Development of a Competitive Immunoassay Microarray for the Detection of the *Staphylococcus Aureus* Enterotoxins A-D and H in Dairy Products

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Dipl. Chem. Simone S. Moises

aus Essenbach

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Prüfungsausschuss:

Vorsitzender:	**Prof. Dr. H. Brunner**
Erstgutachter:	**Prof. Dr. O. S. Wolfbeis**
Zweitgutachter:	**Prof. Dr. J. Wegener**
Drittprüfer:	**Prof. Dr. J. Heilmann**

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1. Introduction

1.1. Food Intoxication

Foodborne intoxications are an enduring risk for public health and, therefore, the feasability of producing and consuming safe foods is considered as one of the major achievements of the last century. Over 200 known diseases are transmitted via food consumption **[A1].** The spectrum of foodborne pathogens includes a variety of viruses, funghi and fungal toxins, chemicals, heavy metals, parasites, bacterial toxins and bacteria, whereas bacteria-related poisoning is the most prevalent. Only less than 20 different bacteria act as originators. Every year, *Staphylococcus aureus, Salmonella, Clostridium perfringens, Campylobacter, Listeria monocytogenes, Vibrio parahaemolyticus, Bacillus cereus,* and entero-pathogenic *Escherichia coli* are causing more than 90 % of all food poisonings that are related to known pathogens. These bacteria are mainly found in raw foods **[A2, A3].** The Centers for Disease Control and Prevention **[A4]** in the United States (U.S.) is collecting data on foodborne disease outbreaks from all states and territories through the Foodborne Disease Outbreak Surveillance System to quantify the impact of these diseases on health. The estimated number of food-related diseases causes approximately 76 million illnesses, 323.914 hospitalizations and 5194 deaths. Only 14 million illnesses, 60.000 hospitalizations and 1.500 deaths are caused by known pathogens, while unknown agents are responsible for the remaining numbers **[A3].** Outbreak data reported internationally for source attribution were collected by Greig et al. **[A5]**. Based on sources of public reports published between 1988 and 2007, 4093 outbreaks are registrated and analyzed. According to this study, 2168 cases are allotted to the United States, 1287 to the European Union (EU), 246 to Australia and New Zealand, 208 to Canada and 184 to other countries. Based on a study and the European Commissions Rapid Alter System for Food and Feed, a total of 11.403 reports were published between July 2003 and June 2007 **[A6].** Controlling bodies and guidelines are necessary due to the large number of outbreaks and their impact on public health. As food safety concerns consumers, food producers and regulatory agencies, widespread concepts through the whole feed and food chain - farm, transport, supply und consumption – are required to protect consumers from pathogen ingestion. Hazard Analysis and Critical Control Points is a systematic preventive approach to food and pharmaceutical safety which addresses physical, chemical, and biological hazards **[A8-A10].** In Europe and the US a considerable number of research

projects aiming for new tools for food safety were funded. Most of the projects unify research and development topics such as improved analytical and sampling methods with modelling and the compilation of databases. Some projects have also a strictly food chain-dominated structure. The large number of generated data sets affords refined statistical informatics. An introduction to practical biotraceability is given by *Barker et al.* **[A11].** The high relevance attributed to consumer protection is documented by the substantial number of integrated EU projects such as BIOTRACER **[A12].** It has 46 project partners from 24 countries, including four International Co-operation countries and has a total budget of 15 million Euro. Its objective is to provide tools and computer models for the improvement of tracing accidental and deliberate microbial contaminations of feed, food and bottled water.

1.2. Protein Microarrays as Rapid Tools in the Food Production Chain

Rapid and reliable detection methods are essential tools to process a large amount of samples that accumulate if a consistent food control shall be achieved. Customary microbiological methods such as cell culture techniques are often laborious and ineffective due to their incompatibility with the speed of the production chain and the distribution of food, its endurance, and the operational costs. Furthermore, bacterial strains can fail regular growth processes and lead to false analysis results. Quantitative polymerase chain reaction (PCR) is an accurate, rapid, specific, and sensitive method for detection of small amounts of pathogen Desoxyribonucleic Acid (DNA) in food samples. Unfortunately, DNA-based assays can only detect the presence of toxin producing organisms and do not quantify the amount of active toxins. On-line detection with PCR methods is also expensive and requires well-trained personnel **[A13].** Typical methods of instrumental analytical chemistry such as mass spectrometry, liquid chromatography, IR or UV/Vis spectrometry are powerful tools for a precise determination of pathogens, but they require time-consuming sample preparation and they are usually not transportable devices, thus not applicable for on-line monitoring, e.g. in the production process. Sensor-based bioassays and microarray techniques are rapid and sensitive tools for on-line detection and automated processes control during food production and the supply chain. They can also be used in extensive research studies, mass tests, or to generate supporting data for modelling programs. The results can be used to

create new International Organization for Standardization or Deutsche Institut fuer Normung standards that are significant for a huge number of food producers.

1.3. Aim of the Work

This work is focusing on two further developments in the field of protein sensors and arrays for the detection of *Staphylococcus aureus* Enterotoxins SEA-SED and SEH in dairy products, especially in raw milk and raw milk cheese.

The first project, a protein microarray for the detection of the Enterotoxins in milk and raw milk cheese, is part of the BIOTRACER project (European Union, 6th Framework Programme). The project consists of all levels of researcher, from mathematic modelers to scientific technicians and is splitted into several levels of research: Its objective is to provide tools and computer models for the improvement of tracing accidental and deliberate microbial contaminations of feed, food and bottled water. The newly developed protein microarray should be part of the tool construction of BIOTRACER creation of data for computer models. The protein microarray provides the possibility of being adapted into the production process control through its rapid processing time and the ability to measure more than just one analyte and/or sample at the same time.

The second part of the work is the co-development of a SPR chip for the same procedure, the online-monitoring of failures in the production process of milk and cheese products.

2. Background

In this chapter, background theory is presented to give a briefly overview of the key technologies used in this work.

2.1. Labeling and Purification of Proteins

The following chapters outline common strategies for labeling and purification of proteins.

2.1.1. Labeling Techniques

Direct protein detection and detection by fluorescence are two possibilities within multiple protein detection methods. Direct protein detection is possible via three aromatic amino acids: Tryptophane, Phenylalanine and Tyrosine. Their absorbance maxima are at 280 nm, 257 nm and 274 nm and they possess intrinsic fluorescence within the range of 270 to 350 nm. Unfortunately, the fluorescence intensities of Tryptophane and Tyrosine are much higher than those of Phenylalanine and the fluorescence of all three amino acids is highly temperature dependent **[B1].** Therefore, proteins are better labeled by fluorescence dyes with functional reactive groups to overcome these disadvantages. During the last ten years, fluorescent labels attained enormous popularity. Their attraction is manifested in a multiplicity of commercially available fluorescence dyes at almost every wavelength. Companies like GE Healthcare **[B2]** or Invitrogen **[B3]**, to name only the major ones supply a broad range of functionalized and therefore "ready-to-use" dyes. The main attraction of these dyes is originated in their high sensitivity after binding to a target. Its continuously signal generation is possible by regeneration of emitted photons. For assay development, the possibility of measuring multiple parameters becomes very attractive: fluorescence intensity, emission spectra, polarization or lifetime are only a small application window **[B4, B5].**

An optimal fluorescent label should fulfill certain requirements and possess the characteristics as follows **[B6]:**

- Stability of the fluorophore in water as well as in organic solvents.

- High molar absorbance of the fluorophore.
- pH - independency of the fluorescence between pH 5-9 (physiological range).
- High photostability is necessary.
- At least one reactive group for coupling steps with the target under mild reaction conditions and medium temperature.
- Weak fluorescence in its unconjugated form and high fluorescence when bound to the biomolecule of interest.
- Large quantum yield to attain a high light intensity.

Now, a large number of labels that can be attached covalently are commercially available.

Due to the regulations of the project „BIOTRACER“ and existing scanning equipment (Affymetrix428 Array Scanner from Affymetrix with lasers for excitation of Cy3/Cy5 and analogues in wavelength), a dye of the cyanine dye group, Cy3, was chosen. The structure of the commercially available Cy3 NHS ester is presented in Figure 1.

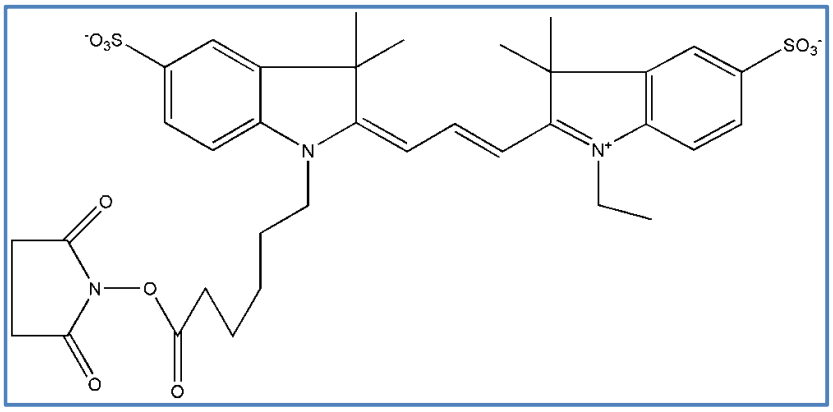


Figure 1. Chemical structure of Cy3 NHS ester (ready for covalent attachment) reproduced from **[B2]**)

The cyanine dyes are long-wavelength dyes and exhibit absorption and emission wavelengths between 530 and 750 nm. The small Stokes´ Shift is characteristically for these dyes. The charged side groups are attached for excellent water solubility and furthermore, for prevention of self-association. Self-association is a conventional reason for tailing in the spectra, self-quenching and multi-exponential decay time **[B5].** The analytes of interest within this work are natural existing proteins. As they are not detectable within the range of visible light, a covalently attached fluorescent label is used for detection. The covalent bond between analyte and label is formed via reaction of two different functional groups, each

located on one partner. The proteins used for labeling here are antibodies. They offer a range of functional groups at the side chaines as well as at the N and C terminus of the protein chain. Thiol groups and amino groups are the most commonly utilized binding sites for labeling.

The thiol group of cystein is reacting in neutral or basic aqueous solution and therefore the iodacetamide, the maleimide and the disulfide exchange reaction are the methods of choice **[B5, B7].** The labeling reaction schemes for protein thiol groups are presented in Figure 2.

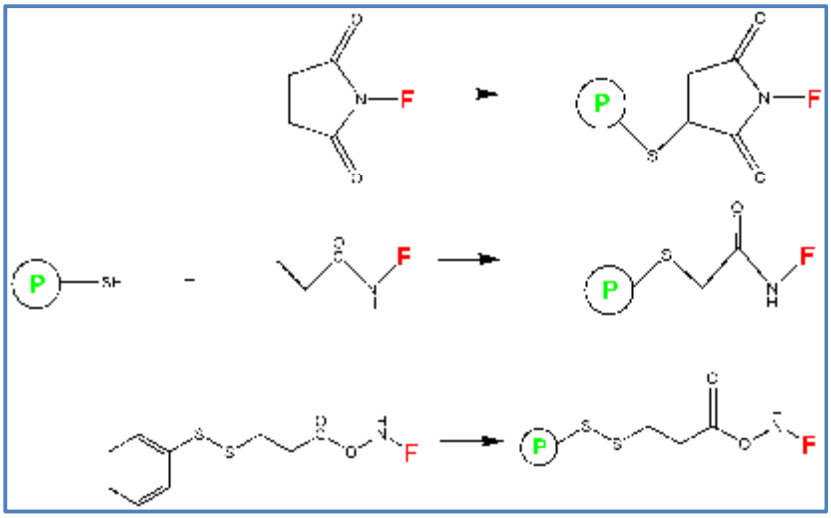


Figure 2. Maleimide labeling, iodacetamide labeling and disulfide exchange reaction of thiol groups on the protein (P: protein, F: fluorophore) (reproduced from **[B7]**)

Protein labeling techniques using amino groups are the most appropriated ones **[B5, B7].** They are presented in Figure 3. The most frequently used coupling reagents for amino groups are reactive esters, especially the N-Hydroxysuccinimidyl ester (NHS ester). Generally, labeling is done within a pH range of 8.5 to 9.5 and over a period of 15 minutes to hours. Labeling is proceeded in this pH range to prevent the hydrolysis of the active ester, which is a competing reaction. Unfortunately, active esters are often insoluble in water due to its uncharged nature. This requires organic solubilizers like dimethyl sulfoxide which can damage or denature the protein of interest. In addition, a change of global charge of the protein is induced via ester coupling and therefore, the solubility characteristics are changed**.** Isothiocyanates are modification reagents with intermediate reactivity. They are more stable in water than the reactive esters and can react optimally at pH 9-9.5.

Sulfonylchlorides are high reactive reagents. Although they are unstable in water, they form extremely stable sulfonamide bonds that can outlast amino acid hydrolysis.

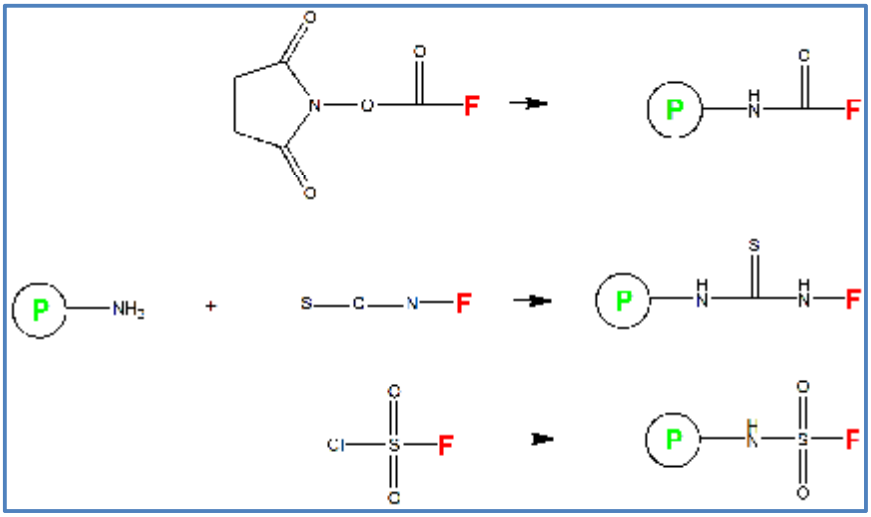


Figure 3. Labeling reactions of amino groups with NHS ester, isothiocyanate and sulfonylchloride (P: protein, F: fluorophore) (reproduced from **[B7]**)

Carboxy acid and hydroxy groups of proteins play only a less relevant role for labeling. Carboxylated functional groups show low nucleophilicity in aqueous solutions, and therefore low reactivity.

2.1.2. Purification Techniques

Several clean-up methods for the labeled proteins were used in this work. A short overview is given as follows:

Gel Filtration with Sephadex medium

Gel filtration also called size exclusion chromatography is the simplest and mildest of all chromatography techniques **[B8].** The separation is based on the differences in size. Group separation is used for the purification of labeled proteins. Sephadex, a trademark of GE Healthcare, is prepared by cross-linking a dextran with epichlorohydrin **[B9]**. There are different types, which vary in their degree of cross-linking and as a result in their selectivity for specific molecular sizes. Sephadex G25 medium, with a size exclusion of 5 kDa, is mostly used. It can be applied at high flow rate and low operating pressure. The liquid inside the particle is the stationary phase, whereas the liquid outside of the particle is termed mobile

phase. Molecules, like proteins, which are heavier and bigger than the exclusion size, do not enter the matrix. Consequently, they elute first, as they pass directly through the column. Small molecules, like dyes, which enter the matrix, elute in order of decreasing size.

Filtering with the Millipore Amicon Ultra Filter Unit

Amicon Filter Units use a 10 kDa cut-off membrane (Ultracel regenerated cellulose) to separate particles with a size smaller than 10 kDa **[B10].** The membrane is integrated into a 50 mL spin tube. The desired fractions are collected within the filter, the smaller fractions remain on the bottom of the tube.

Clean up with the Melon Gel Spin Purification Kit

The Melon Gel IgG Purification System **[B11]** purifies antibodies by removing non-relevant proteins under physiological pH allowing the antibody to flow through in a mild buffer suitable for storage and downstream applications. The system was developed to overcome the drawbacks of commonly used Protein A and G purification methods, which are labor-intensive and requires harsh elution conditions to disrupt the affinity interaction. The gel eliminates the need for an elution step and uses a mild working buffer at physiological pH. In addition, the purified product is in a buffer free of primary amines and can be used directly in amine-reactive conjugation chemistries. The spin-column format is intended for single use and can be completed in less than 15 minutes.

Clean up with the Nab Protein A Plus Spin Kit

NAb Spin Kits **[B12]** are convenient for rapid, small-scale affinity purification of antibodies from a variety of sample types. Each pre-filled microcentrifuge spin column of the immobilized protein resin enables quick purification of 1-13 mg of IgG. Protein A is a bacterial protein that binds with high specificity to mammalian immunoglobulins (Ig). Immobilized types of this protein have been widely used for affinity purification of antibodies. Proteins A is binding to many of the same species and subclasses of IgG, although they have particular differences in affinity and binding capacity. Protein A is generally preferred for affinity purification of IgG from rabbit, pig, dog and cat.

2.2. Protein Microarray Formats and Technology

In this chapter, the current state-of-art of protein microarray technology is presented, with focus on glass-based array formats. An overview of protein array types and their description is given in Table 1.

Table 1. Protein microarray types and their description. Adapted from **[B17]**

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

Protein-binding can occur due to a number of possible capture agents, a short overview is given in Figure 4.

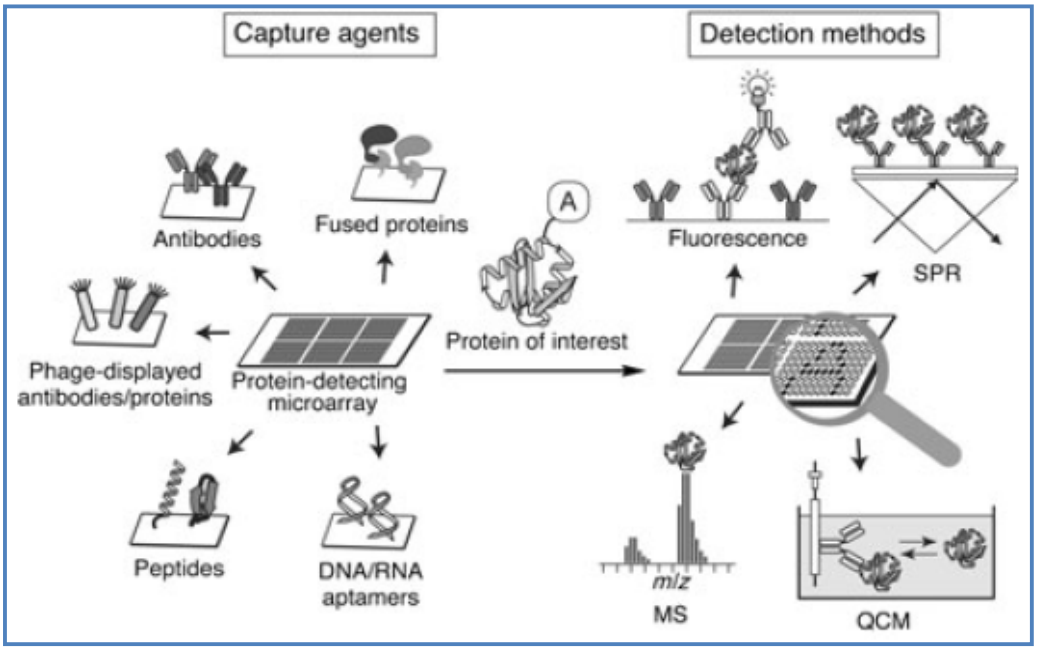


Figure 4. Overview of representative protein capture agents and detection methods for the development of protein-detecting microarray technology. Adapted from **[B15]**. (DNA: desoxyribonucleic acid, RNA: ribonucleic acid, SPR: surface plasmon resonance, MS: mass spectrometry, QCM: quartz-crystal microbalance)

Antibodies are the most prominent capture agents due to their high affinity and specifity to the corresponding targets. Microarray-based enzyme-linked immunosorbent assay (ELISA) techniques have been developed as well to reduce sample consumption and to improve throughput. Introducing affinity tags to C or N terminus of recombinant protein enables fused-protein arrays and facilitates purification of proteins from complex mixtures. Several affinity fusions are available, for example six histidine residues (His tag), strep tag, Gluthathion-S-Transferase (GST), calmodulin-binding peptide, chitin-binding protein, maltose-binding protein or thioredoxin. Correlation between genotype and phenotype is established and libraries of DNA-encoded polypeptides/proteins can be produced and purified by molecular biology methods from *E. Coli*. Aptamers are oligonucleotides and have potential characteristics of both proteins and nucleic acids. They can be easily synthesized and amplified and can compete with antibodies in affinity to targets including proteins. A possible range of protein array applications is presented in Figure 5.

