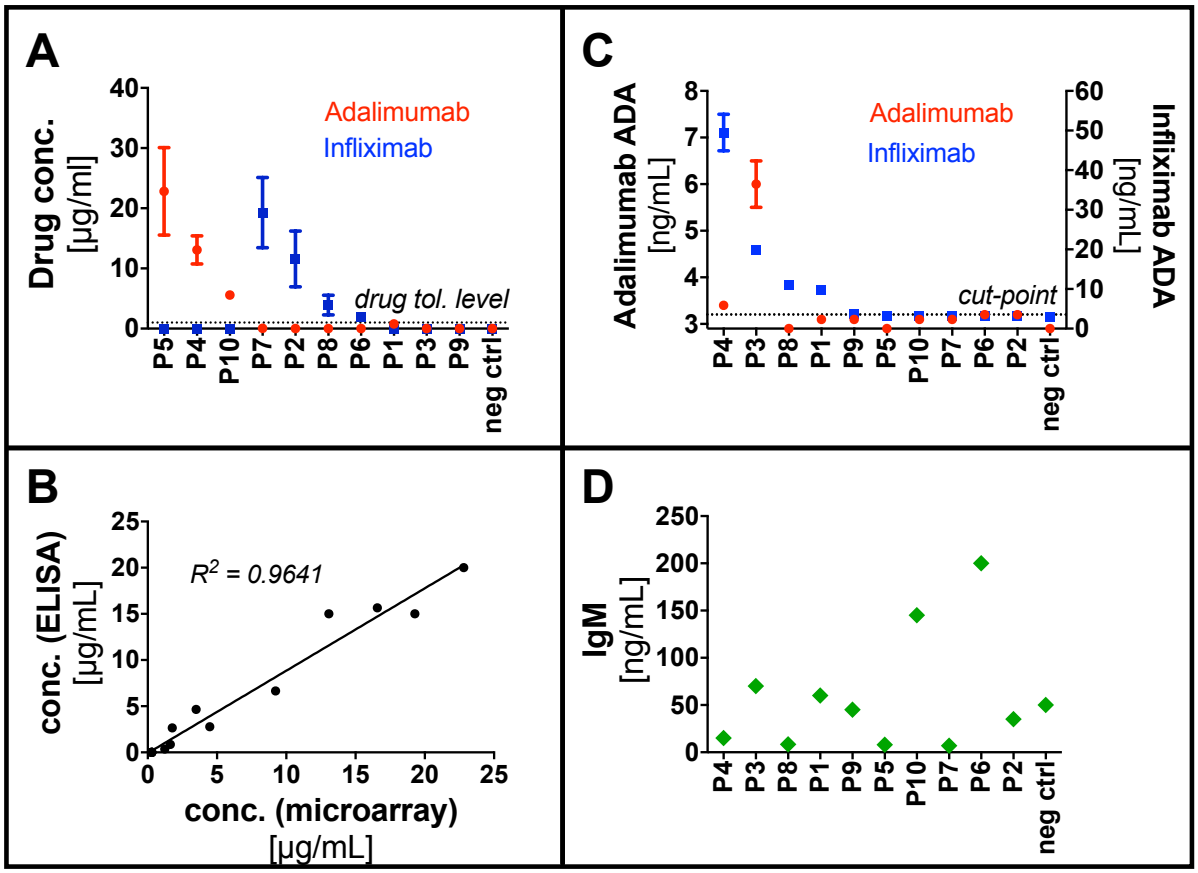
Figure 3.4: Raw fluorescence images of clinical samples
According to the scheme in figure 3.1 B, the test field consisted of five relevant areas: Field 1 consisted of six TNF-α spots. By this, the amount of present anti-TNF-α drugs could be measured in a competitive fashion - high drug concentration, low signal intensity. In combination with field 3 and 5 (surrogate anti-adalimumab /-infliximab antibodies), it was possible to determine which drug was present e.g. low HCA203 intensity, high adalimumab concentration. The spotted drugs in field 2 and 4 indicated whether ADA were present or not (bridging format)
1st Row : negative control, P5, P7
2nd row : P3
3rd row : P1, P4
(from left to right)

In figure 3.4 the acquired raw images of different patient samples are represented. The corresponding test field layout was illustrated in figure 3.1 B. Considering TNF-α (field 1), anti-adalimumab antibody (field 3) and anti-infliximab antibody (field 5) spots, it was already possible to see whether a patient was under treatment and which drug he/she received. Taking adalimumab (field 2) as well as infliximab (field 4) into account, it was further possible to recognize if ADA were present or not.

Calculating with an average weight of 70 kg and a total blood volume of 5 liters, drug concentrations were expected to be around 10 μg/ml in the case of adalimumab (50 mg dosage) or 70 μg/ml in the case of infliximab (dosage recommendation: 5 mg/kg) directly after administration. However, it should be taken into account that the drug titer of patients under continuous treatment might still be increased before a new dosage is given, depending on the individual elimination rate and injection intervals.

With a closer look at the measured drug concentrations in figure 3.5 A, it was possible to stratify and confirm whether a patient had been under treatment with adalimumab (P4, P5, P10) or infliximab (P2, P6, P7, P8). Furthermore, drug titers were found to be in the expected range, except for patient P5. Apart from the regular schedule, this patient was treated on a weekly basis with adalimumab resulting in an elevated plasma concentration of 22.81 μg/ml.

The determined drug concentrations of patient samples were also in good accordance with results derived from ELISA experiments, as seen in figure 3.5 B.



FU = fluorescent units

Figure 3.5: Analysis of clinical samples II

A - Measured adalimumab and infliximab concentrations in each patient sample

B - Correlation of measured drug concentrations; microarray results vs. ELISA results

C - ADA levels measured in each patient sample

D - IgM-associated ADA levels measured in each patient sample

With regard to an appropriate evaluation of ADA titers with the developed method, it was necessary that the sample contained no interfering amounts of free drug. Even though samples were taken prior to a new injection, seven samples featured drug concentrations above the critical drug tolerance level of 1 μg/ml. As a matter of fact, a precise quantification of ADAs could only be ensured for P1, P3 and P9. In comparison with other methods (ELISA, ECL) a drug tolerance of 1 μg/ml represents a regular level.

Determination and explanation of anti-adalimumab and anti-infliximab plasma concentrations

The measured adalimumab and infliximab ADA concentrations for each patient sample are summarized in figure 3.5 C. Calculated as the upper 95^{th} percentile of five negative control samples, a cut-point value of 3.2 ng/mL was determined for anti-adalimumab and 3.58 ng/mL in the case of anti-infliximab antibody concentrations. Concentrations above this level were considered as ADA positive as long as the free drug concentration was below the drug tolerance level. If the free drug concentration was above the drug tolerance level of 1 μg/ml, it had to be assumed that ADAs might still be present, yet remained undetectable.

Regarding the samples from the 4 patients under adalimumab treatment (P3, P4, P5 and P10), 2 patients were classified as ADA positive. The highest anti-adalimumab concentration of 6.0 $\pm$ 0.5 ng/ml was found in patient P3, who received a single adalimumab injection prior to sampling. Similar to a vaccination process, a time-dependent immune response with higher ADA levels in patients under a longer lasting adalimumab therapy were to be expected . Unfortunately, a correlation of the therapy duration and the resulting anti-adalimumab concentration could not be made as the free drug levels of patient P4, P5 and P10 exceeded the drug tolerance level.

In contrast to anti-adalimumab antibodies, which were only found in patients under adalimumab treatment, anti-infliximab antibodies could also be detected in patients treated with adalimumab. Moreover, the infliximab ADA levels exhibited the highest concentrations in the samples of patients P3 and P4 (treated with adalimumab) amongst all ADA positive samples. An earlier treatment with infliximab could not be confirmed in these cases, leading to the assumption that these patients acquired an associated immune response against adalimumab as well as infliximab. It is known that patients who already acquired an immunogenicity against infliximab were also prone to develop antibodies to adalimumab and vice versa [18–20]. The detected ADAs remained drug specific and a cross-immunogenicity against both monoclonal antibodies based on the treatment with just one had not been described so far [21, 22].

Up until now, the method at hand allowed a precise quantification of ADAs. In future, more samples need to be analyzed to deduce clinical conclusions from the calculated ADA levels. Thus, a better and more precise understanding of how to differentiate between ADA positive patients developing a therapy resistance and the ones who do not can be

generated. An approach to gain more information here, would be the specific detection of neutralizing ADAs in addition to the overall amount of ADAs. This could be accomplished by an immobilization of the Fab fragment or an orientated immobilization of the therapeutic antibody.

Quantification of IgM ADAs
The utilized and above mentioned ADA bridging format is a convenient immunoassay to quantify ADAs, no matter which isotype. This is particularly useful because in a comparable setup which would consist of the immobilized drug, bound ADAs and an anti-human detection antibody, one would not be able to quantify the ADAs which are of the same isotype subclass as the humanized monoclonal drug antibody - usually IgG1. Apart from the information whether ADAs are present in a sample or not, a discrimination of the involved antibody isotypes would be a desirable feature.

According to the scheme III in figure 3.1 A, the detection of IgM-associated ADAs was included into our multiplexed assay. The determined IgM concentrations represented in figure 3.5 D could only provide an information on the degree of IgM involvement in the drug-specific immune response. With an elevated IgM concentration of 50 $\pm$ 5.5 ng/mL detected in the negative control sample, it was difficult to assess the calculated concentrations as absolute values. A proper validation with additional IgM negative samples would be needed here. Nevertheless, comparing the IgM levels of each patient sample in which ADAs could already be detected, an IgM involvement was observed for P3 and P1 speaking for an immune response at an early stage. Patient P4 with the highest anti-infliximab antibody level did not show any IgM involvement which gave reason for an IgG-dominated immune response. Observing the highest IgM levels in the samples of patient P6 and P10, lead to the assumption that these patients were just about to acquire a drug-related immune response. Further experiments in order to monitor a time-related ADA development of individual patients are under consideration.

3.1.5 Conclusion

A comprehensive therapeutic drug monitoring of patients under adalimumab or infliximab treatment demands for the investigation of several parameters such as the drug plasma concentration as well as the occurrence and type of ADAs. Moreover, the drug plasma concentration is not only relevant from a pharmacological point of view, but also for the biological performance of the ADA assay (free drug tolerance). In this work, we combined

these individual assays in one protein microarray-based test. In experiments with clinical patient samples (n=10) the applicability of this test could be demonstrated and revealed that ADAs could be detected in 40 % of the patients under treatment. Also, a cross-immunogenicity against both drugs, adalimumab and infliximab, was observed in two cases. The information about an acquired or developing therapy resistance is of high value for the responsible clinician in order to select the future therapy options for the individual patient. Therefore, the microarray platform might be of special interest as it is not restricted to the analysis of adalimumab, infliximab and corresponding ADAs but can also easily be upgraded by many other drug candidates, which might be beneficial especially with regard to the development of biosimilars.

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3.2 Quantification in plasma of the protein drugs infliximab and adalimumab as well as the patients' corresponding immune response using a smartphone-attachable blood glucose meter

3.2.1 Abstract

Therapeutic drug monitoring is an essential component of the treatment of rheumatic patients. In a proof-of-concept study, we describe the use of a blood glucose meter read-out for the quantification of the plasma concentration of the anti tumor necrosis factor alpha (TNF-α) antibodies adalimumab and infliximab. Employing a competitive assay strategy based on the enzymatic activity of invertase - hydrolyzing sucrose into glucose and fructose - plasma antibody concentrations could be measured quantitatively in a range of 0.01 to 47 μg/ml within 50 minutes. Furthermore, a related assay allowed the identification of anti-drug antibodies (ADAs) that may result from a patient's immune response to treatment. ADA concentrations of 1.6 to 1000 ng/ml could be detected within three hours, exhibiting a free drug tolerance of 1 μg/ml. The method was used to characterize clinical samples from inflammatory bowel disease patients to demonstrate its applicability. Utilizing a smartphone attachable blood glucose meter, protein drug concentrations could be determined remotely and transferred to relevant sites by cellular phone.

3.2.2 Introduction

Monitoring of therapeutic drug concentrations is a key component of a patient-specific treatment optimization. In order to achieve the most effective clinical response, a patient's plasma drug level should be adjusted to a certain concentration range - commonly known as the therapeutic window. This applies to small molecules as well as biological drugs. Especially patients with decreased drug clearance capacity could benefit from prolonged treatment intervals; this would also prevent over-dosing. For protein drugs, also the cost factor is particularly high. Therapeutic drug monitoring is therefore cost-effective as demonstrated for the therapeutic antibody adalimumab, for example [1, 2]. Furthermore, the administration of protein drugs could trigger the immune system similar to a vaccination process, leading to the generation of anti-drug antibodies (ADAs) [3]. As a consequence, ADAs can cause therapy resistance, especially if neutralizing antibodies against

the active drug site are formed. It was shown that even drug concentrations below the effective dose can provoke ADA formation [4]. Long lasting and expensive anti-rheumatic therapies with monoclonal antibodies against tumor necrosis factor alpha (TNF-α) tend to loose clinical response effectiveness over time [5]. Their monitoring should therefore not only include the correct adjustment of the administered dose, but also the determination of ADA levels.

Small molecules are typically quantified in blood by employing liquid chromatography coupled to mass spectrometry. This process is also used for the precise quantification of specific proteins, yet it is quite laborious and cost-intensive taking into account the sample preprocessing and the required instrumentation. In contrast, biological drugs, such as antibodies, can be analyzed with immunoassay techniques, which do not necessarily require an extensive infrastructure. Here, we demonstrate that the plasma concentration of the therapeutic antibodies adalimumab and infliximab as well as that of ADAs against these protein drugs can be measured with a simple and portable setup consisting of antigen-coated magnetic beads, an invertase-labeled therapeutic antibody, a sucrose solution and a blood glucose meter. The invertase is used as reporter enzyme. In contrast to classical reporter enzymes, such as horseradish peroxidase and alkaline phosphatase, which produce colored or chemiluminescent molecules, invertase hydrolyses sucrose into glucose and fructose. Thus, a blood glucose meter can be used as a read-out tool for quantifying the amount of glucose, which correlates with the amount of the protein of interest. Similar approaches have been used for the quantification of pathogenic bacteria, protein biomarkers, small molecules as well as mercury [6–12]. Coupling the assay to commercial instrumentation and related software permits a measurement even in remote locations and a direct transfer of the generated data via a cellular phone.