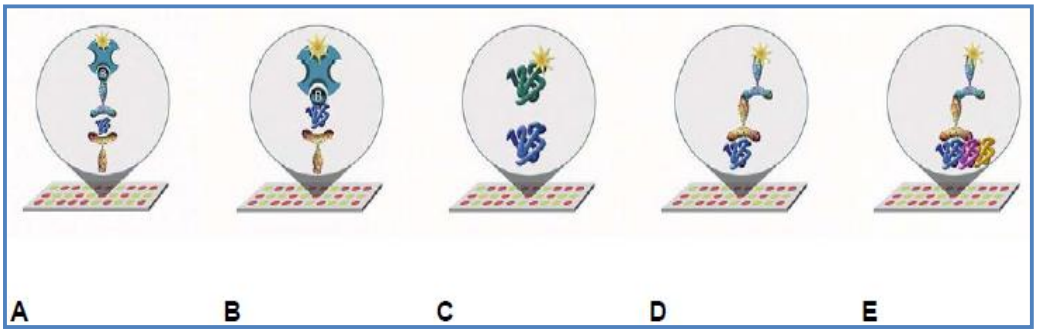


Figure 5. Overview of convenient protein microarray application types: sandwich ELISA (A), directly capturing hapten-labeled antigen (B), protein-protein interaction (C), serum sample probe for antibodies (D) or reverse-phase array (E) (adapted from **[B13]**)

Protein microarrays are attractive for a range of applications: Antibodies can be arrayed as capture molecules for microspot sandwich ELISA-type experiments (Fig. 5A) or for directly capturing hapten-labeled antigens (Fig. 5B). Purified or recombinant proteins are used for studying protein-protein interactions (Fig. 5C) or for probing serum samples for antibodies (Fig. 5D). Reverse-phase arrays are applied for the profiling of hundreds of arrayed samples for the presence of a number of small antigens (Fig. 5E). Examples for surface chemistry classification and its agents **[B15]** are presented in Table 2.

As Table 2 presents, various techniques are available and therefore a range of publications and books **[B16-B18]** exist. Within this work, the focus lies on 2D surfaces, especially from the non-specific category. Silanization with agents from this category is a common treatment for glass-based array applications. Beside these, polyacrylamide or nitro-cellulose coated slides are very popular.

Due to the large number of possible applications and immobilization techniques, a more detailed overview is given in the next two chapters.

Table 2. Surface modifications for protein microarrays (adapted from **[B15]**)

Surface type	Category	Surface (modified with)	Capture agent (with)
2D	*Nonspecific/ noncovalent*	PVDF	Functional group-independent
		Poly-L-Lysine	Functional group-independent
		Calixcrown 5-derivates	Amines
	Nonspecific/ covalent	Aldehyde	Amines
		Epoxide	Amines, Thiols
		Succinimidyl ester	Amines
		Isothiocanate	Amines
		Photoaffinity reaction	Functional group-independent
	Specific/ noncovalent	Avidin	Biotin tag
		Ni-NTA	His tag
		GST	GST tag
		Protein A/G	IgG Fc region
		oligoDNA	oligoPNA
	Specific/ covalent	Maleimide	Thiol group
		Bromoacetyl	Thiol group
		Thioester	Cysteine at N-terminus
		Glyoxylyl group	Aminooxy acetyl group
		Semicarbazide	Glyoxylyl group
		Diels-Alder reaction	
		1,3-Dipolar cycloaddition	
Surface type	**Category**	**Surface (modified with)**	**Capture agent (with)**
3D		Agarose/Polyacrylamide	
		PDMS film	
		Nitrocellulose	Functional group-independent
		Gel pad	
		Supermolecular hydrogel	

PVDF: Polyvinylidenefluoride, NTA: Nitrilotriacetic Acid, PDMS: Polydimethylsiloxane, PNA: Peptide Nucleic Acid

2.2.1. Arrays with 2-Dimensional Surface

A silanization step is recommended when functionalizing of glass slides surfaces. Silanes form 2-dimensional self-assembled monolayers on the glass surface.

Cleaning

Stored glass slides always have contaminated surfaces which affect silanization and create a non-uniform silane monolayer. Therefore, a cleaning step is recommended. Surfaces that are close to 100% hydrophobic are very attractive for silanization due to the fact that nearly all Si-OH groups are deprotonated. The contact angle of water is typically < 5°. Various cleaning methods exist from which the most important ones are explained a little more as follows. The most common methods are **[B18]**: Piranha solution, Hydrofluoric acid, Alkaline, Ultrasonication, UV/ozone and Laser. Piranha solution is a mixture of sulfuric acid and hydrogen peroxide and very effective in cleaning. The solution has always to be prepared freshly. Hydrofluoric acid is fast and effective against contaminants, but also highly toxic and damages the slide when applied for a longer period. Alkaline solution is effective in cleaning but requires time and longer incubation periods. Often, a 1:1:5-mixture of hydrogen peroxide (30% (v/v)), dissolved ammonia (32% (v/v)) and ultra-pure water is used. Ultrasonication in Ethanol cleans the surface with little disposal but is not as effective as Piranha solution. UV/ozone treatment is very fast and effective for removing thin film contaminants but ineffective in cleaning thicker layers. The laser cleans fast and effective but the slides are recontaminated if the slides are not stored under vacuum conditions. For my work, the alkaline solution mentioned above is used.

Silanization Surface Chemistry

Derivatized glass slides are adequate for immobilization of untagged proteins. They are bound via multiple interaction groups and attachment is proceeded in a variety of orientations so that different faces of the protein can interact with other molecules in solution. This random orientation decreases the number of protein interaction sites though inappropriate orientation or inactivation due to conformational change.

The reactive coatings are major based on self-assembling techniques. Silane monolayers require hydroxylated surfaces as substrates for formation. The driving force of self-assembley is the in-situ formation of polysiloxane which is connected to surface silanol groups. These organic films enable the biocompatibility of the surface and protect proteins from structural changes and denaturation in the immobilization step. Commonly used silanization reagents are presented in Figure 6.

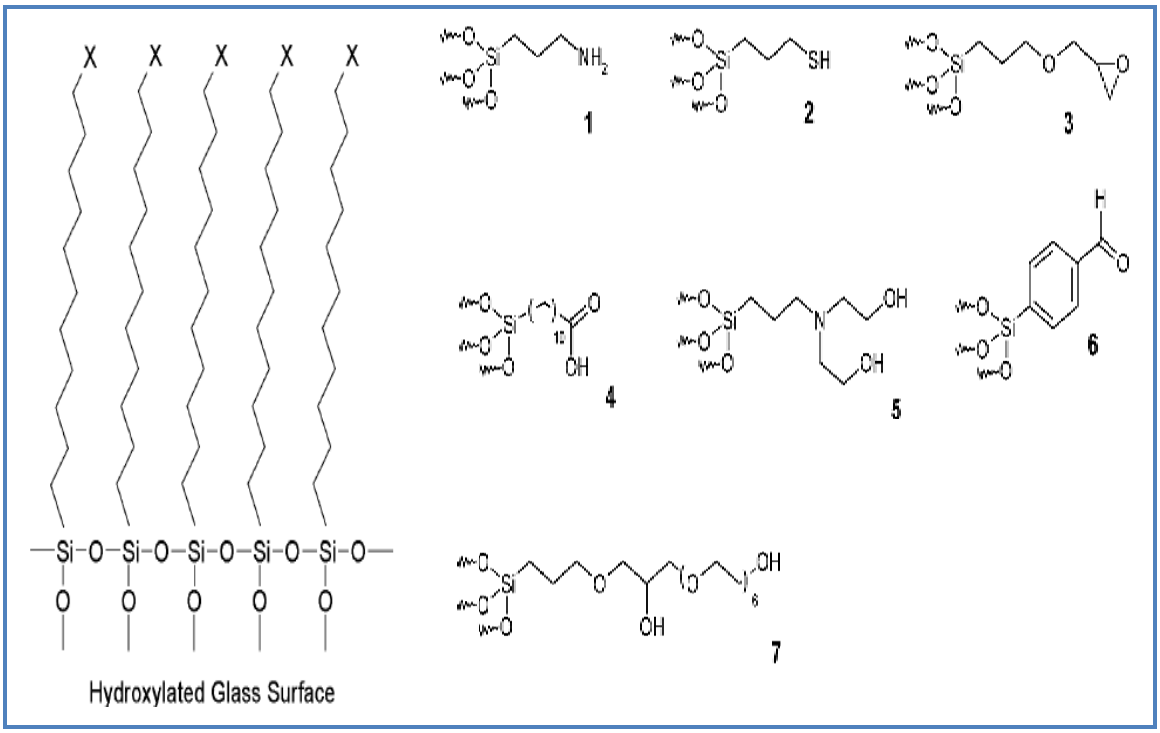


Figure 6. 2D scheme of a polysiloxane monolayer on a glass surface.
(X: terminal functional group) (left).
Silanization reagents: Aminopropyltriethoxysilane (APTES, 1),
3-mercaptopropyltrimethoxysilane (MPTS, 2), glycidoxypropyltrimethoxysilane (GPTS, 3),
Triethoxysilane undecanoic acid (TETU, 4), Bis (hydroxyethyl) aminopropyltriethoxysilane)
(HE-APTS, 5), 4-trimethoxysilylbenzaldehyde (6),
glycidoxypropyltrimethoxysilanehexaethylene glycol (GPTS/HEG, 7) (right).
(reconstructed from **[B17]**)

Various techniques are available and therefore a range of publications exist. The quality of the SAM is influenced by temperature, reaction time, and the amount of silane.

Protein Immobilization

Surfaces for Non-Tagged Proteins

Proteins are bound to the positively charged amino-coated slide via electrostatic interactions. They are simple to prepare and their signal uniformity and reproducibility are good. Proteins are attached via Schiff`s base reaction with side chain amino groups and the more reactive amino groups of the N-terminus on aldehyde-coated slides. These covalent bonds are stronger than the salt bridges on amino-coated slides. These slide type is easy to

fabricate, store and use and results in good signal-to-noise ratios but are not applicable for printing very small proteins. Epoxy-coated slides are derivatized with epoxysilane and proteins are covalently attached via epoxide ring opening reaction. Surface amino, hydroxyl and thiol groups can react with this group, resulting in potentially higher binding efficiency than amino slides. Unfortunately, the epoxy ring is succeptible to moisture and therefore the slides have to be prepared moisture-free **[B17].**

Oriented Surfaces for Tagged Proteins

Expressed proteins can be tagged at the amino or carboxyl terminus for site-specific attachment. This step encourages protein molecules to be oriented in a common direction away from the support surface and reduces structural distortion.

Ni-NTA slides can be used with His-tagged proteins. This interaction is neither very strong nor very stable. Thus dissociation with washing or storage occurs. Chemicals like ethylene diamine tetraacetic acid or dithiothreitol effect the reaction, too. The biotin-avidine reaction is one of the strongest and most stable non-covalent interactions with a dissociation constant of 10^{-15}M. Avidine is toxic to cells. Therefore, the protein is tagged with biotin and the support is functionalized with avidine. Furthermore, non-glycosylated steptavidin is an attractive coating agent, due to the possible non-specifity of the oligosaccharide component of avidine **[B17]**.

2.2.2. Arrays with 3-Dimensional Surface

3D surface slides have substantially deeper coating layers than the planar 2D surfaces which increase the surface and the binding capacity of the slide. 3D slides are difficult in manufacturing and therefore, commercially available slides are expensive. Polyacrylamide slides (Perkin Elmer Life Science or Xan Tec Bioanalytics) have a porous hydrophilic gel layer matrix of 20-30 μm in thickness which reduces the rate of evaporation and minimizes protein denaturation. On agarose slides, proteins are covalently immobilized to activated aldehyde groups in the layer. They are easy to prepare and enable higher sample load but uniformity and reproducibility is not sufficiently assessed. Nitrocellulose slides (15 μm layer)

are a good choice for maintain protein conformation and enable higher sample loading but need prolonged protein incubation time. They tend to have a high intrinsic background level because of light scattering, but this is compensated via higher sample load **[B18].** Pictures of Nitrocellulose Slides and Nitrocellulose surface are presented in Figure 7.

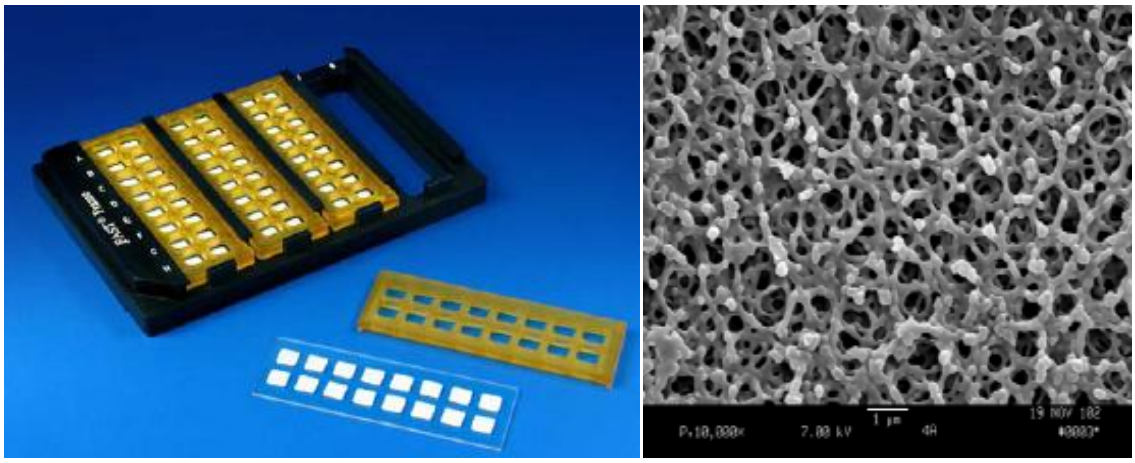


Figure 7. Fast Frame System on 16-pad Fast Slides and 3D structure of Whatman nitro-cellulose (scanning electron microscopy, magnification 10000x). Adapted from **[B13, B14].**

2.3. SPR Technology and Chip Modification Techniques

The physical phenomenon of Surface Plasmon Resonance (SPR) was first observed by Wood in 1902 and is applied in sensitive bio-detectors since 1983 **[B19, B20]**. Wood projected polarized light on a mirror with diffracting grating on the surface and observed a pattern of anomalous dark and light bands in the reflected light. This was the beginning of SPR. The physical interpretion of this phenomenon was initiated by Lord Rayleigh and refined by Fano **[B21, B22]**. The complete explanation was possible in 1968, when Otto and, in the same year Kretschmann and Raether, reported the excitation of surface plasmons. A first biomolecular application of SPR-based biosensors was reported by Liedberg et al. in 1983. **[B23-B25]** In the mid-80`s, Pharmacia Biosensor AB chose SPR as their leading platform technology for direct sensing of biomolecular interactions. They used the Kretschmann configuration which presents advantages in constructional tolerance of the liquid handling system. Light which comes from the prism, the higher refractive index medium, doesn`t pass through the liquid but is reflected at the sensor surface, covered with a metal layer. A thin gold layer was chosen as best inertial film for plasmon resonance. Furthermore, the gold layer was

modified with a self-assembling layer of long-chain thiols to which a hydrogel could be attached. Carboxylated dextrane was coupled to the surface providing a substrate for efficient covalent immobilization of biomolecules. Due to its thickness of 100 nm, the dextrane hydrogel is perfectly compatible with the evanescent field depth of about 200 nm **[B26].** In 1990, Pharmacia Biosensor AB presented the first commercial SPR product, the Biacore instrument **[B27].** This was the most sensitive, advanced, accurate, reproducible, reliable direct biosensor technique and SPR became the golden standard for transducer principles for measuring realtime biomolecular interactions. At the early 1990s, producers struggled to meet the standard set by Biacore. Since then, a range of new Biacore systems was established, which is still setting the standard.

2.3.1 SPR Technology

The Evanescent Wave

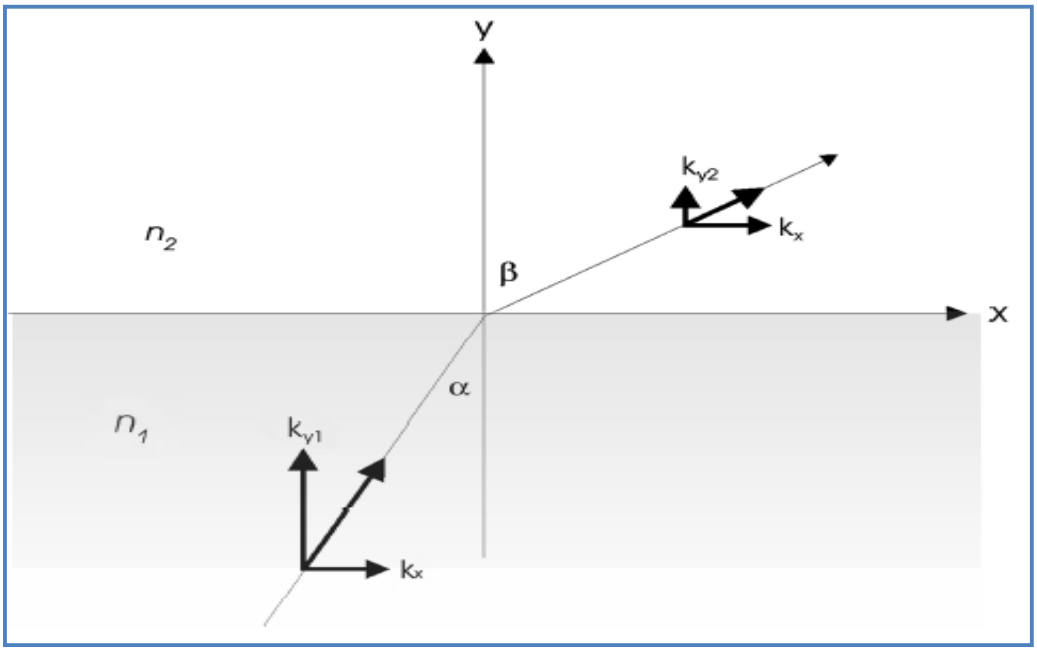


Figure 8. Refraction of light at an incident angle at the interface of two materials with refractive indices n_1 and n_2 (adapted from **[B26]**)

Before discussing SPR technology, it may be appropriate to visualize the evanescent wave, which is the center of SPR sensing, a little more. This is conveniently done by contemplating the phenomenon of total internal reflection.

One has to watch the behavior of light at the interface of two separate media with differing refractive indices to understand this phenomenon. Light is refracted at the interface (Figure 8) after Snellius´ Law:

After supposition of refractive index $n_1 > n_2$, total reflection is observed initiating from a fixed angle α_c:

However, light intensity is not decreasing rapidly to zero at the interface, it is decreasing exponentially with distance. The field in this perpendicular direction, the evanescent field, is reflecting the bound, non-radiative nature of surface plasmons. The exponentially intensity decay of the evanescent field with increasing distance is presented in Figure 9.