3.2.3 Materials and Methods

3.2.3.1 Coating magnetic beads with TNF-α

N-hydroxysuccinimide (NHS)-activated magnetic beads (Thermo Fisher Scientific) were coated with TNF-α (ATGen) according to the manufacture's protocol. A volume of 300 μl bead suspension (bead concentration 10 mg/ml) was activated with 1 ml ice-cold 1 mM HCl and incubated with 2 mg TNF-α in 500 μl 0.1 M $NaHCO_3$, pH 9.0, for 12 hours at 4°C. The TNF-α-coated beads were washed three times in PBST (PBS supplemented with 0.05 % Tween 20 (Sigma-Aldrich)). Beads were stored in PBST containing 0.05 % sodium

azide (Sigma-Aldrich) at 4°C. Measurements of the coupling efficiency via a BCA assay (Thermo Fisher Scientific) revealed a TNF-α amount of 20 μg/mg of bead.

3.2.3.2 Coating magnetic beads with infliximab or adalimumab

According to the manufacture's protocol, infliximab (Remicade, MSD Sharp & Dohme) or adalimumab (Humira, AbbVie.) were dissolved in 500 μl PBS at a concentration of 4 mg/ml. The solution was added to 300 μl bead suspension of protein-A/G coated beads (bead concentration 10 mg/ml; PureProteome Protein A/G Mix Magnetic beads, Merck). After a 12 hour incubation at 4°C, the beads were washed with PBST. Subsequently, bound antibodies were covalently cross-linked to the protein-A/G using bis(sulfosuccinimidyl)-suberate (BS3, Thermo Fisher Scientific). Measurements of the coupling efficiency via a BCA assay (Thermo Fisher Scientific) revealed an adalimumab amount of 182 μg per milligram of bead and an infliximab amount of 176 μg/mg.

3.2.3.3 Elution and recycling of magnetic beads

It was possible to elute infliximab or adalimumab molecules from the magnetic particles with covalently bound TNF-α and reuse the beads for further measurements. Prior to elution, the magnetic beads were washed three times with PBST. They were then resuspended in 1 ml of 1 M glycine, pH 2.6, and incubated for 15 min at 56°C at continuous shaking. After this, the beads were washed five times with PBST and stored in PBST containing 0.05 % sodium azide (Sigma-Aldrich) at 4°C.

3.2.3.4 Invertase-conjugation of infliximab or adalimumab using sodium periodate

For oxidizing the glycosylation moiety of invertase, 36 mg of *S. cerevisiae* invertase (grade V, Sigma-Aldrich) were dissolved in 3.6 ml 0.1 M sodium acetate, pH 4.5, and 3.88 mg of sodium periodate (NaIO4, Sigma-Aldrich). The reaction mix was protected from light and incubated at room temperature. After 2 hours, the solution was filtered through a 0.22 μm pre-syringe filter and dialyzed with 0.1 M sodium acetate buffer, pH 4.5, using centrifugal filter units (Amicon, MWCO 10 kDa, Merck Millipore). An amount of 6 mg infliximab (Remicade, MSD Sharp & Dohme) or adalimumab (Humira, AbbVie) dissolved in 0.5 ml 1 M $NaHCO_3$, pH 9.0, was added to the activated invertase. After an overnight incubation at 4°C, $NaBH_3CN$ was added to a final concentration of 50 mM in order to reduce imines, followed by an additional incubation at room temperature for 30 min.

Finally, the invertase-antibody conjugate was purified by size-exclusion chromatography (Superdex 200 column, ÄKTA Explorer, mobile phase: PBS).

3.2.3.5 Invertase-conjugation of infliximab or adalimumab using glutaraldehyde

Six milligram infliximab or adalimumab and 36 mg invertase were dissolved in 4.2 ml 0.1 M $NaHCO_3$ buffer, pH 9.0. Glutaraldehyde (Sigma-Aldrich) was added to a final concentration of 0.25 % (w/v). The reaction mixture was incubated at room temperature for 20 minutes and purified by size-exclusion chromatography as described above.

3.2.3.6 Activity measurement of the invertase-infliximab conjugate

In order to investigate the pH and temperature dependence of the hydrolytic activity of the invertase-antibody conjugates, 1 μg invertase-conjugate was dissolved in a substrate solution containing 0.5 M sucrose either in 0.1 M sodium acetate at a pH ranging from 3.5 to 5.5, or in sodium phosphate at pH 6 to 8. Furthermore, the incubation temperature was varied between 20°C and 60°C. iBGstar blood glucose test stripes and the iBGstar blood glucose meter (Sanofi) were used for quantifying the amount of glucose as recommended by the manufacturer.

For an analysis of the invertase-infliximab conjugate, the conjugate was serially five-fold diluted in PBS in a concentration range from 4.2 mg/ml to 10.7 ng/ml. A volume of 20 μl of each concentration was added to 0.2 mg of TNF-α-coated magnetic beads and incubated at room temperature for 1 hour. After three washing steps with PBST, the beads were resuspended in the substrate solution (0.5 M sucrose in 0.1 M sodium acetate, pH 4.5) and incubated at 40°C. The invertase activity was detected with the iBGstar system. A volume of 0.8 μl was added to a test stripe. Following the manufacturer's recommendations, we aimed at reaching a signal equivalent to a glucose concentration of around 100 mg/dl as it represents the most accurate region for signal intensity measurements.

3.2.3.7 Competitive quantification of infliximab or adalimumab

A serial dilution of infliximab or adalimumab was prepared in pure plasma (pooled plasma of 5 healthy individuals). Ten microliter of each dilution was mixed with 10 μl PBST containing 1.5 μg/ml infliximab- or adalimumab-invertase conjugate. The mixture was then added to 0.2 mg of TNF-α-coated magnetic beads and incubated for 1 hour at room temperature. After three washing steps with PBST, the beads were resuspended in 0.5 M

sucrose in 0.1 M sodium acetate, pH 4.5, and incubated at 40°C for 50 minutes. BGstar blood glucose test stripes and the smartphone attachable iBGstar blood glucose meter were used as read-out instrument to quantify the amount of glucose. Experiments were repeated three times, generating a triplicate of individual measurement points. A four-parameter logistic curve fit was performed to visualize the dynamic quantification range. For calibration purposes, a semilog line fit (logarithmic x-axis and linear y-axis) was applied to data points in the dynamic quantification range using GraphPad Prism 7.

3.2.3.8 Quantification of anti-drug antibodies (bridging format assay)

Five-fold serial dilutions of anti-adalimumab and anti-infliximab antibodies (HCA203, HCA213; AbD Serotec - Bio-Rad Laboratories) were prepared in pure plasma (pooled plasma of 5 healthy individuals). The samples were diluted 1:10 with PBST and 20 μl of this solution were added to 0.4 mg of adalimumab- or infliximab-coated beads. After an incubation for 3 hours at 4°C and three washing steps with PBST, 20 μl of adalimumab- or infliximab-invertase conjugate at a concentration of 1 μg/ml in PBST was added to the beads for 1 hour at room temperature. Finally, the beads were washed three times using PBST, resuspended in the substrate solution (0.5 M sucrose in 0.1 M sodium acetate, pH 4.5) and incubated at 40°C for 3 hours. The amount of glucose was measured as described above.

3.2.3.9 Drug tolerance

Plasma samples were spiked with anti-infliximab antibody HCA213 at a constant concentration of 200 ng/ml and different infliximab concentrations from 1 mg/ml down to 10 ng/ml. Samples were then further processed as already described for the quantification of anti-infliximab antibodies

3.2.3.10 ELISA

ELISA experiments were used as reference for the competitive quantification of infliximab and the comparison of the drug tolerance of the anti-infliximab antibody assay. 96-well microtiter plate (Maxisorb, Nunc) were coated by adding 5 μg/ml of TNF-α or infliximab in 50 μl PBST and incubated overnight at 4°C. The following day, the protein solution in the wells was discarded and each well was blocked with 100 μl of 5 % (w/v) skimmed milk for 3 hours at 4°C. Test solutions were prepared as described above. Instead of invertase-

conjugated infliximab, biotinylated infliximab (EZ-Link NHS-PEG_4-Biotin, Thermo Fisher Scientific) was used. After an incubation time of 1 or 3 hours and three washing steps with PBST, 20 μl of a 1 μg/ml streptavidin-horseradish peroxidase (BioLegend) solution were added to each well. After 30 minutes at room temperature, the read-out was performed by adding TMB chromogenic substrate (3,3',5,5'-tetramethylbenzidine, Thermo Fisher Scientific) and a plate reader (Infinite M200, Tecan) to measure the absorbance at 450 nm.

3.2.3.11 Plasma samples

Plasma samples of 10 inflammatory bowel disease patients treated with adalimumab (n=4) or infliximab (n=6) were collected at the Department of Gastroenterology and Endocrinology, University Hospital, Philipps-University Marburg, Germany (see table 3.2). All patients had given written informed consent. Analysis of the collected samples was approved by the ethics committee of the University Hospital of Marburg and Gießen and carried out according to standardized laboratory work procedures for potentially infectious materials.

Table 3.2: Clinical characteristics of the patients, whose plasma samples were analyzed

Patient ID	Sex	Age [y]	Disease	Drug treatment	Therapy duration [m]
P1	m	26	UC	Infliximab	7
P2	m	20	MC	Infliximab	3
P3	f	54	CI	Adalimumab	1
P4	f	32	MC	Adalimumab	3
P5	m	22	UC	Adalimumab	9
P6	m	39	MC	Infliximab	8
P7	m	30	MC	Infliximab	8
P8	m	57	UC	Infliximab	13
P9	m	29	CC	Infliximab	86
P10	f	44	UC	Adalimumab + MTX	25

Clinical characteristics of the patients, whose plasma samples were analyzed.
M= male; f = female; MC = Crohn's disease; UC = ulcerative colitis; CI = Colitis indeterminata; CC = Crohn's colitis; MTX = Methotrexate.

3.2.4 Results and discussion

As illustrated in Figure 3.6, we used a competitive strategy for the quantification of adalimumab as well as infliximab in plasma. A defined amount of invertase-conjugated infliximab or adalimumab, respectively, was mixed with a patient's plasma sample and added to magnetic beads coated with TNF-α. Depending on the molar ratio of free and invertase-conjugated antibody in the sample, more or less invertase-conjugated antibody bound to the TNF-α on the magnetic beads. The amount of TNF-α-bound invertase determined how much glucose was generated once the sucrose solution was added. The glucose level was measured with a commercial read-out system. The glucose acts as a surrogate for the amount of protein-drug as it correlates inversely with the infliximab or adalimumab concentration in the plasma sample. The magnetic beads simplify the handling during the entire analysis process as they - and thereby the analytes bound to them - can be collected quickly and easily after each washing step.

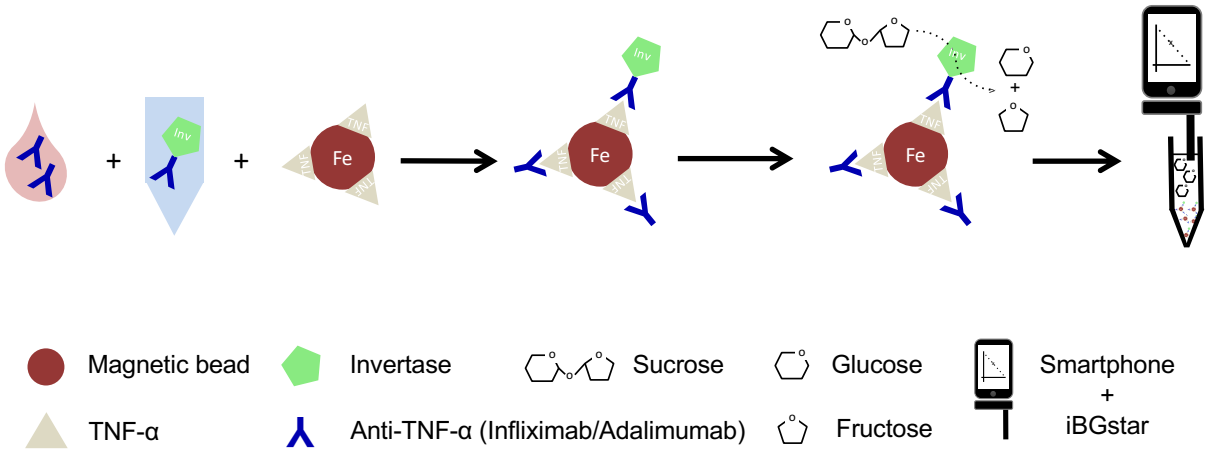

Figure 3.6: Schematic representation of the competitive quantification process for the detection of infliximab or adalimumab.
A plasma sample is mixed with TNF-α-coated magnetic particles and invertase-conjugated drug antibody. During incubation, free therapeutic antibody (infliximab or adalimumab) of the plasma sample and the invertase-conjugated antibody of the detection solution compete for the available TNF-α binding sites on the magnetic beads. Subsequently, a sucrose solution is added as a substrate and the bead-bound invertase hydrolyzes the sucrose into fructose and glucose. Using a blood glucose meter, the glucose concentration is measured, which inversely correlates with the amount of antibody in the plasma sample.

3.2.4.1 Optimization of assay conditions

Using a blood glucose meter as read-out instrument requires a glucose concentration in the range of 20 to 600 mg/dl. Since it was our aim to perform measurements quickly, several experimental conditions were studied and optimized in order to enhance the enzymatic hydrolysis of sucrose into glucose and fructose and thus the speed of the measurement.