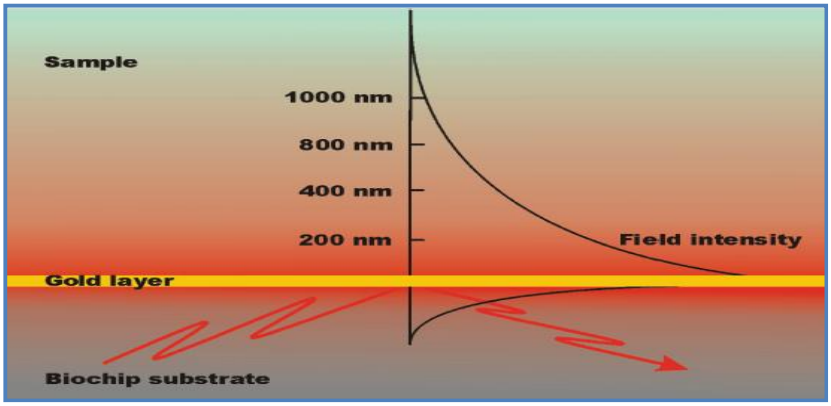


Figure 9. Exponentially intensity decay of the evanescent field with increasing distance from the metal layer (adapted from **[B26]**)

If the surface of a glass substrate is coated with a thin metal film, a part of the incident light can refract into the metallic film. Typical coatings consist of noble metals such as silver, gold, copper, titanium, and chromium. In this assembly, a second critical angle exists that is greater than the angle of total reflection. At this angle, the surface plasmon resonance angle, a loss of light appears and the intensity of reflected light reaches a minimum. This results from the interaction of the incident light with oscillation modes of mobile electrons at the

surface of the metal film. These oscillating plasma waves are called the surface plasmons. If this metal surface is coated by a thin layer of affinity ligands, the binding of biomolecules, e.g. proteins, causes a change of the refractive index. This is detected by a shift in the resonance angle.

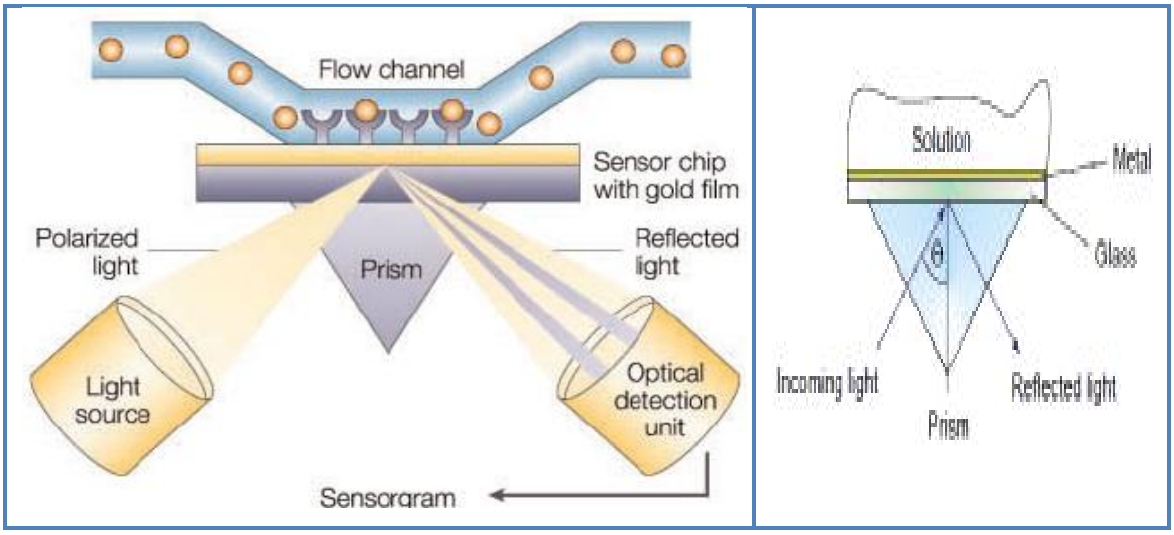


Figure 10. Scheme of the Surface Plasmon Resonance (left) and Kretschmann configuration for SPR sensors (right) [adapted from **[B28]** and **[B29]**)

The frequently used Kretschmann configuration (Figure 10) is based on a metal film which is evaporated on one face of a glass prism. The light is coupled into the prism above the critical angle of total reflection, and the resulting evanescent wave penetrates the metal film. The plasmons are excited at the outside of the film. The angle of resonance is dependent on the refractive index of the surface. SPR reflectivity measurements can be used for the detection of specific molecular interactions of bound receptor molecules on the metal surface with their corresponding targets (e.g. DNA or proteins) **[B30, B31].** The greatest attraction of SPR measurements is due to direct, label-free and real-time measurement of the refractive index at the surface. These sensors offer the measurement of low levels of biological and chemical compounds near the sensor surface. The sensor recognition of a biomolecular binding event happens when these molecules accumulate at the sensor surface and change the refractive index by replacing the background electrolyte. Water molecules have a lower refractive index than protein molecules **[B26].**

Assay Process: from Buffer to Analyte

A binding cycle observed with an optical biosensor is presented in Figure 11. Prior to the experiment, receptor molecules are immobilized on the surface via adequate coupling chemistry. At t=0 s, the cell containing the receptor is floated with running buffer to have a reliable baseline before capturing starts. At this point, active receptors are on the surface, ready for analyte binding. An analyte solution in running buffer is passed over the receptor at t=100 s. The refractive index of the medium adjacent to the surface is increasing after binding of analyte to the surface. This is monitored by increasing resonance signal. When analyzing this step of the binding curve, the observed association rate k_{obs} is received. Furthermore, the association rate constant k_{ass} is determinable if the analyte concentration is noted. At the equilibrium, the amount of analyte that interacts with the receptor by association and dissociation is equal. The response level at this point is related to the active analyte concentration in the sample. At t=320 s, the analyte solution is replaced by buffer, the receptor-analyte complex dissociates. The dissociation rate constant k_{diss} can be obtained here. At t=420 s, a pulse of regeneration solution (high salt or low pH) is used to disrupt binding and regenerate the free receptor. The binding cycle is repeated several times with varying analyte concentration to receive a data set for global fitting to an adequate binding algorithm **[B26, B28].**

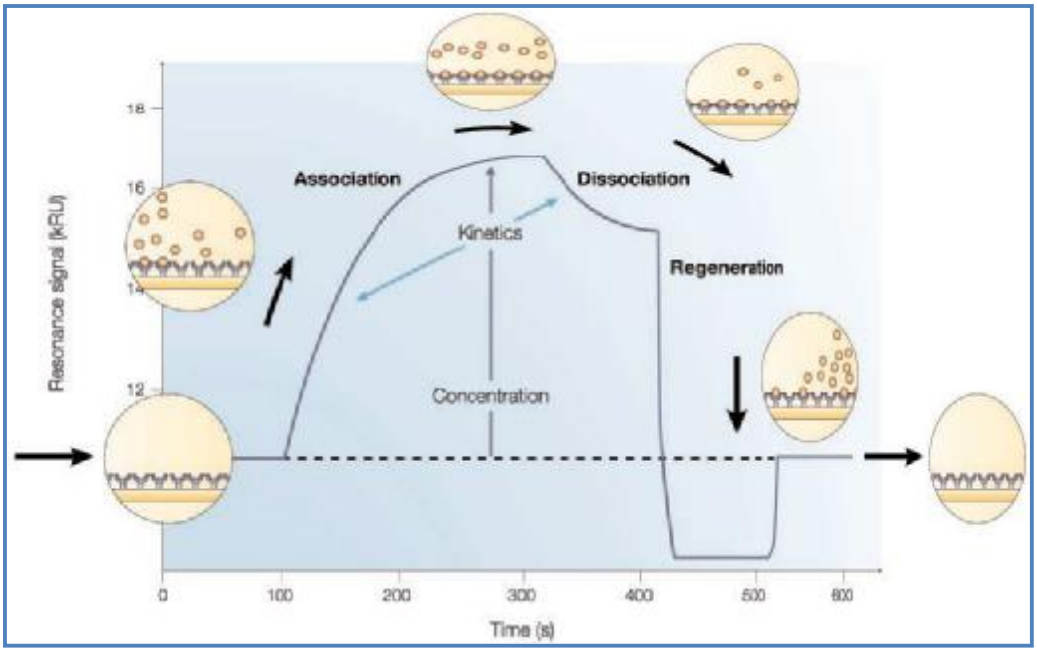


Figure 11. Binding cycle observed with an optical biosensor (adapted from **[B28]**)

Kinetics

Interaction affinity can be calculated from the ratio of dissociation and association constant ($K_D = 1/K_A = k_{diss}/k_{ass}$) or by linear or nonlinear fitting of the response at the equilibrium of varying analyte concentrations. Again, buffer is injected to condition the surface for the next analysis circle. If regeneration is not complete, remaining mass causes an increased baseline level. Typical values for K_A are within the range of 10^5-10^{12} L/mol, the values for K_D within 10^{-5}-10^{-12} mol/L. The dimensions for both rates are different and vary with stochiometry of the complex. Typical ranges show large variations and depend most on temperature. When starting, no product is present at the surface. At this point, the association rate is highest and dissociation rate is lowest. More and more of complex is produced and enhances the rate of dissociation during the process. Paralelly, the association rate might decrease. Equilibrium is reached when both rates are equal **[B26, B28].**

2.3.2. SPR Chip Modification Techniques

Adhesion Layer and Self-Assembled Monolayers (SAMs)

For SPR applications, it`s necessary to protect the sensitive bio-receptors from the uncompatible chip substrate material. Furthermore, functional groups for receptor immobilization were introduced by coating the substrate with a hydrophilic bioinert layer. Unfortunately, this layer would be washed away without the use of an adhesion layer. The adhesion layer provides a stable link between substrate and immobilization matrix and shields the substrate from buffer. Preferably, the thickness lies between 2 and 5 nm. Due to their chemical interness and easy functionalization options, gold or platin are used. Within this work, a gold layer is used. Alkyl derivates of thiol groups with chains lengths of 4-10 carbon atoms assemble spontaneously on golden SPR chips under formation of high packing density self-assembled monolayers. Typical derivates are 16-hydroxyhexadecane-1-thiol and 16-Mercaptohexadecanoic acid. The adsorption is done in 300 µM ethanolic solutions for 8-24 h. Long incubation time guarantees more ordered and packed SAMs.

Coupling Chemistry for Receptor Immobilization

Now, the receptors can be coupled to the SAMs. Therefore, coupling chemistry is approached. In general, four different groups of coupling approaches exist: adsorption, covalent activation chemistry, electrostatic immobilization and directed immobilization. The most prominent examples of coupling variants are presented in Figure 12 and 13.

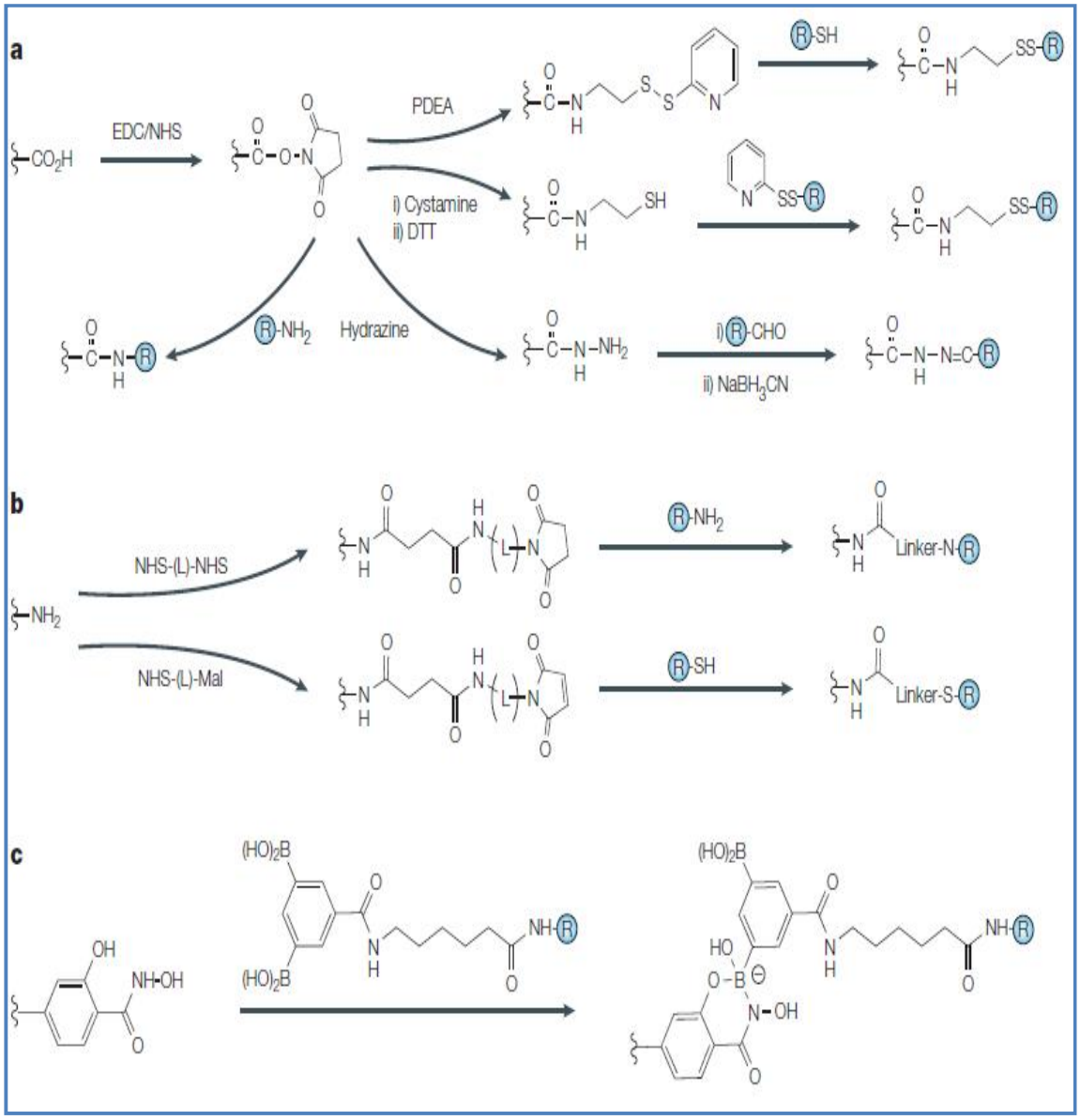


Figure 12. Coupling methods for receptor immobilization: covalent attachment (R = residue) (adapted from **[B28]**)

Water-soluble 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)-mediated activation of a carboxymethylated support is presented in Fig. 12 box (a). The resulting reactive NHS ester can be coupled directly with amino residues of the receptor. Further derivatization with sulphydryl-reactive reagents allows reaction with free surface thiols for reversible disulfide linkage. Amino-presenting surfaces can be processes with commercially available bifunctional linkers to effect coupling with free amino or sulphydryl groups on the receptor (Fig. 12 box (b)). Surfaces that are derivatized with salicylhydroxamic acid can be used to produce reversible complexes with phenyldiboronic acid-activated receptors (Fig. 12 box (c)).

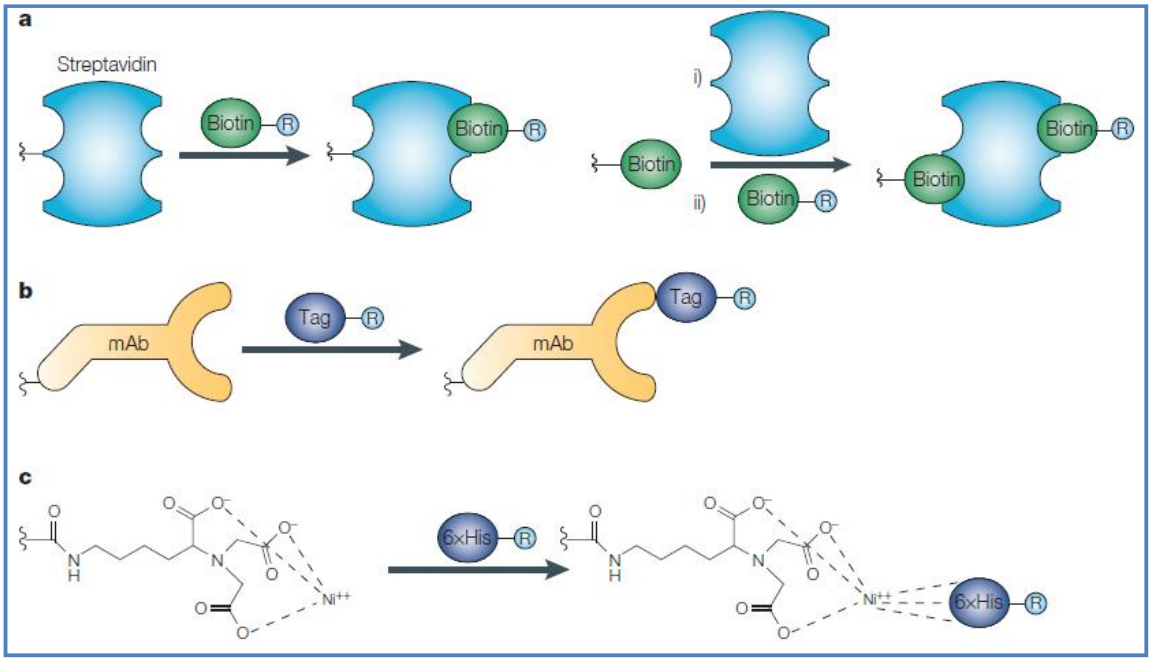


Figure 13. Coupling methods for receptor immobilization: non-covalent attachment (adapted from **[B28]**)

Biotin- or streptavidin-presenting surfaces are applicable for biotinylated receptors (Fig. 13 box (a)). Monoclonal antibodies can be covalently attached to a solid support by means of amine coupling. Epitope-tagged or fusion proteins are reversibly and directly coupled to the surface through antibody antigen interactions (Fig. 13 box (b)). Metal-coordinating groups such as iminodiacetic acid and NTA are used for immobilization of 6-His- and 10-His-tagged receptors (Fig. 13 box (c)).

Within this work, EDC coupling chemistry is used and will be presented more in detail here. The receptors, Staphylococcal enterotoxins A and B, are proteins and build stable covalent

bound with the modified SAM. Due to the carboxyl groups on the surface of the SAM, it is not reactive with amino groups yet, so activation with 1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimid is recommended. EDC is very reactive and highly water soluble as hydrochloride. The EDC coupling mechanism is presented in Figure 14.

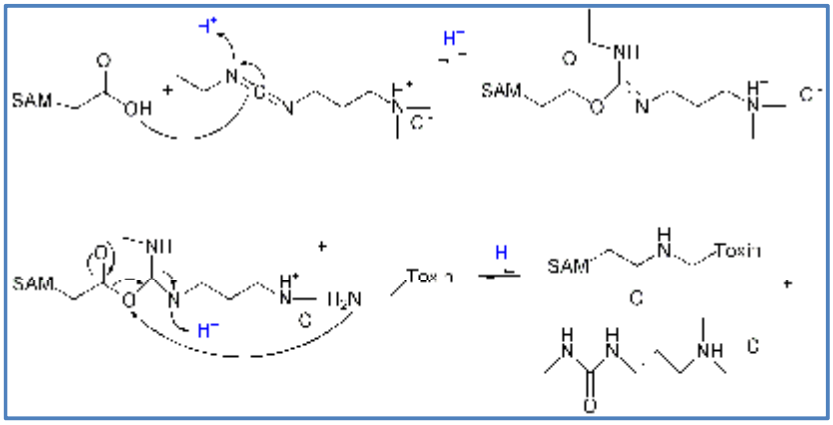


Figure 14. EDC protein coupling mechanism (reproduced from **[B7]**)

Unspecific binding of proteins to polar surfaces (carboxy groups) is prevented via blocking step. EDC is highly reactive, so toxins can react with each other or EDC is inactivated with water. Binding of toxins to the surface changes conformation and therefore influences biological activity. Failed recognition of the antibody can result from this.

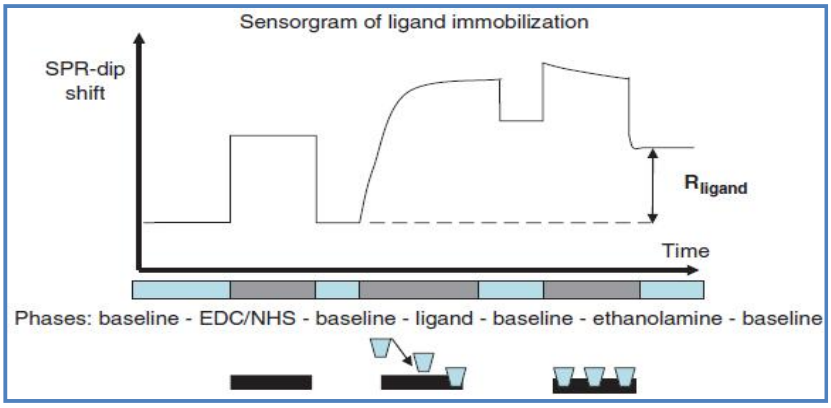


Figure 15. Sensorgram of a receptor immobilization (adapted from **[B28]**)

A typical SPR coupling experiment with EDC/NHS, a receptor/ligand and ethanol for blocking free binding sites is presented in Figure 15 **[B26, B28].**

2.3.3. SPR Assay Types

Common immunoassay formats are presented in Figure 16.