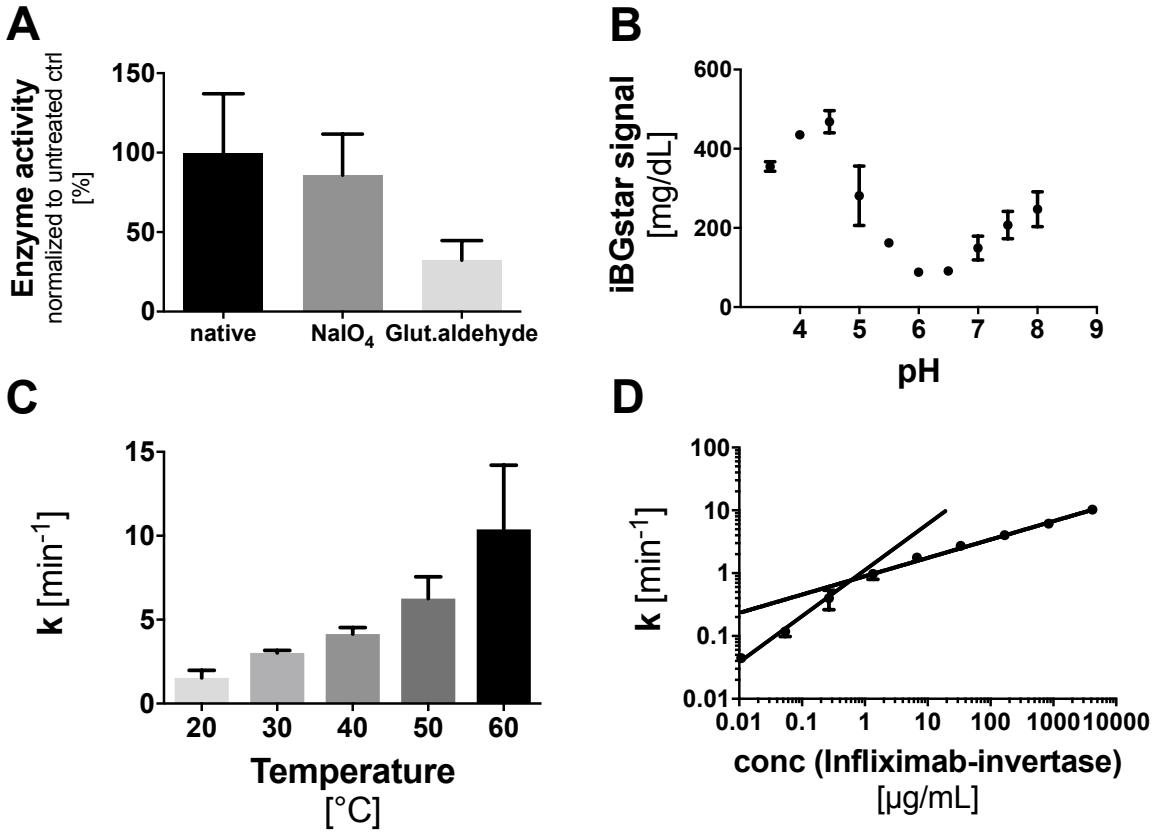

Figure 3.7: Activity of the invertase-antibody conjugates.
(A) Comparison of invertase activity of the free enzyme (native) to that of the conjugate after coupling to infliximab by sodium periodate ($NaIO_4$) or glutaraldehyde activation. (B) The invertase activity was determined at different pH. (C) Enzyme kinetics at different incubation temperatures. (D) Quantification of the activity of the infliximab-invertase conjugate at different concentrations. Each value in the four panels is the mean of two individually repeated experiments with two measurements per experiment sample; the standard deviation was calculated based on all four measured values.

Antibody-invertase conjugation:
The covalent coupling of the antibody to the invertase may impact the invertase activity. Therefore, conditions had to be chosen carefully not to affect the enzymatic activity by this conjugation but still to produce sufficient conjugate yield. Two coupling procedures were tested, using either glutaraldehyde or sodium periodate for invertase activation. The enzyme activities of the resulting, purified conjugates were compared with untreated invertase (Figure 3.7 A). A higher yield of antibody-invertase conjugate was achieved with 0.25% glutaraldehyde (data not shown), yet the invertase activity of the respective conjugate was reduced by 67.7% compared to untreated invertase. In contrast, activity of the conjugate decreased by only 14.2% if sodium periodate was used as coupling activator.

Incubation pH and temperature:
The activity of invertase-antibody conjugates was analyzed at different pH. The enzyme exhibited its highest activity at pH 4.5 (Figure 3.7 B). Not surprisingly, activity also changed with temperature (Figure 3.7 C). The findings were in good agreement with results for unconjugated invertase [6–8]. Even though an incubation temperature of 60°C yielded the highest hydrolysis rate, we chose a temperature of 40°C for all subsequent experiments so as to reduce any kinetic variation in glucose generation over time, which is indicated by the increasing standard variations at 50°C and 60°C. Protein denaturation is substantially slower and basically negligible at 40°C. Also, working with a small volume of 20 μl, evaporation might have an influence on the glucose concentration during incubation. However, no significant effect was found at 40°C.

Sucrose/glucose concentration:
The invertase's enzymatic activity is dependent on the sucrose concentration. Similar to reports of other groups [13], a maximal generation of glucose was achieved at a sucrose concentration between 0.25 M and 1 M (data not shown). It should be mentioned that the incubation time could not be reduced by the addition of glucose to the sucrose solution, thereby starting the measurement at a glucose concentration that is better for detection, because glucose serves as a catabolic inhibitor of the invertase activity [13]. In summary, using sodium periodate for the antibody-invertase conjugation, a substrate solution with 0.5 M sucrose and performing the hydrolysis reaction at pH 4.5, in a volume of 20 μl and at a temperature of 40°C were found to be the most appropriate assay conditions.

3.2.4.2 Determining the range of quantification with invertase-conjugated infliximab

For performing competitive quantification analyses, it was important to determine the range of invertase-labeled infliximab concentration that could be analyzed quantitatively. The reaction was found to be linear across about five orders of magnitude ranging from 0.05 to 5,000 μg/ml (Figure 3.7 D). Also, concentrations above 5,000 μg/ml would be detectable as no saturation of the TNF-α binding sites on the beads was observed. If longer incubation periods were employed, also lower concentrations could be covered. Yet, for the intended purpose, there was no need to aim at plasma concentrations below 0.05 .μg/ml infliximab.

3.2.4.3 Competitive quantification of adalimumab and infliximab

The competitive strategy allowed an adjustment of the dynamic quantification range by varying the amount of added invertase-conjugated infliximab. A concentration of 30 μg/ml invertase-conjugated infliximab resulted in a detection limit at 12 μg/ml (Figure 3.8 A). In contrast, a concentration of 1.5 μg/ml invertase-conjugated infliximab yielded a dynamic range between 0.7 and 46 μg/ml. Apart from affecting the detectable concentration range, a closer look at the molar ratios of free and invertase-conjugated antibody was interesting. One would assume that a 1:1 molar ratio represents the half-maximal inhibitory concentration (IC50), if free and invertase-conjugated antibody feature the same affinity. However, instead of the IC50, the lower limit of detection marked the point at which a 1:1 equilibrium of free and invertase-conjugated antibody was reached. Since there is no full saturation of all the TNF-α binding sites on the beads with infliximab (Figure 3.7 D), a molar excess of free infliximab is needed to displace bound invertase-conjugated antibody. As an adalimumab or infliximab plasma concentration of at least 5 μg/ml is necessary for an effective therapeutic response [14], we adjusted our quantification range accordingly to 0.01 to 47 μg/ml (Figure 3.8 B). The measured and plotted data points were fitted as a semilog line within the dynamic quantification range (0.01 - 46.8 μg/ml) and used as calibration curves for the quantification of adalimumab and infliximab in plasma.

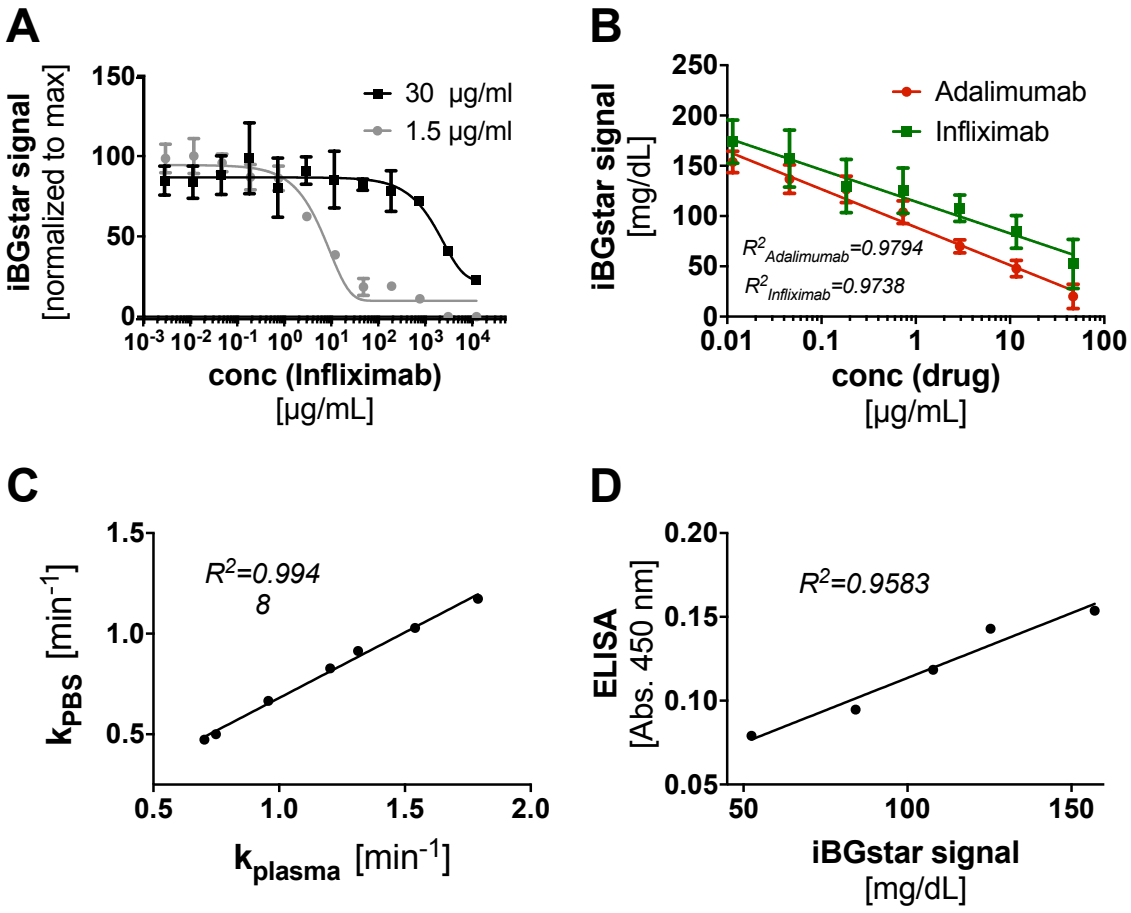

Figure 3.8: Investigating effectors of the competitive adalimumab/infliximab quantification.
(A) The dynamic quantification range is dependent on the ratio of free and invertase-conjugated therapeutic antibody after mixing the patient sample and the detection solution. The analysis strategy allows an adjustment of this range by varying the amount of the invertase-infliximab conjugate. Results are shown for two concentrations; the graphs were produced with a four-parameter logistic curve fit. (B) Generation of calibration curves for the quantification of adalimumab and infliximab by spiking known concentrations into plasma samples. (C) Correlation of results achieved by performing the experiments in PBS buffer or patient plasma. (D) Correlation of values obtained by employing for quantification ELISA and the glucose detection process.

3.2.4.4 Matrix effects and comparison with ELISA quantification

In order to demonstrate the applicability of the method, experiments regarding matrix effects were carried out. Signals obtained with glucose detection in PBS buffer correlated very well with data generated in plasma samples (Figure 3.8 C). This proved that neither any glucose nor TNF-α in plasma samples disturbed the quantification of the therapeutic antibodies. In addition, results were confirmed with ELISA as a reference method that still represents a gold standard in the quantification of protein concentrations. The process resulting in the read-out of glucose levels provided results, which were in agreement with data obtained in ELISA experiments (Figure 3.8 D).

3.2.4.5 Quantification of anti-adalimumab or anti-infliximab antibodies

Apart from the quantification of the therapeutic antibody concentration itself, an appropriate therapy monitoring also incorporates the quantification of ADAs. Their occurrence, especially of neutralizing ADAs, is a common problem limiting the effectiveness of therapies with biologicals. In order to investigate whether the detection of ADAs is possible using the glucose read-out, we modified the competitive quantification strategy into a bridging format (Figure 3.9). We prepared infliximab- and adalimumab-coated magnetic beads, incubated plasma samples containing surrogate anti-adalimumab and anti-infliximab antibodies and finally added the corresponding invertase-conjugated drug antibody. The used surrogate ADAs were designed to bind either the antibody in complex with TNF-α (HCA203, anti-adalimumab, binding type 3, non-inhibitory) or the antibody's paratope (HCA213, anti-infliximab, binding type 1, inhibitory and neutralizing). A concentration dependent detection of HCA203 and HCA213 was achieved (Figure 3.10 A). Employing a semilog line fit, a quantification performance across nearly three orders of magnitude from 1.6 to 1,000 ng/ml could be demonstrated. Also, similar slope values of the trend line indicated an equal binding kinetics of the paratope-specific and the anti-drug/TNF-α complex antibody.

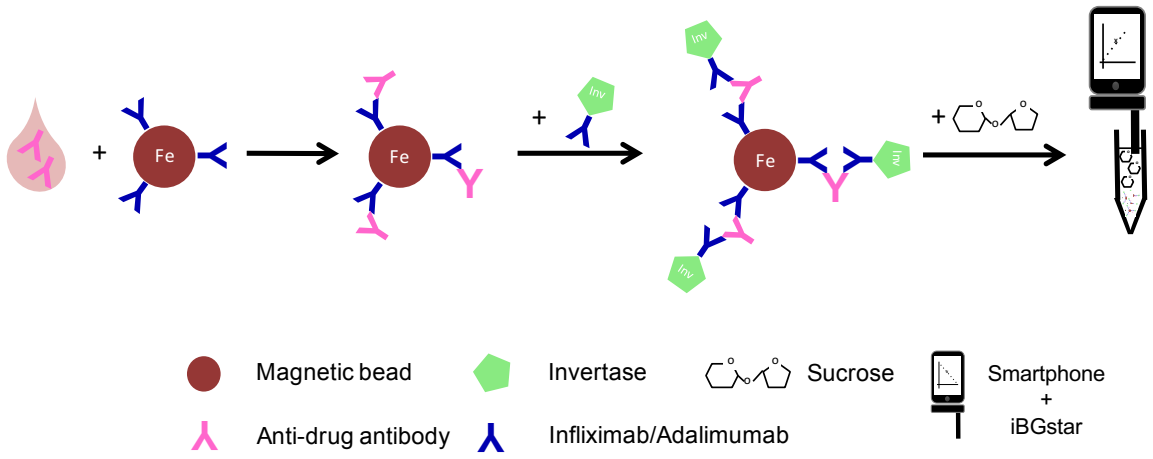

Figure 3.9: Schematic presentation of the process of anti-drug antibody (ADA) quantification (bridging format).
A plasma sample with anti-drug antibodies is added to adalimumab- or infliximab-coated magnetic beads. Bound anti-drug antibodies are subsequently detected with the corresponding invertase-conjugated antibody, upon which sucrose hydrolysis is used as a means for detection and quantification.