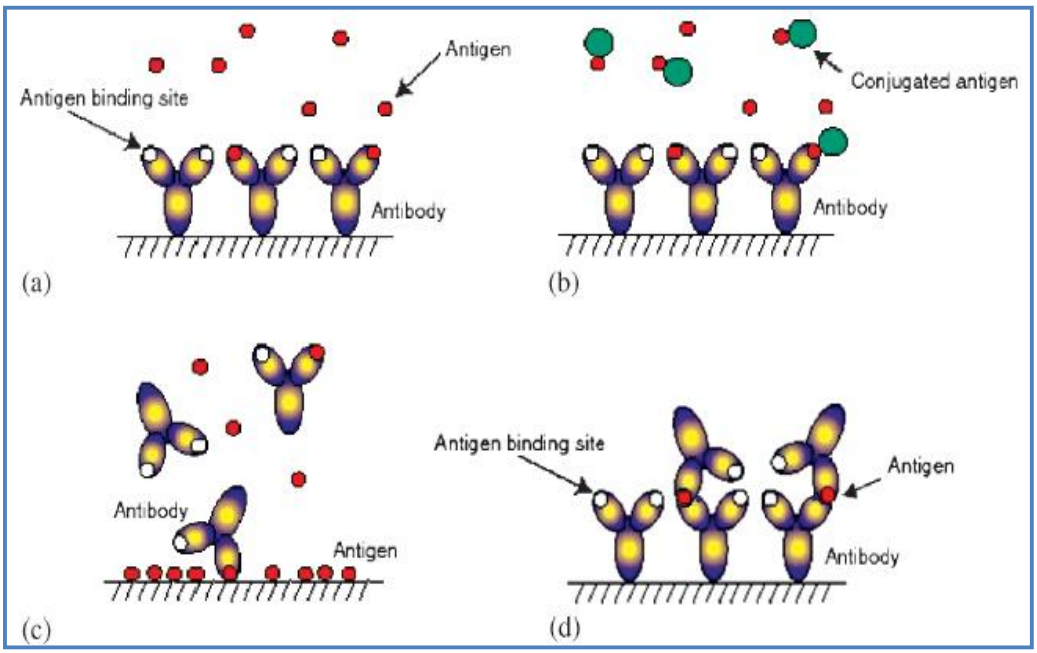


Figure 16. Common Immunoassay Formats for SPR (adapted from **[B28]**)

(a) Direct Assay

Within this assay type, antibodies (= receptor) directed against the antigen are located on the surface. The sample solution, which contains the analyte, is incubated with the sensor surface. The signal increase is antigen-binding dependend and is directly correlated with the analyte concentration in the sample. Direct assays can also act with antigen-modified surfaces for the detection of binding the specific antibody.

(b) Competition Assay

Competitive assays are optimal for the recognition of small antigens with low weight that do not generate an acceptable signal while accumulating at the surface. Herein, specific antibodies are immobilized on the sensor surface. The sample solution contains the antigen and antigen conjugate. Due to its high molecular weight, the conjugate enhances an SPR

angle shift. The mixture of antigen with its corresponding antibody is incubated with the surface. The signal difference between a reference sample (only conjugated antigen) and the sample solution indicated the amount of antigen in the sample. High antigen concentrations produce low signal (less conjugate bound). Signal maximum is achieved when no free analyte is present. In competitive immunoassays, the conjugated antigen is often attached to a large refractive index label (latex bead or gold nanoparticle) loaded with antigen.

(c) Inhibition Assay

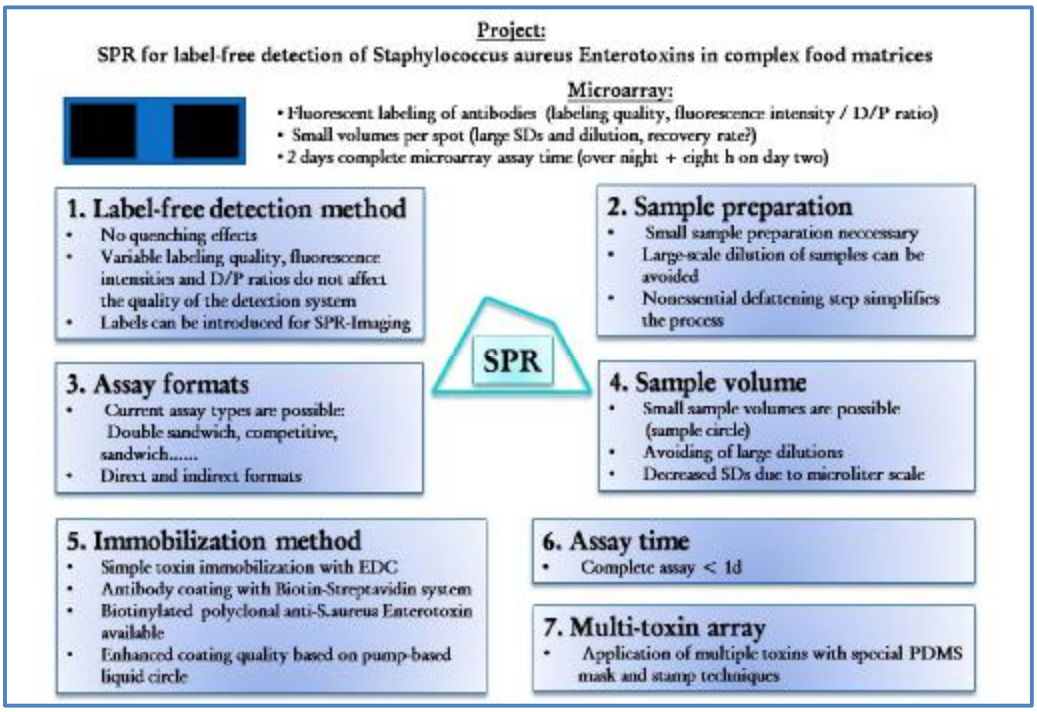
In this format, target antigens are immobilized on the sensor surface. The sample solution which contains the antigen is mixed with specific antibodies in excess and incubated with the sensor surface. Antibodies are binding to antigen that is bound on the surface and in solution. Signal difference between a blank sample without antigen and the sample solution indicates the antigen amount in the sample. Herein, high antigen concentrations in sample result in low signals, resulting from a less number of antibodies to bind. The binding is detected directly, due to the high molecular weight of antibodies.

(d) Sandwich Assay

In sandwich assays, antibodies against the analyte are immobilized on the array surface for capturing the analytes after sample incubation. A secondary antibody is binding specifically with either the antigen or the primary antibody. The antigen is captured by a sandwich of two antibodies. A high affinity capture antibody is required here. The increase in signal is proportional to the amount of antigen in the sample. The surface is washed with buffer followed by injection of a secondary antibody. The binding process can be monitored by the high weight of the secondary antibody. For further enhancement, antibody conjugates with colloidal gold or latex particles as refractive index label can be applied.

SPR: Adavantages of a Label-Free Method for the Application of Real Samples

The adavantages of SPR in combination with food samples are presented as follows.



2.4. *Staphylococcus Aureus* **Enterotoxins**

Foodborne Diseases (FBD) are defined as “diseases of toxic or infectious nature caused by, or thought to be caused by the consumption of food or water” (World Health Organization, **[B32]**). Over 250 FBDs have been described and the symptoms vary widely depending on the etiological agents. Diarrhea and vomiting are the most common symptoms. Among the FBDs, there are 2 different types: food-borne infections and food-borne intoxications/poisoning. The infections are caused by many different disease-causing pathogen agents that can contaminate food whereas intoxications are caused by poisonous chemicals or other harmful substances that are present in food **[B33].** In case of infections, the food is the carrier of pathogen bacteria, a reproduction within the food is not necessary. Transgression of the infection barrier induces the break-out of the infection. The barrier is species-dependent. Typical examples are salmonellosis and infections with *Campylobacter, Yersinia* and *E. Coli*. In case of intoxications, bacteria are reproduced in the food. Toxins are biosynthesized from their metabolic products with the help of chemical substances out of food. Outbreak of illness is given after the consumption of toxin-containing food, with or without living bacteria. Typical examples are *Staphylococcus aureus, Bacillus cereus* and *Clostridium perfringens.* The toxins are preformed in the food, and so the intoxications have

short incubation times/onsets of few hours (S. aureus: 15-30 minutes!). In most cases, the symptoms leave after at least 24 h **[B34].** Outbreaks are frequently traced back to situations where food preparers did not comply with hygiene and safety regulations or to incidences where food, that has to stay freezed, was exposed to room temperature. Unfortunately, alignments containing enterotoxins usually taste, smell and look unsuspicious and not adulterated **[B35]**.

2.4.1. *Staphylococcus aureus* - General Information

Staphylococcus aureus affiliates to the species "Staphylococcus" and therefore to the family of *Micrococcaceae*. It was found in 1883 from Sir Alexander Ogston which gave him the name due to the winegrape-like cluster look. S. aureus can be differed from other Staphylococcus types with the coagulase test, where it is coagulase-positive. Staphylococcus aureus is a non-motile, gram-positive and catalase-positive coccus bacterium with a diameter of 0.5-1.5 µm. The cocci are arranged in grape-like clusters, as singles, as pairs or in short chains. The cell wall is resistant to lysozyme and sensitive to lysostaphin which cleaves the pentaglycin brigdes of *Staphylococcus spp.* Some strains are capable of building toxins and unlike to other bacteria, it is non-spore forming. *S. aureus* is characterized as fast-growing in aerobic or non-aerobic conditions on a broad spectrum of growing media. The colonies are clearly separated and smooth after 18-24 h of growing at 35°C. The golden pigmentation, originated from carotinoides, is visible in most cases. S. aureus possesses the capability to grow at high salt concentrations (5-15% NaCl), between 7-48.5° C (optimum 30-37° C), at a pH between 4.2 and 9.3 (optimum 7.5) and builds carotinoide pigments. Furthermore, glucose can be dissipated under formation of lactid acid **[B33, B34].** It grows on simple growing media, under high temperatures (45°C) and preferentially in foods with low water activity (cooked or strong salted food), where it outcompetes other microorganisms **[B36, B37]**. The bacterium is disseminated ubiquitarily, man and animals possess it on the mucosa of nose and mouth without getting ill. Thus, contamination paths are often irreproducible. More, it belongs to the resident flora and washing hands does not improve the situation. Staphylococcus aureus is obtained as one of the major agent for food intoxications and has clinical importance due to the ability of building heat-stable enterotoxins and enzymes. It is an important pathogen because of toxin-mediated virulence,

invasiveness and antibiotic resistance. Furthermore, it is a major cause of nosocomial infections as well as community-acquired diseases. The spectra of infections range from furuncles and pimples to toxic shock syndrome and sepsis, most of which depend on multiple virulence factors. But some infections like food poisoning rely only on one single type of factor. Its prevalence in food is considered as a sign for absent hygiene. Illnesses due to these toxins are real intoxications and marked via acute gastroenteritis. One to six hours after ingestion, persistent vomiting occurs. Diarrhea, abdominal cramps, headache, muscle cramps and sweating can appear, too. Normally, remission is achieved in less than 24 h, only the elderly and children have to be very careful **[B34, B36, B37].**

2.4.2. Staphylococcal Enterotoxins - A characterization

Staphylococcal Enterotoxins (SEs) are proteins providing high water and saline solubility. Their molecular mass ranges between 20-30 kDa with a pI between 5 and 8.6. The major biochemical characteristics are listed in Table 3. Enterotoxins comprise single polypeptide chains with a high content of lysine, trypsine, aspartic acid and glutamic acid. The secondary structure is built of α-helices and ß-sheets. Crystal structure studies monitored that all enterotoxins possess similar 3-D structures. They are ellipsoid and comprises two domains, A and B. Domain B has, at its end, the characteristic cystine loop with a disulfide bridge. The cystine loop is required for proper conformation and probably involved in the emetic activity. The larger domain A comprises C- and N- terminus. Both domains are linked together with a chain of α-helices **[B33, B35].**

Table 3.
Biochemical characteristics of the staphylococcal Enterotoxins (partially adapted from **[B34]**)

SE Type	Molecular mass (kDa)	Gen size (bp)	Isoelectric point
SEA	27.100	774	7.26
SEB	28.336	801	8.6
SEC1	27.531	801	8.6
SEC2	27.589	801	7.0
SEC3	26.900	801	8.2
SED	27.300	777	7.4
SEE	26.900	774	7.0
SEG	27.042	777	5.7
SEH	25.210	726	5.7
SEI	24.928	729	not defined
SEJ	28.565[1]	806	8.65[1]
SEK	25.539	729	6.5
SEL	24.593[1]	723	8.66[1]
SEM	24.842[1]	722	6.24[1]
SEN	26.067[1]	720	6.97[1]
SEO	26.777[1]	783	6.55[1]
SEP[2]	not defined	782	not defined
SEQ	28.200	728	7.5
SER[2]	bigger than 27.000	779	not defined
SEU[2]	not defined	785	not defined

[1]: Molecular mass and isoelectric point are postulated due to gen size and gen sequence; [2]: Existence postulated due to molecular biology tests, toxin not realized/sequenced

Staphylococcal enterotoxins cause severe gastroenteritis with symptoms like diarrhea and vomiting within one to six hours after ingestion and act as superantigens that stimulate non-specific T-cell proliferation **[B38]**. Staphylococcal enterotoxins can act undesirably in food due to their extreme resistance and stability. In general, these proteins are resistant to chymotrypsine, papaine and rennin and keep their activity in the digestive tract after ingestion. The inactivation of SEB with pepsin affords pH values ≤2! Furthermore, the toxins are insensitive to freeze-drying and irradiation. The most important fact concerning food safety is their extreme heat-stability. The bacteria itself is inactivated at high temperatures, in contrast to the toxins **[B39-B41].** Thus, prevention of bacteria growth at production and storage processes is recommended! Their physical and chemical characteristics were summarized by *Jay*. **[B42]** Detection and analysis of enterotoxin existence was started with *S. aureus* strains from the first food intoxications. This method originated the first three

toxins, SEA-SEC. New SE types were identified from data of genome sequence analyses. A comparison of sequence homologies is presented in Figure 17.

Toxin	SEA	SEB	SEC1	SED	SEE	SEG	SEH	SEI	SEJ	SEM	SEN	SEO
SEA	100	33	30	50	83	27	37	39	64	35	39	37
SEB		100	68	35	32	43	33	31	33	29	32	36
SEC1			100	31	29	41	27	26	30	26	29	33
SED				100	52	27	35	33	51	41	38	39
SEE					100	27	35	35	63	37	39	37
SEG						100	34	28	29	28	31	30
SEH							100	33	35	38	34	31
SEI								100	34	31	31	57
SEJ									100	38	42	33
SEM										100	28	31
SEN											100	42
SEO												100

Figure 17.
Percentage of amino acid identity in different enterotoxin types (adapted from **[B33]**)

Only SEA-SEE and SEH could be produced as pure proteins and identified as toxins. Beside these classic types a new selection, named SEG-SEU, was found. Among these, SEA-SED are the most common food pathogens. Particularly, enterotoxin A is involved in most of the disease outbreaks, whereas SEB is a potential bioterrorism agent. Minimal toxic dose data is quite rare and exists only for prominent representants of the group. The corresponding oral dose for SEA, SEB and SEC1 is specified by less than 1 µg/kg for humans. SED, SEE and SEH are less toxic, 10-30 µg/kg are afforded for monkeys. SEA is the most toxic one, all other types are less toxic **[B33, B34].**

2.4.3. Formation and Prevalence of Enterotoxins in Food

Toxin production in food requires two parts. On the one hand, the food must be contaminated with toxin-producing *S. aureus* and on the other hand, special factors are needed to reach the bacterial count for the production. There are a lot of environmental factors that affect staphylococcal enterotoxin production: nutritive value, pH, water activity, temperature, oxygen content and interactions with other microorganisms. There is a complex interaction of all factors within the food, so detection of a simple and major factor is really difficult **[B34].** Concretely, glucose inhibits production of toxins, especially for SEB and SEC. Probably, the drop in pH, due to the glucose metabolism, induces the inhibition. The production of toxins has its optimum in neutral pH, decreases in acidic pH and is inhibited in pH below 5. The inhibition effect of acidic pH is increased by high salt concentrations. No more production is achieved at salt concentrations above 12%, with no dependence on pH. Furthermore, alkaline pH decreases the production of SEB-SED. In addition, Staphylococcus is very sensitive to competing microflora **[B33].** As example, a level of 10^6 S. aureus/g food must be reached in cheese to build an adequate amount of enterotoxins. The incidence of enterotoxins is much more determined via bacterial counts than via progression. The higher bacterial counts at the beginning of the cheese process, the more probable is the overcome of competitive parameters. Concerning cheese, long storage times are unfavorable for the prevention of bacterial reproduction. Unappropriate cooling or high water contents of cheeses are disadvantageous, too. Competitive inhibition of *S. aureus* through surrounding microflora prevents large reproduction of the bacteria and therefore toxin production. Cheese starting cultures contain milk acid bacteria which build lactose from milk acid. This process decreases the pH value and inhibits staphylococcal growth. Concentration, activity and composition of the starting culture are significant for the progression of *Staphylococcus aureus* counts. Staphylococcal enterotoxins are predominantly generated in the post-exponential growth phase of the bacteria. Around 40-50% of all strains from humans are capable of building enterotoxins **[B34].** Commercially available detection kits are listed in Table 4.

Table 4. Commercially available test kits for Staphylococcal enterotoxins in food

TEST TYPE	BOMMELI (Dr. Bommeli AG, Bern)	RIDASCREEN (R-Biopharm GmbH, Darmstadt)	TECRA (Tecra Diagnostics)	TRANSIA (Microgen Bioproducts, Camberley)	SET-RPLA (Oxoid, Basingstoke)	VIDAS (BioMerieux, Nürtingen)
Detectable toxins	A - D (H)	A - E	A - E	A - E	A - D (E)	A - E
Differentation toxins	Yes	Yes	No	No	No	No
Sensitivity	High	High	Medium	Medium	Low	Low
Specifity	High	High	Low	Low	High	Medium
Test time (h)	24	3	4.5	1.5 - 2	16	1.5
Complexity Test	High	Medium	Medium	Medium	Low	Automated
Complexity Extraction	High	Medium	Medium	Medium	Low	Low
Costs	Medium	High	Medium	High	Medium	High
Tests per kit	10	12	12 - 44	10 - 40	20	30
Format	ELISA	ELISA	ELISA	ELISA	RPLA	ELISA
	PS - balls	MT - Strip	MTP	Tube		MT - Strip
LOD (ng / g)	**0,6 [B43] 0,1 - 1[B34]**	**0,5 – 0,75 [B44] 0,2 - 0,7 [B47]**	**1 – 3 [B45] 1 [B46]**	**> 0,6 [B43]**	**4 [B43] 1 [B48]**	**0,05 - 0,1 [B49]**

Due to high cost per test and the insufficient separation specifity for different types of toxins, additional tests are necessary.

2.5. Fluorescence: an Application in Protein Microarrays

Protein microarray applications demand high detection sensitivity, especially the ability of differentiating potentially low analyte signals from the background. The surface coating should contribute only minimally to the intrinsic background of the substrate. A number of detection strategies are used for imaging protein microarrays, but the majority of laboratories currently rely on fluorescence-based scanners. All available microarray scanners are configured to detect the most conventional fluorescent dyes for microarray analysis, Cy3 and Cy5. Certainly, most of them are additionally equipped with other lasers and filters to enable the use of a wider range of dyes. In this work, an Affymetrix 428 array scanner with

two appropriate lasers for Cy3 and Cy5 is used. Fluorescence intensity emission can be detected with the help of photomultiplier tubes within the system.