Compared to the competitive quantification of drug antibodies, an increased background was noticed in the bridging format. To investigate whether this effect was due to the utilization of protein A/G-coated magnetic particles, as the invertase-conjugated antibodies could also bind to both the ADAs and free protein-A/G sites on the magnetic particles, infliximab and adalimumab were immobilized on NHS-magnetic particles. Yet, the same background intensity was obtained. It is possible that the drug antibodies also form bridges with TNF-α. However, an elevated plasma concentration of TNF-α in rheumatic patients, which is reported to reach up to 1.2 ng/ml, seems to be too low to result in any pronounced background [15]. The slightly increased background signal is probably due to unspecific binding. According to the FDA guidance for industry 'Assay Development and Validation for Immunogenicity Testing of Therapeutic Protein Products' (https://www.fda.gov/downloads/Drugs/Guidances/UCM192750.pdf), a suitable ADA assay should exhibit a sensitivity of at least of 250 to 500 ng/ml. This requirement is met with the antibodies in this proof-of-concept study.

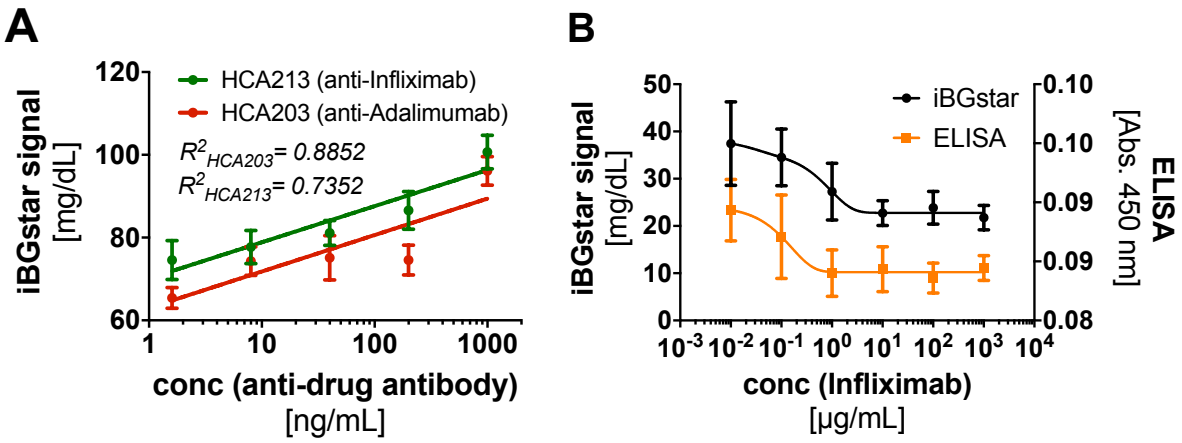

Figure 3.10: Quantification of anti-drug antibodies and investigation of the drug tolerance value.
(A) Dilution series of surrogate anti-drug antibodies were prepared in plasma. Each sample was processed and measured according to the bridging format illustrated in figure 3.9. (B) A serial dilution of infliximab with a constant concentration of 200 ng/ml HCA213 was prepared in plasma. The amount of HCA213, which was able to bind to the solid support - infliximab-coated magnetic particles or ELISA microtiter plate - was measured using the bridging format either with invertase- or biotin-conjugated infliximab. Each data point represents the mean value of two repeated experiments with two measurements per sample. The standard deviation was calculated from the four measurement values.

3.2.4.6 Free drug tolerance

While the quantification of ADAs could be realized technically, further physiological and biological conditions of the patient sample needed to be taken into account, too. It is not certain that one is able to quantify ADAs even if they are present. The reason for this is the fact that a patient might exhibit a high therapeutic antibody titer due to a recent administration or a slow metabolism. ADAs will then bind to the drug molecules making it difficult to detect the ADAs as the immuno-detection is also based on the interaction of the ADAs and the therapeutic drug, the latter immobilized on the detection platform. The higher the amount of free drug, the less ADAs will be available for detection. The free drug tolerance indicates at which concentration of free therapeutic antibody the ADA assay would be disturbed.

In order to determine the tolerance value, plasma was spiked with infliximab at different concentrations and 200 ng/ml of HCA213. Figure 3.10 B shows that both methods, glucose detection and ELISA, tolerated an infliximab concentration of 0.1 μg/ml. However, the addition of infliximab had a major impact on the standard deviation of the signals even at low concentrations.

It is possible to increase assay sensitivity as well as the tolerance to free drug, if an acid dissociation that dissolves immuno-complexes and a subsequent neutralizing step is included in the workflow [16]. Accordingly, we performed such dissociation with 300 mM acetic acid. However, the process led to a strong variation of signal intensities (data not shown) and was therefore not implemented. In order to achieve solid results, the ADA concentration should preferably be measured in the absence of detectable drug levels [17].

3.2.4.7 Analysis of clinical samples

Samples of ten patients under a current adalimumab or infliximab treatment were analyzed (Table 3.2). As a negative control, the plasma samples of five healthy individuals were used. Comparing the drug concentrations measured by ELISA and the process of glucose detection, a positive correlation with a coefficient of determination of 0.877 was observed (Figure 3.11 A). Employing TNF-α to quantify adalimumab and infliximab, it was not possible to distinguish, which of the two drugs was present in a sample. Such a differentiation could be implemented in a separate test. However, this was not our primary objective, since the information about the actual treatment was known from the clinical information.

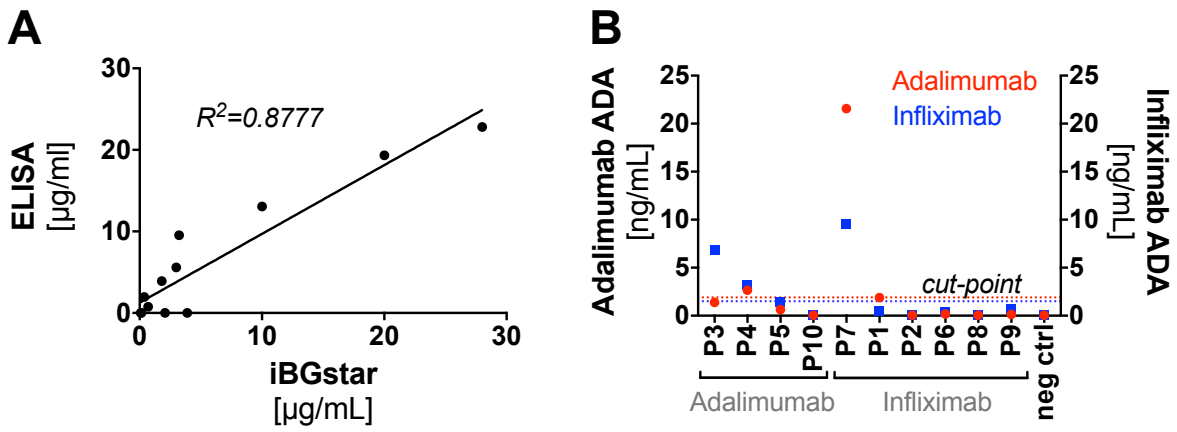

Figure 3.11: Analysis of patient samples(A) Correlation of drug concentrations as determined by ELISA and measured by the competitive invertase-conjugate iBGstar assay. Each data point represents the mean value of two measurements. (B) The concentrations of anti-adalimumab and anti-infliximab antibodies were determined in samples of patients under adalimumab treatment (P3, P4, P5, P10) or infliximab treatment (P1, P2, P6, P7, P8, P9). ADA levels above the threshold (cut-point) line were considered as ADA positive.

According to the recommended administration schedule, adalimumab and infliximab concentrations were expected to be below 10 μg/ml and 70 μg/ml, respectively. For an accurate quantification of ADAs, however, drug concentrations should be below 1 μg/ml (free drug tolerance), which was the case only for samples P1 and P6. Nevertheless, threshold values of 1.9 ng/ml and 1.5 ng/ml for anti-adalimumab and anti-infliximab antibodies were calculated (as the upper 95th percentile of five negative control samples) and samples above this threshold were considered to be ADA-positive. It has to be mentioned that due to the protein-A/G oriented immobilization of drug antibodies in the used setup, only ADAs against the fragment antigen binding (Fab) region and therefore preferably neutralizing antibodies could be detected.

Even though patient P3 and P4 were under a current treatment with adalimumab, anti-infliximab antibodies could be detected in both, also. The opposite was found in patient P7; in his plasma anti-adalimumab antibodies were found despite a current treatment with infilixmab. The information whether these patients had previously also been treated with either adalimumab or infliximab was lacking. The development of a cross-immune-response would be possible in principle, but seems to be unlikely and has not been reported to date. Another possible explanation could be a very high TNF-α concentration in the plasma

samples (> 1.2 ng/ml) resulting in a TNF bridging in the assay, which would also provide an explanation for the relatively high ADA concentrations in P4 and P7.

3.2.4.8 Smartphone connectivity

One advantage of the presented method is the possibility of transferring the measurement results onto a smartphone and thus to any location worldwide. A system that is directly attachable to a smartphone was used in our experiments. Most available blood glucose meter manufacturers offer smartphone connectivity via Bluetooth. The fact that these devices are portable, robust, sensitive and sufficiently precise opens up a wide field of applications, which could be adapted to the glucose measurement as demonstrated in the applications shown here. Such a diagnostic tool can provide tests, which can be carried out at home or somewhere remote and could reduce the patient's need of seeing a medical doctor. At the same time, privacy is protected. On the other hand, the data could be transferred immediately if needed, as for serious diseases or when the results may be too complex to be interpreted by the patient and therefore require immediate evaluation by medical professionals.

3.2.5 Conclusion

In this study, we optimized an invertase-mediated strategy for the quantification of both protein drugs as well as anti-drug antibodies. It can be operated with a relatively simple assay format consisting of a smartphone-attachable blood glucose meter. Optimal invertase activity conditions allowed to generate measurable glucose concentrations within one or three hours, respectively. An accuracy was achieved that was similar to that of ELISA measurements. Further reduction in incubation times could be achieved with larger amounts of magnetic beads, thus capturing more enzyme molecules, or by more sensitive glucose meters. However, with the current setup the quantification ranges achieved meet clinical requirements. Moreover, the option of performing this assay in combination with a smartphone offers an additional advantage with respect to convenient patient monitoring.

3.2.6 Acknowledgements

We are grateful to Agamatrix for providing 1,000 BGstar test stripes for free.

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Summary

The investigations made in this thesis describe the development and optimization of miniaturized microarray-based immunoassays, which provides sensitive and robust results in a short amount of time to be easily applicable in a clinical setting.

In chapter 1, it was demonstrated that the microarray offers a convenient platform to investigate the quality of antibodies. Rather than characterizing antibody by antibody method by method in order to determine its specificity, cross-reactivity and affinity, the microarray can effectively be used to select the most promising candidates. In our experiments, 456 scFv-Fc features of unknown quality were printed on an antibody microarray. This antibody microarray was incubated with clinical tissue samples isolated from pancreatic ductal adenocarcinoma and healthy pancreas, as well as recurrent and non-recurrent bladder tumors. A significant variation in the expression of the E3 ubiquitin-protein ligase (CHFR) and the glutamate receptor interacting protein 2 (GRIP2) was observed by more than one scFv-Fc binding to these targets. The relevant candidates were then further characterized with regard to cross-reactivity as well as affinity by antigen microarray and surface plasmon resonance experiments. The characterized binders were finally used to confirm the microarray results by immunohistochemistry analysis.

In chapter 2.1., the protein microarray method was modified to be suitable for an absolute protein quantification. The intention of this investigation was to develop a microarray-based protein quantification method, which could be integrated into a portable point-of-care diagnostic system. In this respect, emphasis was placed on applicability requirements such as convenient handling, reduced number of incubation steps and short incubation durations in the first place and secondary on a tuning of the limit of detection. The prostate specific antigen (PSA), as an established, low abundant biomarker, was used as model protein. By the optimization of the detection antibody labeling as well as its concentration, a measurement in the range of 1 ng/ml - 1 μg/ml within 15 minutes was possible employing a competitive quantification strategy, which required just a single incubation step. To further improve the preciseness of measurements at low concentrations, different

protein modification and presentation strategies were tested, yet no advantage could be pointed out. In a simultaneous quantification experiment with PSA and TNF-α, it could finally be demonstrated that a multiplexed quantification with more than one protein is possible utilizing this format.

As the intactness of a protein is a prerequisite for the generation of valid results in quantification experiments, a simple method to characterize the on-chip protein stability was developed as described in chapter 2.2. For this purpose, the extrinsic fluorescent dye thioflavin T was considered as probe to determine the degree of function loss. Four different protein types (PSA, TNF-α, infliximab and a scFv-Fc fusion protein) were immobilized on a microarray surface and stored at different temperatures (-20, 4, 25 and 60 °C). After one, seven, thirty and ninety days the capability to be bound by detection proteins as well as the thioflavin T-associated fluorescence were measured. Depending on the type of protein, a correlation between a decreasing protein recognition (as a result of denaturation) and an increasing thioflavin T fluorescence (as a result of incorporation into denaturation sites) could be pointed out. A significant negative correlation was observed for the β-sheet rich proteins TNF-α as well as infliximab, whereas a significant positive correlation was determined for PSA. No significant correlation was visible for the scFv-Fc fusion protein. With this method at hand, it was possible to identify and select appropriate spotting matrices as well as storage conditions to preserve the stability of each protein.

The acquired knowledge was finally used to design a microarray-based anti-drug antibody (ADA) assays, as described in chapter 3.1. This assay combines the determination of drug type and drug concentration, as well as the separate quantification of IgG- and IgM-ADAs. The test performance allows a drug quantification within a range of 0.01 to 4 μg/ml (competitive strategy) and an ADA quantification starting from 10 to 10,000 ng/ml (bridging format) with a free drug tolerance of 1 μg/ml. The clinical applicability of this assay was further demonstrated by analyzing samples from a clinical trial. This assay can be integrated into a portable read-out system and thereby fulfills the initially set requirements.

An additional method, which is based on a smartphone-attachable blood glucose meter, was developed to further optimize the existing strategy in terms of usability. The invertase-mediated and smartphone connected method, described in chapter 3.2., allows the quantification of adalimumab as well as infliximab plasma concentrations from 0.01 to 47 μg/ml within 50 minutes. A corresponding assay, enables the quantification of ADA concentrations from 1.6 to 1000 ng/ml within three hours, exhibiting a free drug tolerance of

1 μg/ml. This smartphone connectable method offers a convenient solution to facilitate a therapeutic drug monitoring for both, the medical professional as well as the patient.

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