For the analysis of the appropriate spot intensity, two possibilities are given: total signal intensity and the mean signal intensity. The total signal intensity is the sum of all intensity values of all analyzed pixels in the spot area. Unfortunately, total signal intensity is sensitive to the variation in the amount of biological material which is deposited on the spot, contamination and anomalies in the image processing operation. These problems occur frequently, so the total intensity may not be an accurate mode of measurement analysis. The mean signal intensity is the average intensity of the signal pixels. This parameter has certain advantages over the calculation of the total intensity. Often, spot size is correlated to the concentration of the sample in the wells during the spotting process. Measurement of the mean value decreases the error which is caused by the variation in the amount of immobilized decoy deposited on the spot. Advanced image processing permits accurate distinction of contamination pixels from signal pixels, so the mean value method should be the parameter of choice **[B17].**

When using older laser scanners, the planarity of the entry funnel and the measuring plane is often a problem. This results in differentiating scanner plots of the same array type. Furthermore, a comparison of two or more different arrays is only possible with exact slide measurement geometries.

3. Microarray Surface Preparation

This chapter describes the cleaning, preparation and quality control of the homemade microarray slide silane surfaces.

In the following chapters, anti-SEX (X = A, B, C, D or H) is replaced by <SEX>.

3.1. Control of Cleaning via Contact Angle

Microarray surfaces were cleaned prior to silanization, followed by sessile drop contact angle measurement as quality and efficiency control. Water is used as measurement liquid. A repertory of methods is published in **[C1].**

The contact angle is specific for any given system. If a liquid is strongly attracted to a solid surface (e.g. water on very hydrophilic substrate), the droplet is completely spread out and the angle is close to 0°. Less hydrophilic surfaces will have contact angles up to 90°. Water exhibits angles of 0-30° on many hydrophilic surfaces. On hydrophobic surfaces, contact angles are above 90°. Before cleaning, contact angles were higher than after the process. This monitors the increasing hydrophilic character of the cleaned slides which is required for silanization.

The results of the cleaning step are presented in Table 5.

Table 5. Contact angles for H_2O before and after the cleaning step

Slide type	Contact angle Θ_b / [°] (before cleaning)	Comment	Contact angle Θ_a / [°] (after cleaning)	Comment
Plain glass	19,92 ± 11,04	inhomogenous distribution	12,25 ± 3,7	relatively homogenous distribution
Hydrophobic pattern slide glass	63,75 ± 3,75	-	32	-

All contact angles are higher than expected from literature **[C1]**, which might be caused from to short cleaning time.

3.2. Control of Silanization via Contact Angle Measurement

Four silanes were applied for surface treatment:

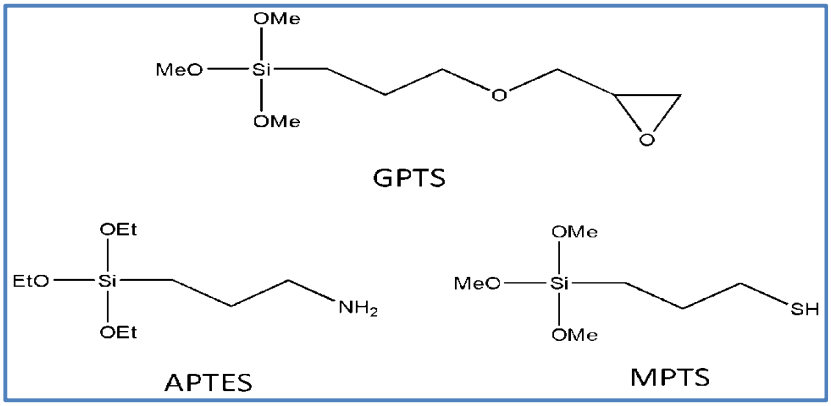


Figure 18. Common glass slide silanization reagents (reconstructed from **[B17]**): Aminopropyltriethoxysilane (APTES), 3-mercaptopropyltrimethoxysilane (MPTS), glycidoxypropyltrimethoxysilane (GPTS).

The silanization step is proceeded under nitrogen atmosphere. For APTES, contact angles of 55-63° in water **[C2-C4]** are published, whereas GPTS-coated surfaces exhibit angles around 57° **[C5].** Contact angles are measures with the sessile drop method after silanization. The results are outlined in Table 6 and Figure 19.

Table 6. Contact angle values for H_2O after the silanization of plain glass slides and hydrophobic patterned slides with various silanization agents

Silanization method	Reagent	Parameters	Contact angle θ_s / [°]
A	100 % GPTS	3h, RT	62,56 ± 5,57
B	2.5 % GPTS	1h, RT	56,83 ± 3,88
C	100% APTES	2h, RT	39,33 ± 4,01
D	5% APTES	1h, RT	56,48 ± 4,81
E	1% MPTS	1h, RT	60,41 ± 3,67
F	100% Aldehyde silane	2h, RT	63,50 ± 4,01
G	2% Aldehyde silane	1h, RT	57,31 ± 6,90
H	2% APTES	3h, 115°C, N_2	66,38 ± 1,84
Nexterion Slide E (NEX)	-	published: 57	51,29 ± 1,11
Erie Super Amino Slide (ERA)	-	published: 40 ± 5	41,30 ± 3,60

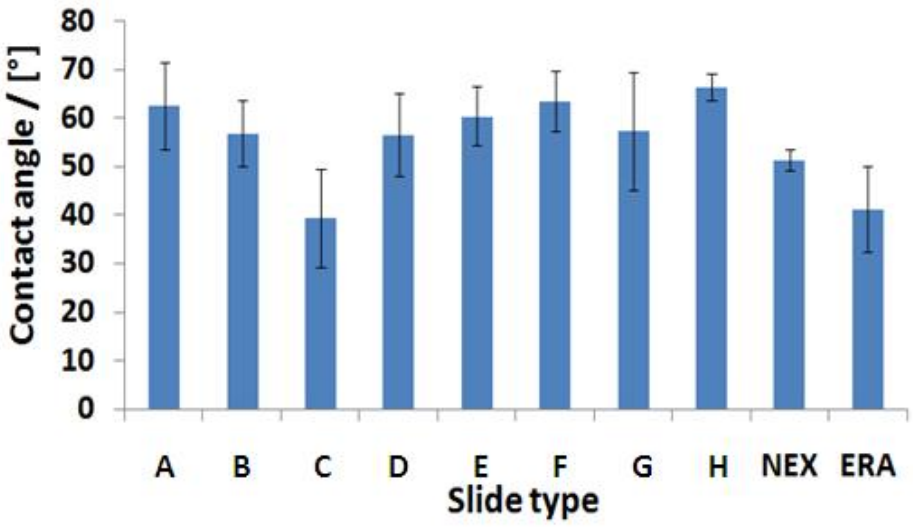


Figure 19. Contact angle data for different slide modification types

Nexterion Slide E and Erie Super Amino Slides were used as standardization for the contact angle measurement method to guarantee correct proceeding. For MPTS and aldehyde slides, there was no contact angle data available in literature. Due to the high standard deviation of the angle (F, G) and high costs for the silane, aldehyde silane was no longer used for further experiments. MPTS covered slides (E) require a linker for binding antibodies or toxins, which prolonges the processing time of the array and harbors the risk of random alignment. As expected, methods using undiluted silane show similar values for epoxy- and aldehyde groups (A and F), independent of incubation time. GPTS slide A exibits a contact angle above the cited literature (**[B5]** and NEX literature) of 57° and a relatively high standard deviation. This assumes that a 100 % solution of silane is not the appropriate choice. GPTS slide B is according to the reference of 57°. Amino slide C is analog, the value lies near the ERA reference and literature of 40°. Herein, a 100% solution of APTES is applicable. Slides D and H are near the expected values of 56-63°. The disadvantage of using APTES is its rapid aging in liquid as well as on the array. Furthermore, a linker is needed (see MPTS). Therefore, GPTS slides are chosen as preferred surface. GPTS -modified slides are best to use 3 months up to 1 year after silanization, so long-time storage is applicable. All further experiments are proceeded with a variance in number H, 3 h at 115° C with 2% GPTS (instead of APTES) in dry toluene in N_2 atmosphere.

3.3 Control of Immobilization via Fluorescence Detection

Control is proceeded with a Cy3-labeled staphylococcal antibody array of ascending toxin layer concentration rows (from left to right each) in Figure 20.

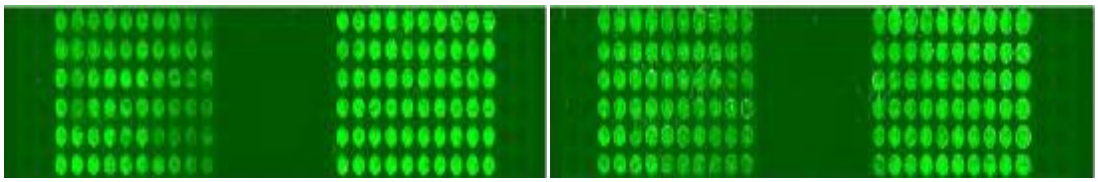


Figure 20. Quality control of silanization with increasing concentrations of competitive toxin: SEA (left) and SEB (right) (0-40 ng/mL, in columns from left to right)

All columns have identical concentration and so they represent the quality level of the silanization. With exception of certain surface defects, the quality of the slides (standard deviation: 8-15%) is acceptable.

4. Labeling *Staphylococcus aureus* Enterotoxin Antibody

This part of the thesis focuses on the fabrication of the detector elements – the fluorescently-labeled antibodies of *Staphylococcus aureus* Enterotoxins. These are applied to quantify the level of competitive toxins in sample and standard solutions.

4.1. Targets: GST- and *Staphylococcus aureus* Enterotoxin (A-D and H) Antibodies

For working efficient and saving money, anti-Glutathione-S-Transferase (anti-GST) was used for the first labeling experiences. The results are transmittive due to the general properties of all antibodies concerning labeling positions. For final determinations of the Dye-to-Protein (D/P) ratio and the alternative Dye Chromeo 546, different anti-Staphylococcus aureus Enterotoxins were applied. Labeling experiments with anti-GST and anti-*Staphylococcus aureus* enterotoxin gave the results presented in the following chapters.

4.2. Dyes: Cy3 and Chromeo 546

Fluorescence dye with optimal properties: Cy3-NHS ester

<GST> and <SEX> (X: A, B, C, D and H) are labeled with the Cy3-NHS ester. Alternative labeling experiments were proceeded with the Chromeo 546-NHS ester.

4.3. Determination of the Appropriate Molar Ratio (MR)

A series of nine different molar ratios (MRs) is used to label <GST> with Cy3 dye followed by a cleaning step using a Sephadex G-25 column. At a MR of one and three, the labeled and unlabeled fractions were marginally separatable, so the protein has to be used without cleaning. With increasing MR, the length of the zones is increasing and separation is easier. Unfortunately, the dilution factor is also increasing with increasing zone length. Optimal Dye-to-Protein (D/P) ratios are in the region between one and two with the highest fluorescence signal that is possible. After measuring the absorbance, best values are expected for ratios from ten to 18. After calculating D/P from the absorbance values, optimal D/P results are

provided at a MR between ten and twelve followed by a decrease at MRs above 15. The fluorescence-to-protein (F/P) ratio is increasing at ratios higher than ten. An MR of twelve is chosen for all further labeling experiments, providing most of the criteria (acceptable fluorescence intensity and D/P) to be fulfilled. The results are presented in Figure 21.

$$\frac{D}{P} = \frac{Concentration(Dye)}{Concentration(Protein)} \qquad \frac{F}{P} = \frac{Fluorescence(Protein)}{Concentration(Protein)}$$

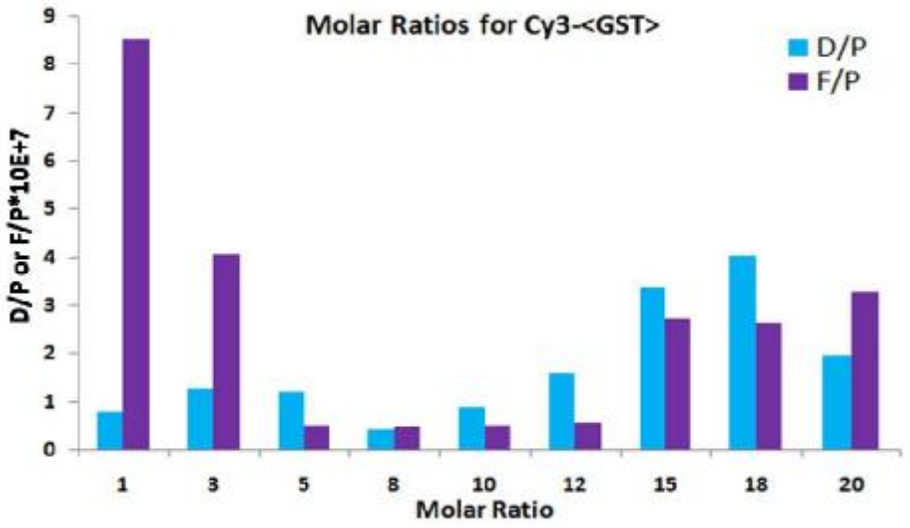


Figure 21. Dye-to-Protein and Fluorescence-to-Protein ratio of the labeled <GST> fractions

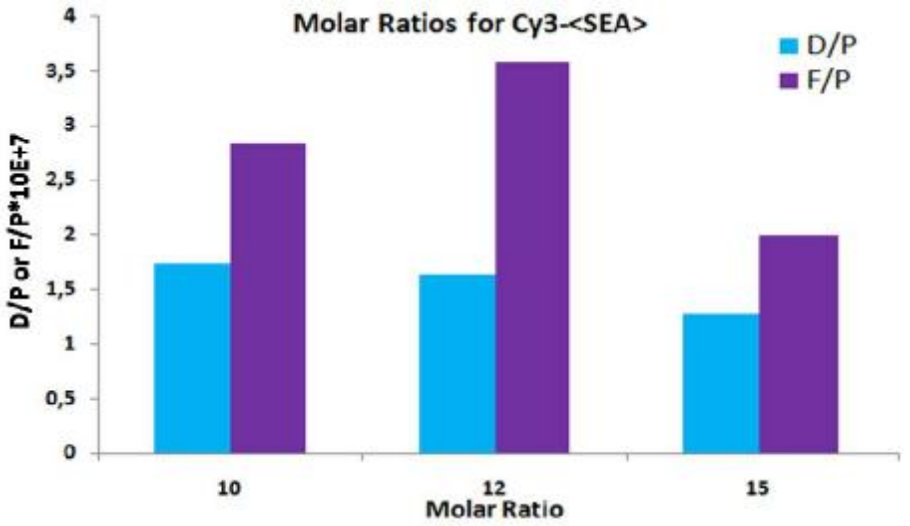


Figure 22. D/P and F/P ratios of the labeled <SEA> batches

A series of three different molar ratios is used to label <SEA> with Cy3 dye followed by a cleaning step using the Melon Gel Kit. After measuring the absorbance, D/P provides optimal results at MR = 10 and 12 and is decreasing at MRs above 12. F/P is increasing at ratios higher of 10 and 12. An MR of 12 is chosen for all further labeling experiments. The results are presented in Figure 22.

4.4. Purification of Labeled Dyes

Three identically labeled <GST> samples (MR20; this MR is considered to be sufficient in **[D2]**) that are cleaned up using three different methods: a Sephadex-filled glass column, a Sephadex-filled plastic column and Millipore Filter Spin Units. The received fractions of each clean-up method were picked up for a D/P comparison as presented in Figure 23. All clean-up methods yield in acceptable D/P and F/P ratios, but the dilution factor is still a problem which cannot be solved. Among column-based clean-up types, the plastic column is best due to the combination of medium D/P ratios and its practicability. Filter units show similar results but were not used again due to their expensiveness.

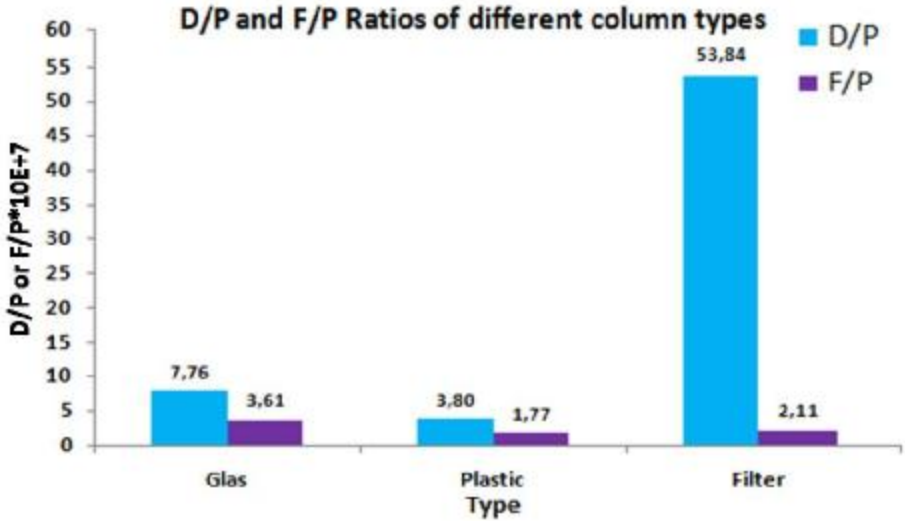


Figure 23. D/P and F/P ratios of the different column types

For further comparison of the methods above with advanced cleaning procedures, five identically labeled <GST> samples (MR15) that are cleaned up with five different methods. Melon Gel Kit and the Nab Spin Plus Kit are used additionally to Filter, Sephadex G15 and

G25 plastic columns. A pure protein reference is used as well. Fractions of all methods were picked up for a D/P comparison as presented in Figure 24. The NabPlus Spin Kit uses harsh elution conditions of pH 3 and therefore, the linkage between the dye and the protein is destroyed during cleaning. This results in very low D/P and F/P values. G25 and the filter units show similar reaction as above. G15 seems to be the best choice when using columns, due to the higher F/P ratio. Among all clean-up types, the Melon Gel Kit is best due to the nearby optimal D/P ratio, a high F/P ratio and its simple and time-consuming handling. Melon Gel is used for all further labeling with the Staphylococcal antibodies now.

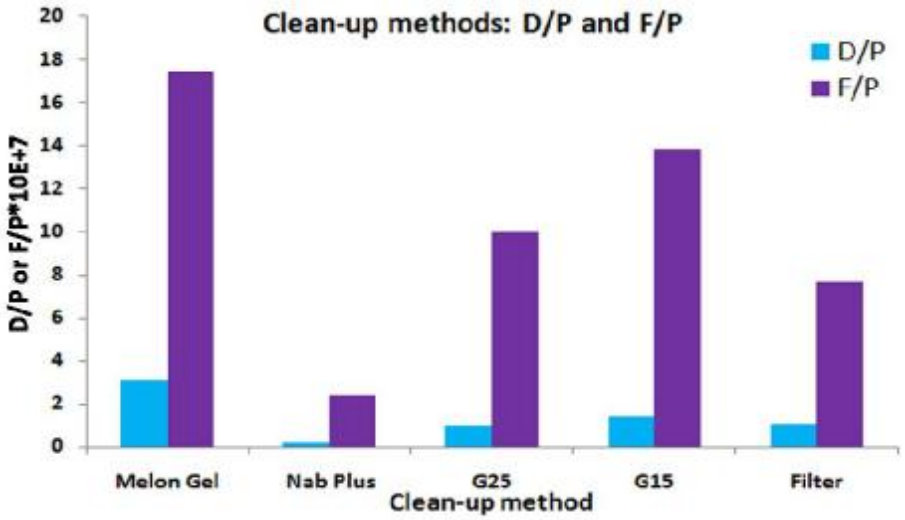


Figure 24. D/P and F/P ratios of the different clean-up types

4.5. Determination of Optimal Dye-to-Protein Ratio by Condition Variation

Enhancements with Cy3 and the Target Antibodies from Staphylococcus aureus Toxins

A series of five different pH values and two reaction times is proceeded in a labeling experiment using <SEA> and <SEB>. Clean-up is done with the Melon Gel Kit. pH values lower than eigth require prolonged reaction time because the reaction product is built much slower at low pH. At pH values of or above eight, the reaction time can be reduced from 19 to 1.75 h. As Figure 25 monitors, a pH of 8.3 seems to be the best option for labeling, due to the time-saving argument and the near to the physiological pH range. But the F/P is lower than the one of pH 8, so there must be partial biological degradation or fluorescence

quenching at a pH of 8.3. Due to this fact, a new approach with a shortly prolonged reaction time of 4h and pH values nearby the physiological range is started with <SEA> and <SEB>. Using shorter reaction time for lower pH values has a positive effect on the D/P of both antibodies (Figure 26). Shortening the reaction time from four to three hours decreases D/P and F/P of both antibody types, but the results are still in acceptable ranges. Therefore, a pH of 8 and a reaction time of three hours set as standard now (Figure 27).

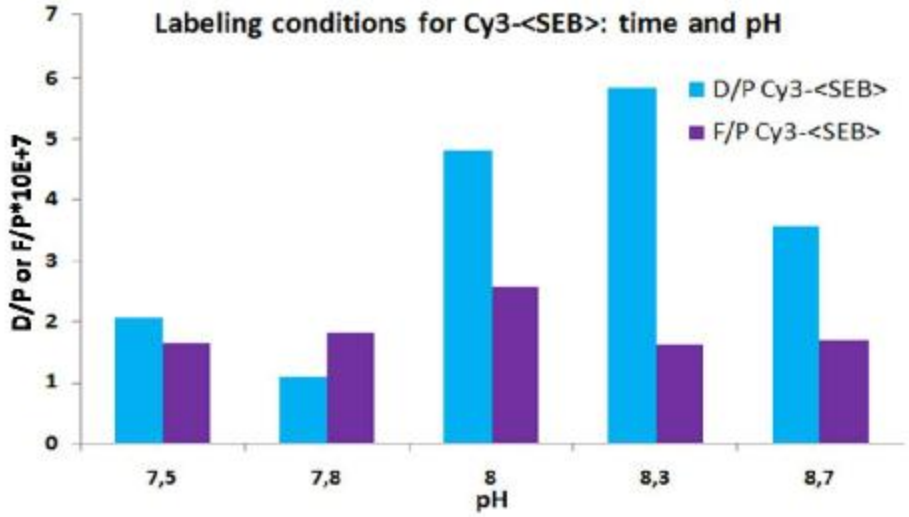


Figure 25. D/P and F/P ratios of the different reaction conditions for <SEB>

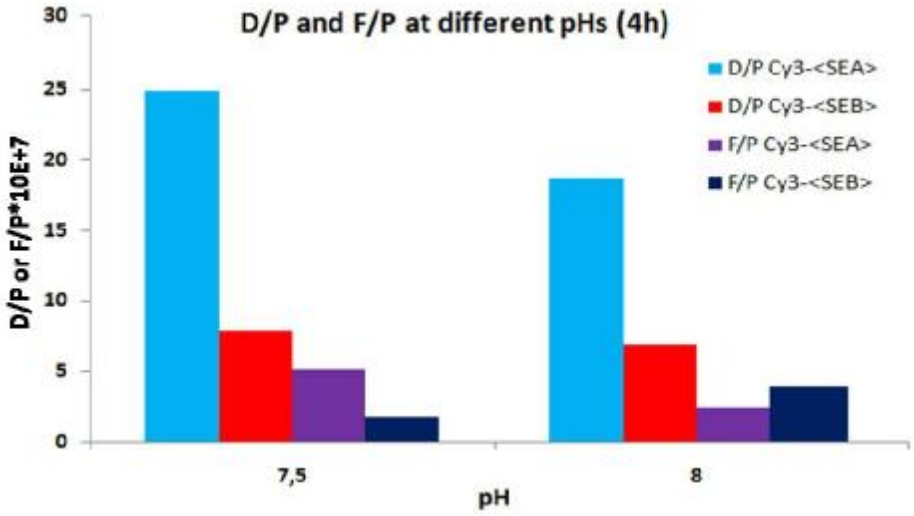


Figure 26. D/P and F/P ratios of the different reaction conditions for <SEA> and <SEB>

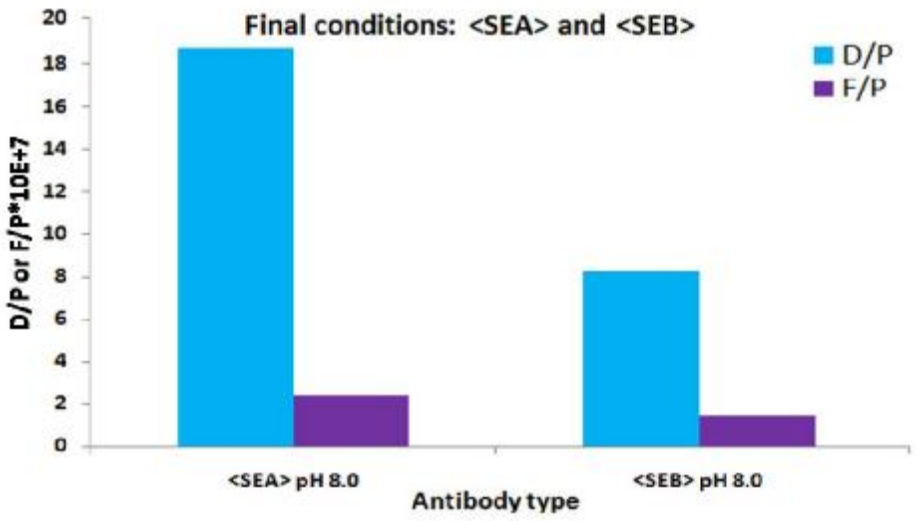


Figure 27. D/P and F/P ratios of the final reaction conditions: pH 8 and 3 h labeling time

Competitive Dye Contest: Cy3 vs. Chromeo 546

<SEA> and <SEB> are labeled with Cy3 and Chromeo 546 to compare both dyes on similar protein targets (Figure 28). At standard conditions, D/Ps for Cy3 are much higher but the effective fluorescence intensity of both antibody types is lower. Chromeo 546 has a lower ε, but better properties concerning effective fluorescence intensity. This might be due to the substituted side group (related to Cy3) and the protection of the surrounding.

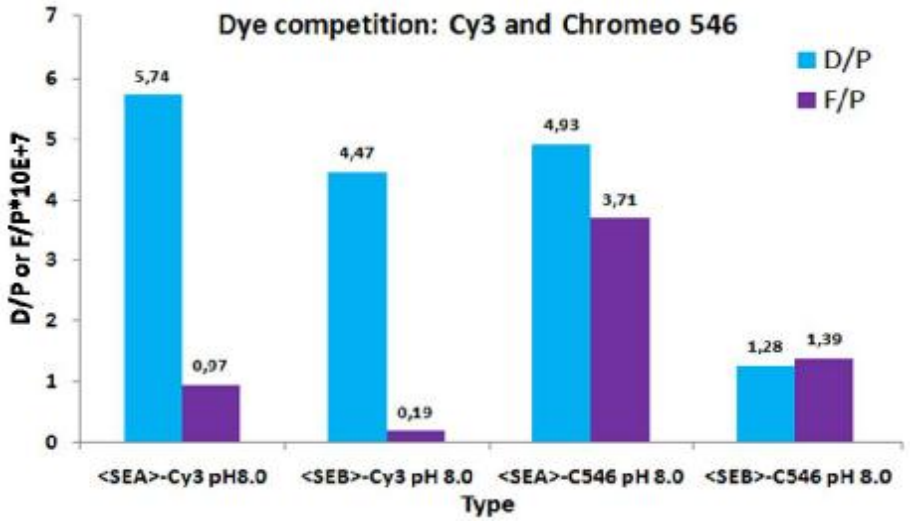


Figure 28. D/P and F/P ratios of the dye competition: Cy3 and Chromeo 546

Competitive Antibody Contest: Toxin Technology (TT) vs. Acris Antibodies (AA)

Within this experiment, antibodies from two suppliers (Acris and TT) are tested (Figure 29). At standard conditions, all D/P values are all relatively similar and ideal. The F/P values of TT antibodies are much lower, but due to the fact that they are all relatively similar, they are better to apply on the array all together. So they are used for the microarray experiments.

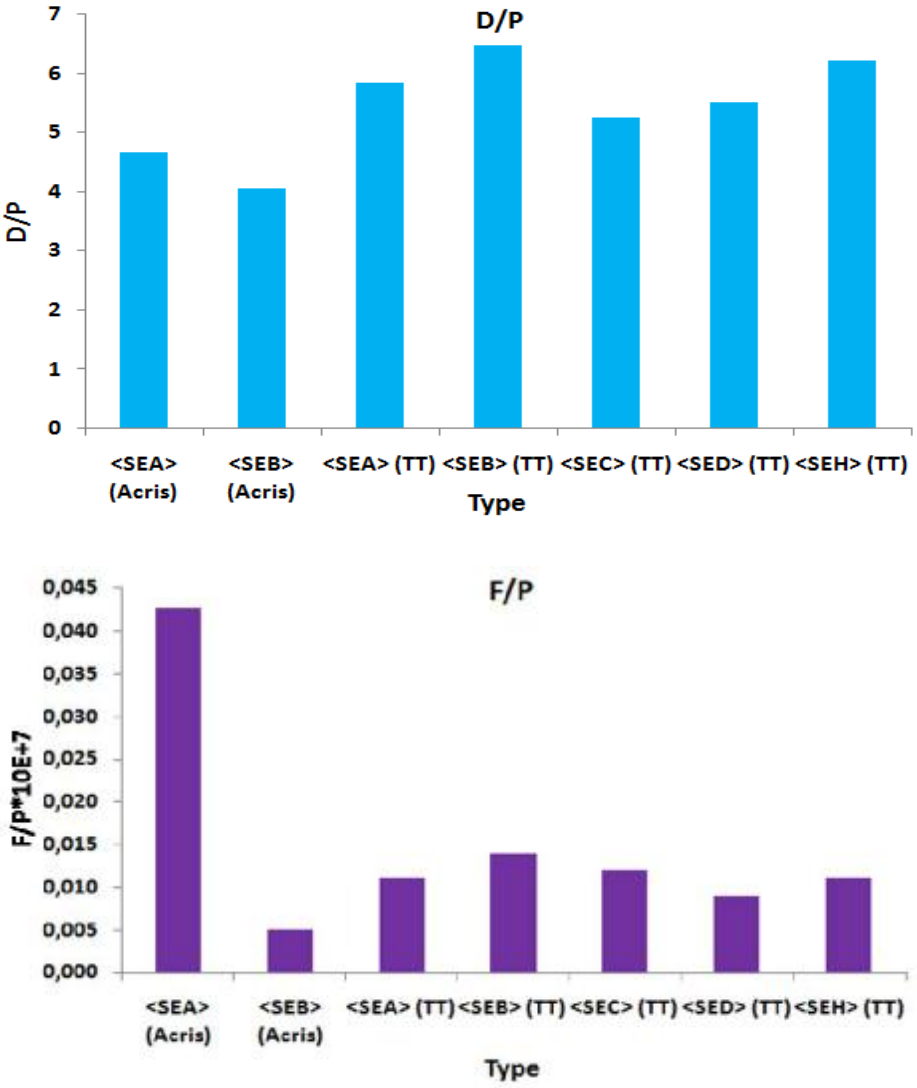
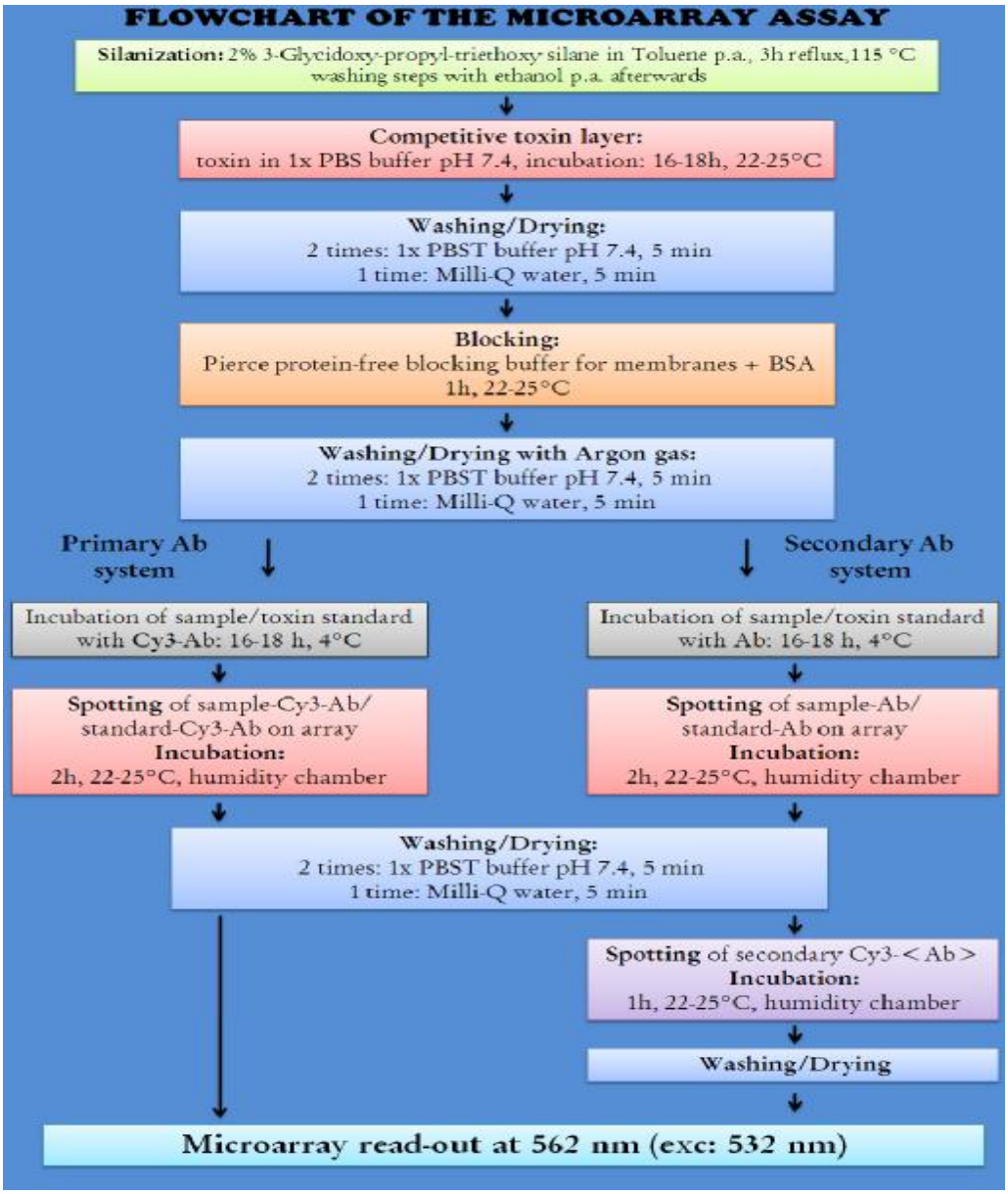


Figure 29. D/P and F/P ratios of the antibody competition: Acris Antibodies and Toxin Technology

5. Competitive Enterotoxin Microarray

The aim was to develop a microarray system with low unspecific binding and high signal-to-background ratios as a detection platform for different enterotoxins with high sensitivity. Determination of the binding constants of <SEA> and <SEB> was performed by means of SPR (chapter eight) to verify a high specific system.

A general overview of the applied assay types, using primary and/or secondary antibodies, is given in Figure 30.



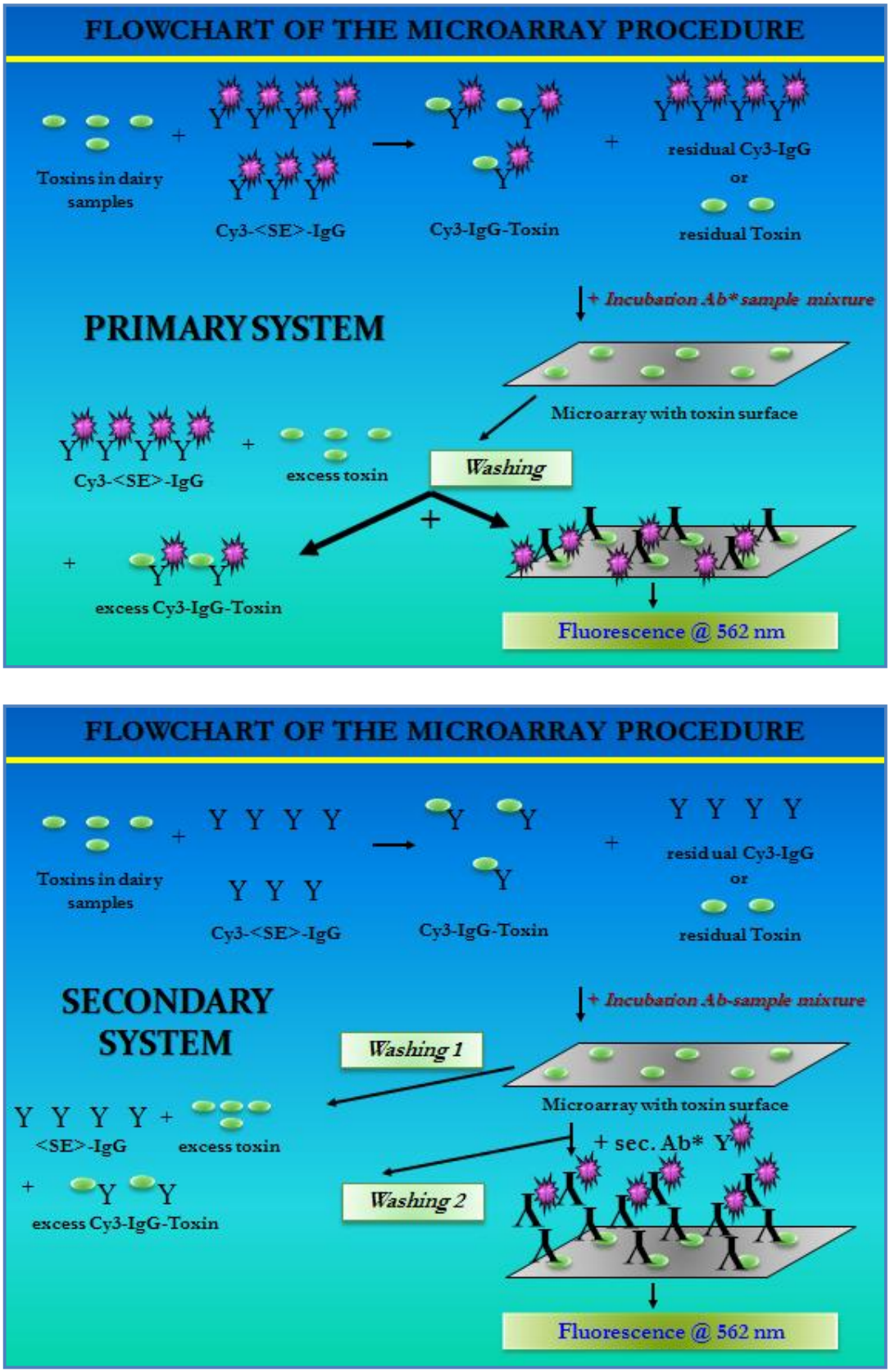


Figure 30. Scheme of the microarray procedure for primary and secondary systems

5.1. Primary System: Labeled Primary Antibodies as Detection Elements

Within this chapter, the development of the primary antibody array system for the EU project “BIOTRACER” is described. This first array system is used for the detection of toxins in real raw milk samples shown in chapter 5.5.

5.1.1. Antigen Layer Tests on Hydrophobic Patterned Slides and Nitrocellulose Slides

Chapter 5.1.1 presents the results of the antigen layer tests for hydrophobic patterned and nitrocellulose slides. Precise array construction with regard to antigenic surface saturation and low surface defect structures is very important as the competitive toxin layer is building the binding region for the unbound antibodies within the sample.

The fluorescence intensity is measured as mean signal intensity for all further analyses. The mean signal intensity is the average intensity of the signal pixels. Measurement of the mean value decreases the error which is caused by the variation in the amount of immobilized decoy deposited on the spot. Herein, the mean signal intensity of Cy3 at 532 nm excitation wavelength, F532 Mean, is plotted. All plotted intensities are background-corrected (BG corr).

Hydrophobic Patterned Epoxy Slides

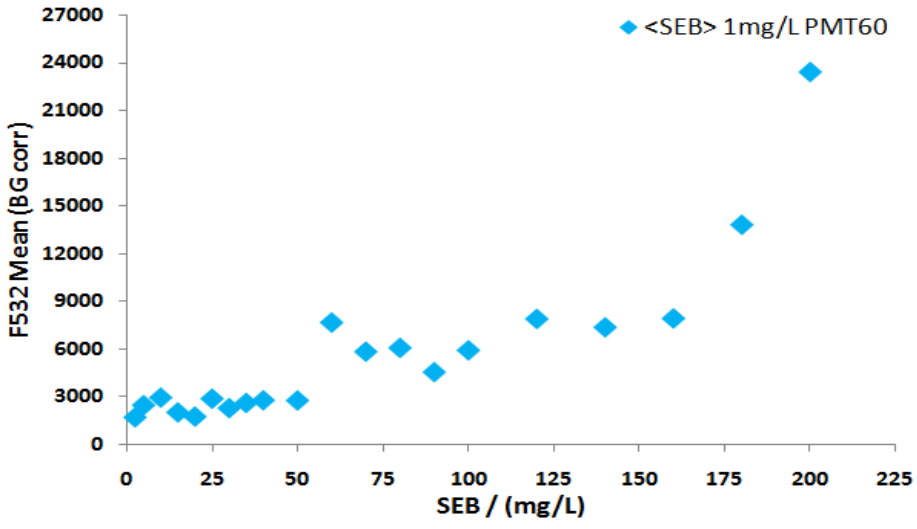


Figure 31. Antigen layer test with SEB on patterned epoxy Slides

Table 7. Standard deviation and Signal-to-Background data for Figure 31

(F532Mean Background: 442)

ß(SEB)/(mg/L)	2.5	5	10	15	20	25	30
Standard deviation (F532)	374	680	917	603	546	1008	743
Signal-to-Background ratio	3.89	5.61	6.70	4.59	3.99	6.54	5.20
ß(SEB)/(mg/L)	**35**	**40**	**50**	**60**	**70**	**80**	**90**
Standard deviation (F532)	794	594	598	2684	1973	677	1362
Signal-to-Background ratio	5.93	6.31	6.30	18.38	13.24	13.77	10.33
ß(SEB)/(mg/L)	**100**	**120**	**140**	**160**	**180**	**200**	
Standard deviation (F532)	3244	4050	890	2864	6462	11705	
Signal-to-Background ratio	13.44	17.89	16.71	17.97	31.30	53.05	

A first SEB/Cy3-<SEB> test system for GPTS surfaces was processed on hydrophobic patterned slides (Figure 31 and Table 7). Therefore, SEB concentrations from 0-200 mg/L were applied to a GPTS-silanized slide. Fluorescence detection of SEB was done by addition of 1mg/L Cy3-<SEB> and scanner read-out at 562 nm with an excitation wavelength of 532 nm. Excitation and emission parameters of the scanner are kept constantly for all further array experiments of this thesis. The photomultiplier tube (PMT) voltages of the scanning system reach from 10-70, but a mid-range value around 40 is preferred. Herein, a value of 60 was applied for analysis. The optimal competitive toxin concentration is resided nearby the beginning of the fluorescence saturation region of the system. Herein, a SEB concentration of 50-60 mg/L seemed to be adequate to form a layer where the fluorescence intensity of the Cy3-labeled SEB antibody layer is near the saturation region. On GPTS slides, the signal-to-noise ratio is optimal and the background is very low at the emission wavelength. These two parameters are very important for constructing highly sensitive arrays. Unfortunately, it is often very complicated to keep them constant and reproducible. So array construction is mainly based on their optimization.

Nitrocellulose Slides

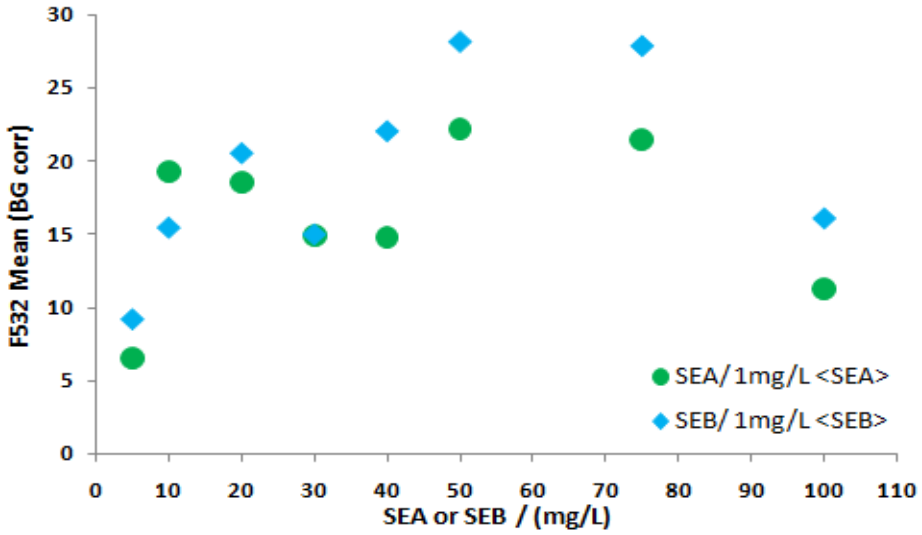


Figure 32. Competitive layer test with SEA and SEB on nitrocellulose slides (PMT37)

Table 8. Standard deviation and Signal-to-Background data for Figure 32

(F532Mean Background: 2.35)

ß(SEA)/(mg/L)	**5**	**10**	**20**	**30**	**40**	**50**	**75**	**100**
Standard deviation (F532)	1.33	0.59	2.36	1.01	0.73	0.59	1.74	1.41
Signal-to-Background ratio	2.91	8.33	8.03	6.48	6.43	9.57	9.26	4.92
ß(SEB)/(mg/L)	**5**	**10**	**20**	**30**	**40**	**50**	**75**	**100**
Standard deviation (F532)	1.88	0.08	3.27	1.32	2.00	0.07	1.77	1.21
Signal-to-Background ratio	4.02	6.69	8.86	6.49	9.50	12.10	11.98	6.96

First test systems for SEA/Cy3-<SEA> and SEB/Cy3-<SEB> were made on two separate areas on one 1-pad nitrocellulose slides (Figure 32 and Table 8). SEA and SEB concentrations from 0-100 mg/L were incubated on nitrocellulose slides. Fluorescence detection of both types was done by addition of 1mg/L of the appropriate Cy3-antibody followed by scanner read-out. Toxin concentrations of 40-50 mg/L seemed to be adequate to form a competitive layer where the fluorescence intensity is near the saturation region. Both toxin types were

screened on one slide to monitor similar fluorescence intensity regions. This is basically necessary for all further array experiments, which should be measurable with similar PMT voltage. The signal-to-noise ratio is not optimal because the background of the nitrocellulose layer is very high at 562 nm emission wavelength. Therefore, nitrocellulose slides cannot be scanned at high PMT voltages which results in lower F532 values, compared to GPTS slides. The combination of low PMT voltage, low F532 values and comparably high background values at low PMT voltages results in reproduction problems. Furthermore, the 3D-structure of this slide type causes high standard deviations due to immobilization failures and irregular fluorescence intensity distribution within the spot core.

Other Slide Types

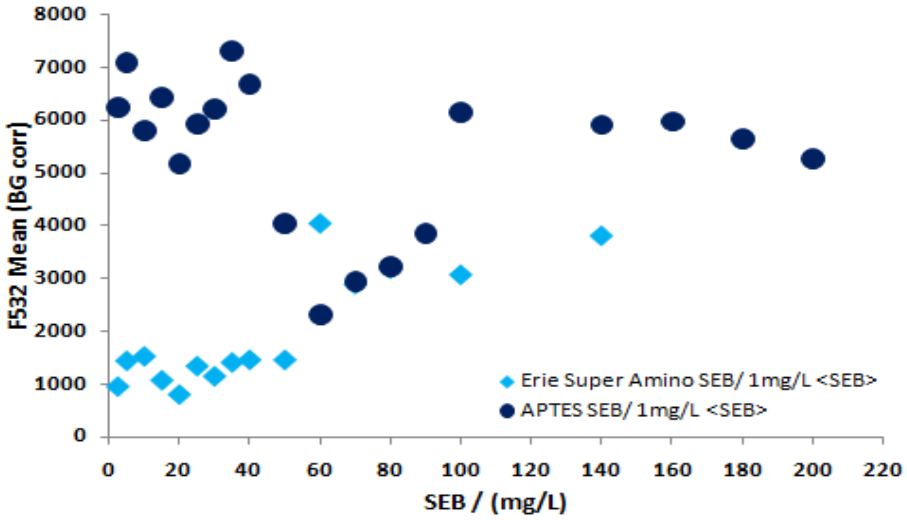


Figure 33. Competitive layer test with SEB on two different amino slide types

The third test system contains homemade APTES slides and Erie Super Amino Slides. They were incubated with SEB concentrations from 2.5-200 mg/L followed by the incubation of 1 mg/L Cy3-<SEB> to guarantee comparability of the slide types (Figure 33). Unfortunately, both slide types require a further linker system with disuccinimidyl suberate, which is the main source of deviations in the reactive layer and extends the array processing time. PMT voltage, signal-to-noise ratio and background signal are comparable to GPTS slides for both APTES slide types. The result of the homemade APTES slide is not satisfying, the best SEB concentration cannot be clearly defined. For Erie Super Amino slides, a SEB concentration

between 50 and 60 mg/L seems to be adequate. Due to these negative facts, ATPES slides were not used for further array construction.

5.1.2. Labeled Primary Antibody Layer Tests: The Detection Unit on both Slide Types

Chapter 5.1.2 presents the results of the primary antibody layer tests for hydrophobic patterned and nitrocellulose slides. Defining the antibody layer clearly is necessary due to fluorescence quenching effects and false-positive or false-negative detection of analytes in samples. For all further experiments, all five toxin types should be measurable on one slide with one PMT voltage, so the fluorescence intensities should be relatively similar or at least in the same order of magnitude. The ambitious trial is to find appropriate antibody concentrations for <SEA> to <SEH> and to establish antibody-toxin layer concentration pairs.

Hydrophobic Patterned Epoxy Slides

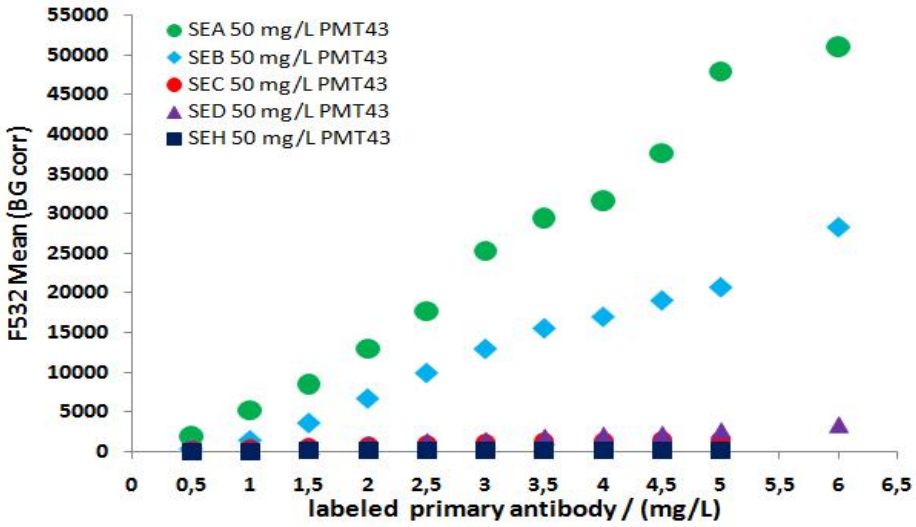


Figure 34. Cy3-antibody layer test for all five toxin types on GPTS slides

Following the results of the previous chapter, a competitive layer concentration of 50 mg/L was chosen for all toxin types. As a detection layer, Cy3-labeled antibodies for SEA-SED and SEH were tested from 0.5-6 mg/L (Figure 34 and Table 9). Every antibody-toxin pair was tested on a separate slide. Cy3-<SEA> and Cy3-<SEB> provide proper fluorescence intensity values at an optimal PMT voltage of 43.

Table 9. Standard deviation and Signal-to-Background data for Figure 34

(F532Mean Background: 71.09 (<SEB>), 54.15 (<SEA>))

ß(<SEA>) /(mg/L)	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**6**
Standard deviation (F532)	390	2097	1379	3405	1742	2794	3623	2267	3361	6128	6541
Signal-to-Background ratio	33.6	94.8	155.8	238.6	326	466.5	543.3	583.2	695	885.2	943.8
ß(<SEB>) /(mg/L)	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**6**
Standard deviation (F532)	241	636	1893	2784	2739	1546	3577	3957	4671	2620	2080
Signal-to-Background ratio	5.3	20.2	50.9	94.4	139.7	182.6	219	239.3	268.6	291.9	398.5

For Cy3-<SEC> - Cy3-<SEH>, a competitive layer concentration of 50 mg/L is not enough to provide similar fluorescence intensity ranges compared to the SEA/Cy3-<SEA> and SEB/Cy3-<SEB> system. Higher toxin and antibody layer concentrations are applied in the further array development to reach the goal of parallel scanning read-out at one PMT voltage. The desired antibody concentration for A and B is resided nearby the beginning of the fluorescence saturation region of the system. Herein, a concentration of 5 mg/L for A and B seemed to be adequate to form a layer where the fluorescence intensity of the Cy3-labeled antibody layer is near the saturation region.

Basically, the A and B-type antibody/toxin system provides increased fluorescence intensity values compared to the residual types. This might be caused by increased binding constants or more effective labeling with less self-quenching on the antibody or in solution.

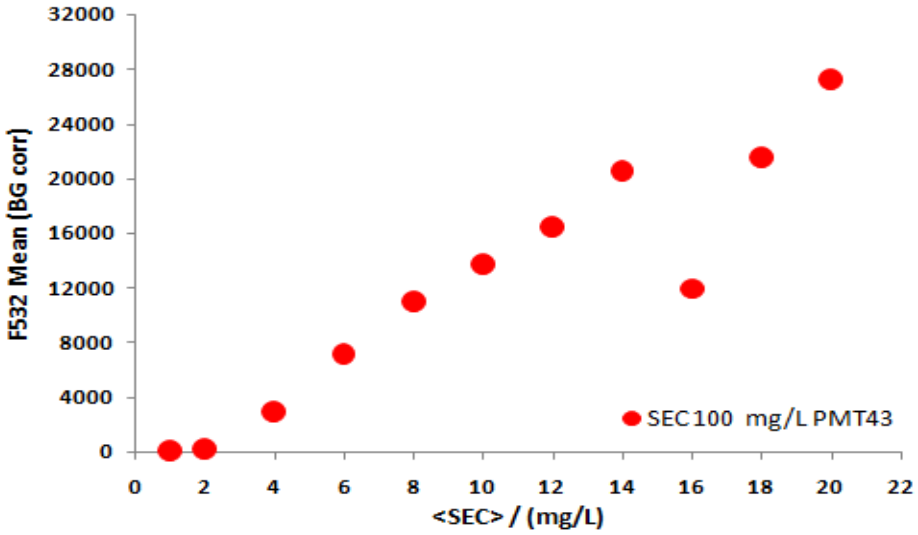


Figure 35. Cy3-antibody layer test for SEC on GPTS slides

Table 10. Standard deviation and Signal-to-Background data for Figure 35

(F532Mean Background: 55.83)

ß(<SEC>) /(mg/L)	1	2	4	6	8	10	12	14	16	18	20
Standard deviation (F532)	77	187	429	1116	2061	2298	2762	3830	1068	2828	6932
Signal-to-Background ratio	2.2	4.8	54	129	197	246	296	369	214	364	489

For SEC, the toxin layer concentration was increased to 100 mg/L and a Cy3-<SEC> concentration from 1-14 mg/L was applied. A SEC concentration of 100 mg/L combined with 14 mg/L Cy3-<SEC> is providing an adequate pair to fulfill the criteria of the given SEA/SEB system PMT voltage, fluorescence intensity range and saturation region (Figure 35 and Table 10). After analysis of the labeling experiments of <SED> and <SEH>, both antibody types monitor decreased D/P and F/P ratios compared to the other three types. The first antibody experiments on GPTS arrays are confirming these results additionally due to lower intensity values. Therefore, increased toxin layer concentrations of 100-200 (SED) and 100-250 (SEH) were incubated. Furthermore, increased antibody concentrations from 1-30 (<SED>) and 1-20 (<SEH>) were applied (Figure 36 and Table 11). Finally, concentration pairs of 200 mg/L SED/25 mg/L Cy3-<SED> and 250 mg/L SHE/20 mg/L Cy3-<SEH> were chosen. Unfortunately,

within this concentration ranges the fluorescence intensity values of SEA-SEC could not be reached at PMT43. Furthermore, labeling efficiency could not be improved and the toxin and antibody concentrations could not be increased any more due to the extremely high prices.

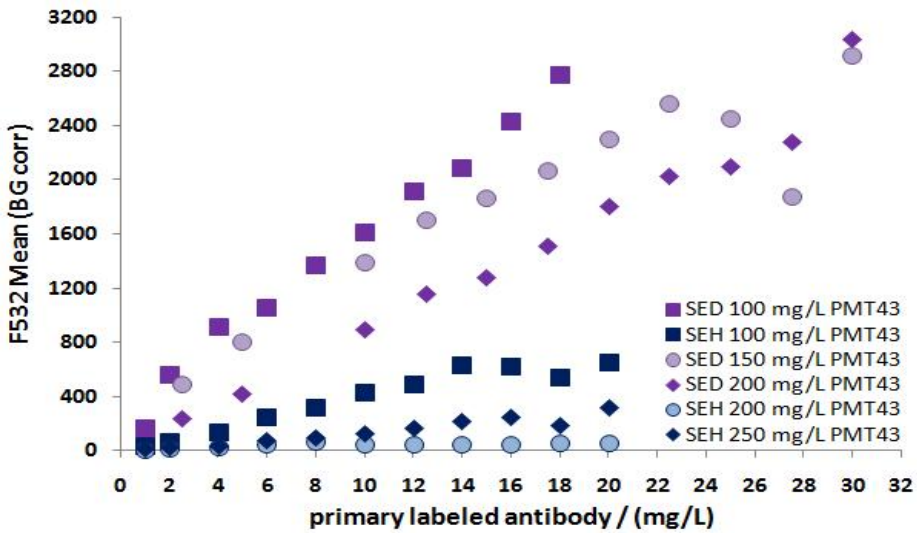


Figure 36. Cy3-antibody layer test for SED and SEH on GPTS slides

Table 11. Standard deviation and Signal-to-Background data for Figure 36

(F532Mean Background: 37.71 (<SED>), 17.67 (<SEH>); ß(SED)=200 mg/L, ß(SEH)=250 mg/L)

ß(<SED>) /(mg/L)	**2.5**	**5**	**10**	**12.5**	**15**	**17.5**	**20**	**22.5**	**25**	**27.5**	**30**
Standard deviation (F532)	66	111	293	374	484	232	350	591	393	435	557
Signal-to-Background ratio	6.2	11.2	23.8	30.8	33.8	40.1	47.7	53.7	55.7	60.4	80.4
ß(<SEH>) /(mg/L)	**1**	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**	**18**	**20**
Standard deviation (F532)	6.95	16.6	15.97	11.43	28.34	15.88	23.54	28.95	19.94	102.2	49.35
Signal-to-Background ratio	0.77	1.15	1.97	3.95	5.27	6.80	9.54	12.18	13.66	10.37	17.84

For SEC, the toxin layer concentration was increased to 100 mg/L and a Cy3-<SEC> concentration from 1-14 mg/L was applied. A SEC concentration of 100 mg/L combined with 14 mg/L Cy3-<SEC> is providing an adequate pair to fulfill the criteria of the given SEA/SEB system PMT voltage, fluorescence intensity range and saturation region (Figure 35 and Table 10). After analysis of the labeling experiments of <SED> and <SEH>, both antibody types monitor decreased D/P and F/P ratios compared to the other three types. The first antibody experiments on GPTS arrays are confirming these results additionally due to lower intensity values. Therefore, increased toxin layer concentrations of 100-200 (SED) and 100-250 (SEH) were incubated. Furthermore, increased antibody concentrations from 1-30 (<SED>) and 1-20 (<SEH>) were applied (Figure 36 and Table 11). Finally, concentration pairs of 200 mg/L SED/25 mg/L Cy3-<SED> and 250 mg/L SHE/20 mg/L Cy3-<SEH> were chosen. Unfortunately, within this concentration ranges the fluorescence intensity values of SEA-SEC could not be reached at PMT43. Furthermore, labeling efficiency could not be improved and the toxin and antibody concentrations could not be increased any more due to the extremely high prices.

Nitrocellulose Slides

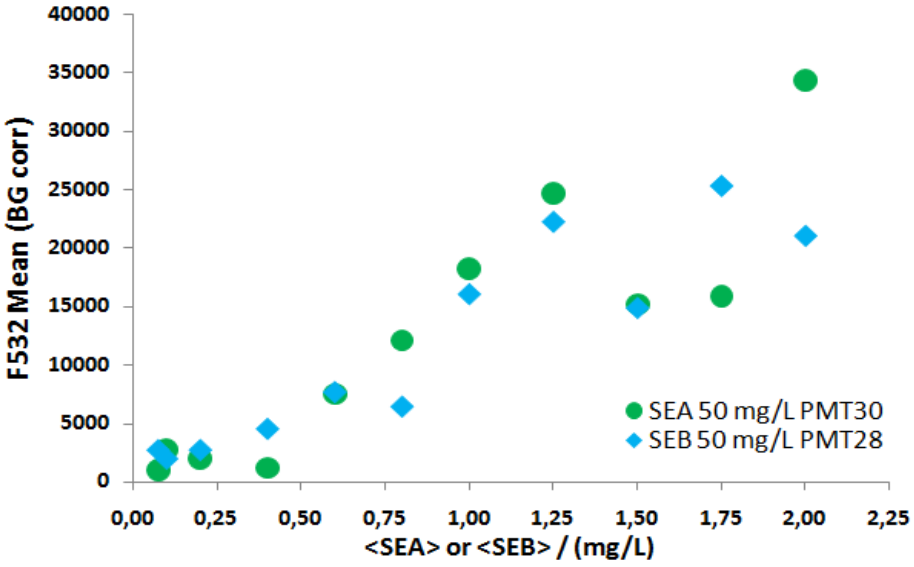


Figure 37. Cy3-antibody layer test for SEA and SEB on two 16-pad nitrocellulose slides

Following the results of the previous chapter, a competitive layer concentration of 50 mg/L was chosen for both toxin types. As a detection layer, Cy3-labeled antibodies for SEA and

SEB from 0.1-2 mg/L (Figure 37 and Table 12). Every antibody-toxin pair was tested on a separate slide. Cy3-<SEA> and Cy3-<SEB> provide proper fluorescence intensity values at an optimal PMT voltage of 30 and 28. Herein, a concentration of 1.25 mg/L for A and B seemed to be adequate to form a layer where the fluorescence intensity of the Cy3-labeled antibody layer is near the saturation region.

Table 12. Standard deviation and Signal-to-Background data for Figure 37

ß(<SEA>) /(mg/L)	**0.075**	**0.1**	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**	**1.25**	**1.5**	**1.75**	**2**
Standard deviation (F532)	867	592	420	536	2964	942	2910	2236	1164	6076	1519
Signal-to-Background ratio	1.06	1.23	1.12	1.05	1.29	1.59	1.87	2.18	1.68	1.56	2.17
ß(<SEB>) /(mg/L)	**0.075**	**0.1**	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**	**1.25**	**1.5**	**1.75**	**2**
Standard deviation (F532)	174	188	242	331	2.07	333	2132	2750	2635	4340	462
Signal-to-Background ratio	1.21	1.15	1.21	1.34	1.58	1.49	1.21	2.68	2.12	2.91	2.59

5.1.3. Competitive Assay Development on Nitrocellulose Slides and Hydrophobic Patterned Slides

Competitive tests on all microarray types are made to determine the region, where the fluorescence intensity decreases linearly with increasing concentration. This is called the “Linear Range” or the “Linear Concentration Range”. Within this linear region, the toxin content of samples with unknown toxin concentration can be determined via linear regression. This principle works similarly for all competitive measurements on SPR chips, where the Linear Range has to be defined, too.

Competitive Toxin Standards in PBS buffer: the Comparison

Nitrocellulose Slides

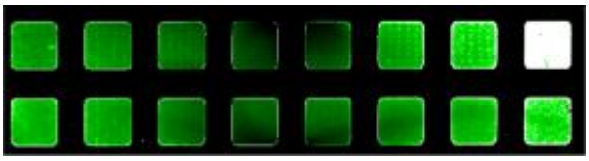


Figure 38. Scan of a competitive 16-pad Fast Slide at PMT40

On this 16-pad nitrocellulose array picture, the small 16-pin constructed toxin layer spots are visible (Figure 38). Often, the value of 0 mg/L toxin is not measurable due to the high fluorescence intensity compared to the other pads.

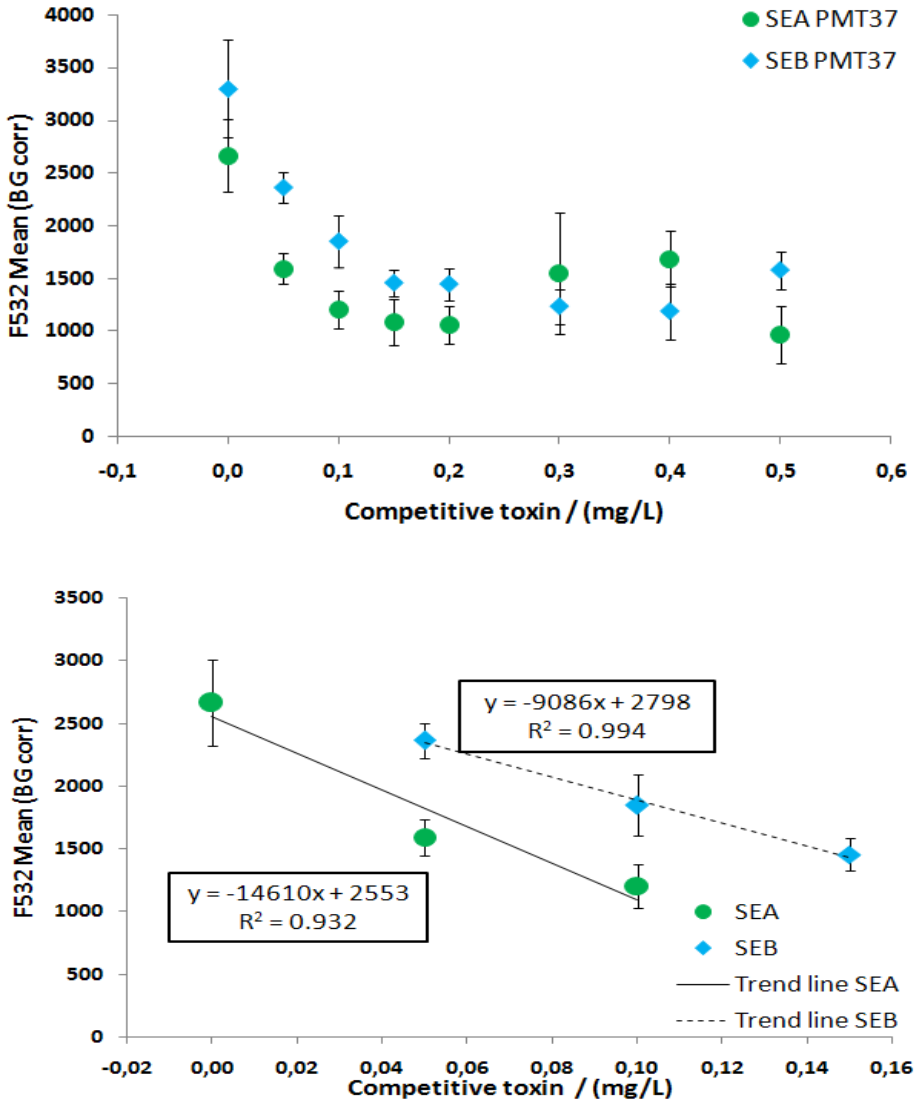


Figure 39. Competitive toxin assay for SEA and SEB on one 16-pad nitrocellulose slide

A concentration of 50 mg/L competitive SEA or SEB was applied on 16-pad nitrocellulose slides followed by an antibody layer of 1.25 mg/L. Toxin sample concentrations from 0-0.5 mg/L SEA or SEB in PBS buffer were used. Both arrangements monitor a decrease for competitive toxin between 0 and 0.2 mg/L, which corresponds to a region of 0-200 ng/mL (Figure 39). This region is defined generously for real sample trials. For SEA, a linear region from 0-0.1 mg/L (0-100 ng/mL) can be defined with R^2=0.932. For SEB, this region is monitored between 0.05 and 0.15 mg/L (50-150 ng/mL) with R^2=0.994. Competitive SEA and SEB experiments show excellent accordance of fluorescence intensities at 562 nm emission wavelength. Unfortunately, the signal-to-noise ratios are low again and the linear range is too far away from the BIOTRACER requirements of 0.1-10 ng toxin/mL sample. Because of this, all further experiments are applied on GPTS slides only.

Hydrophobic Patterned Epoxy Slides

A concentration of 50 mg/L competitive SEA or SEB was applied on GPTS slides followed by an antibody layer of 5 mg/L. These are the standard concentrations for all further SEA and SEB-based GPTS arrays.

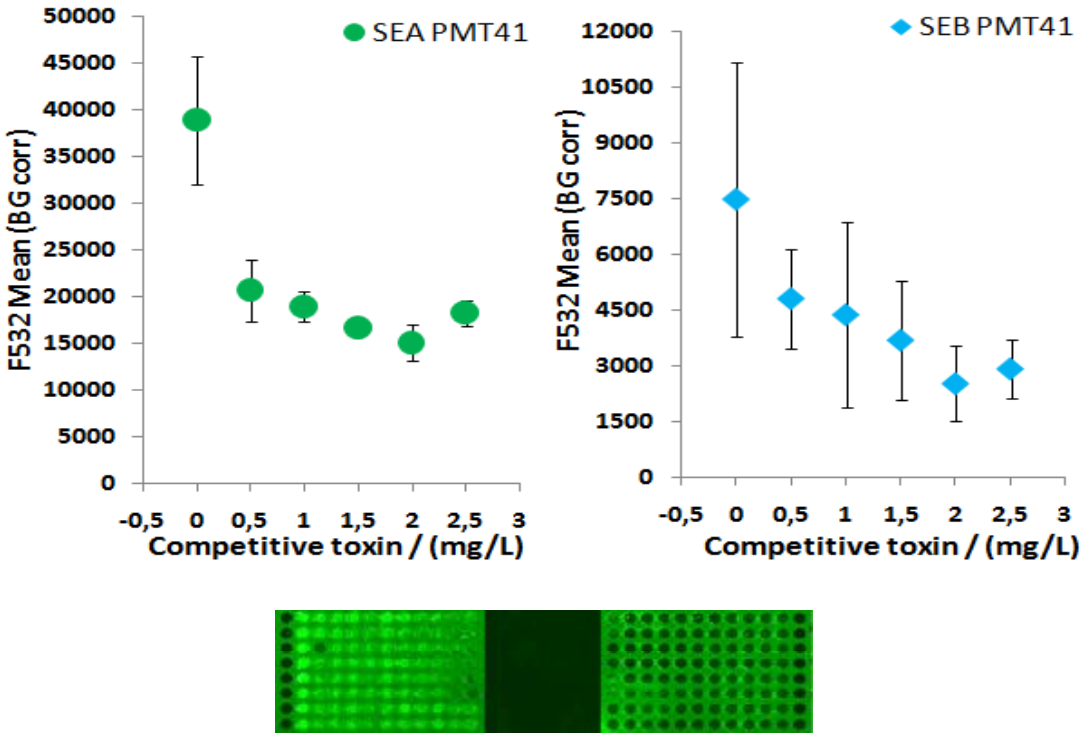


Figure 40. Graph and scanning picture of the first competitive trial with SEA and SEB on hydrophobic patterned epoxy slides

As a first trial, SEA and SEB samples in PBS buffer were applied in the range of 0-5 mg/L to get an overview of the general fluorescence intensity trend of the epoxy array system (Figure 40). Application of 5 mg/L toxin is the upper limit, due to a “labeled antibody”- layer of 5 mg/L. Fortunately, the fluorescence intensity is in a relatively high range at medium PMT voltage of 41. This is a very desirably appearance and accomplishes excellent starting conditions for the competitive array development. Both curves, for SEA and SEB, are decreasing until 2 mg/L. This indicates an accurate reaction process of antibody and toxin. Within the region of 0-2 mg/L competitive SEA or SEB, the slope is decreasing by trend. This enables the standardization as calibration region and has to be proven by further experiments.

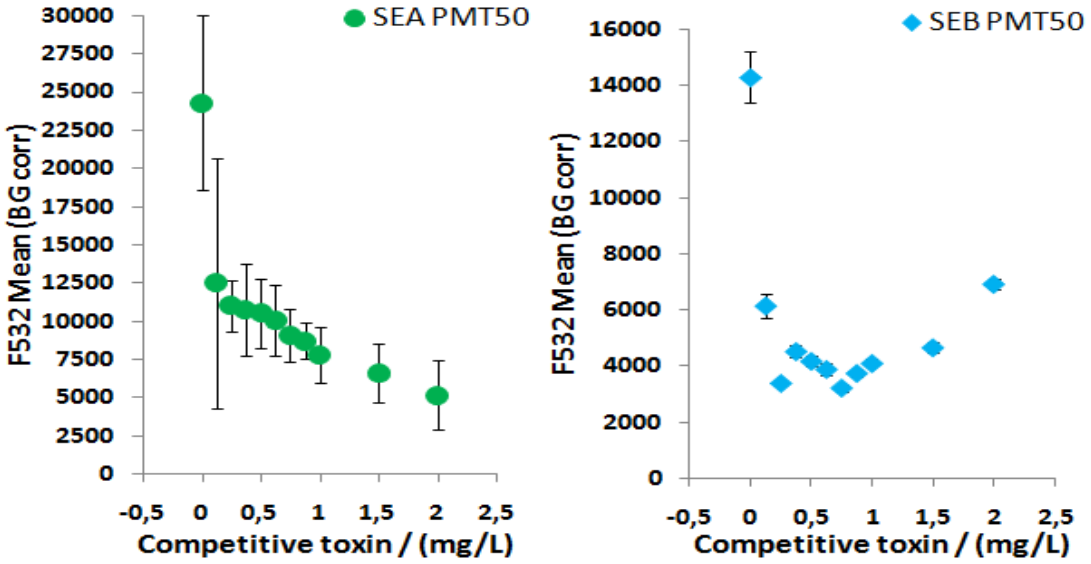


Figure 41. Results of the new concentration range for competitive SEA and SEB (0-2 mg/L)

As already determined on the former experiment, the concentration range of 0-2 mg/L of competitive toxin decreases the fluorescence intensity at medium PMT voltages (Figure 41) and provides good possibilities for being used as calibration region. Due to the high risk of being intoxicated at low toxin concentrations of 0.1 µg per ingested sample, the range has to be further decreased. A new range of 0-0.25 mg/L was chosen for SEA and SEB.

A concentration of 100 mg/L competitive SEC and 14 mg/L Cy3-<SEC> was used for GPTS slides. SEC samples in PBS were applied in the range of 0-14 mg/L to get an overview of the general fluorescence intensity trend of the epoxy system (Figure 42). Application of 14 mg/L toxin is the upper limit, due to a labeled antibody input of 14 mg/L. Unfortunately, the

fluorescence intensity is only in a mid range at medium PMT voltage of 41. The curve is decreasing over the progression of 0-8 mg/L and ends in a saturation region. Within the region of 0-1 mg/L competitive SEC, the slope is decreasing linearly by trend. This potential dynamic calibration region has to be proven by further experiment.

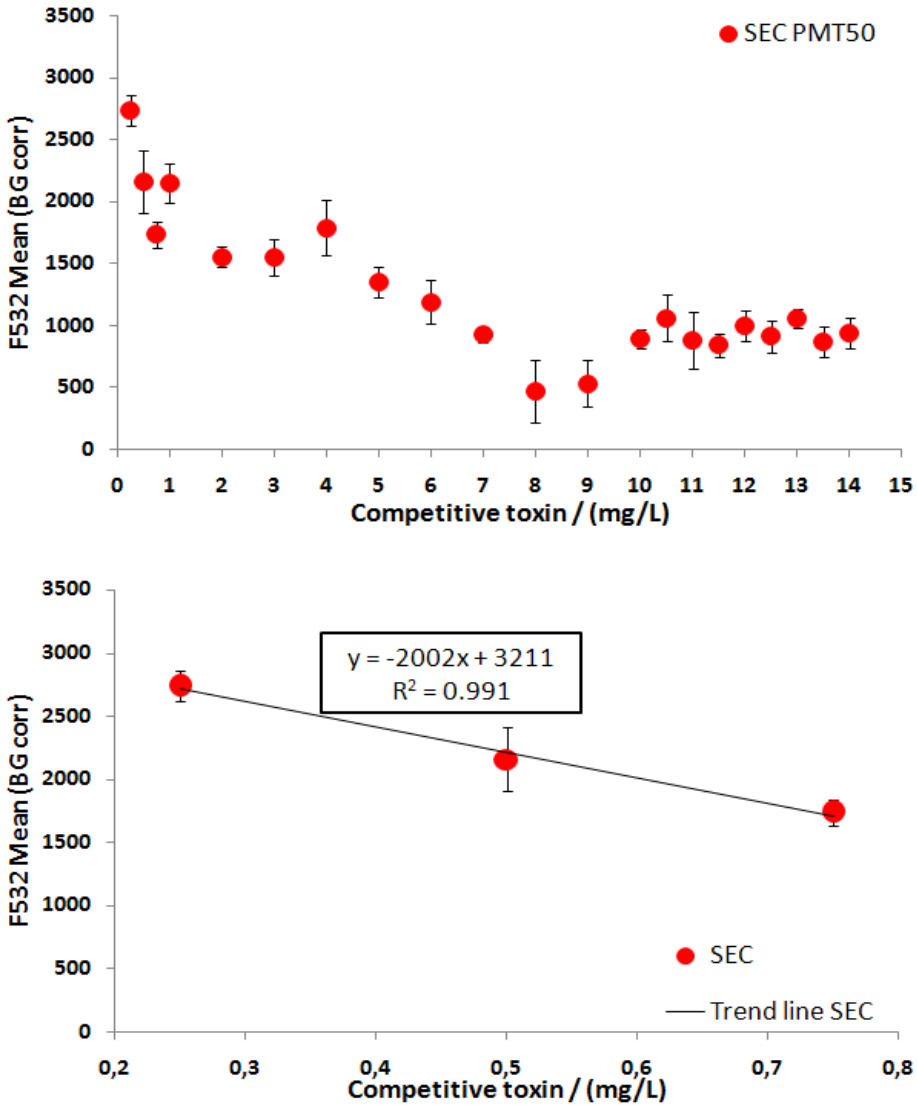
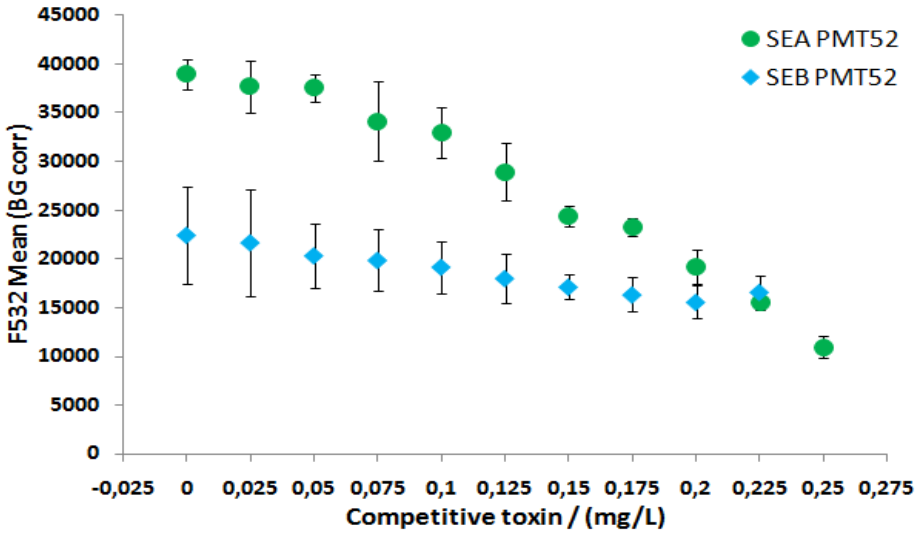
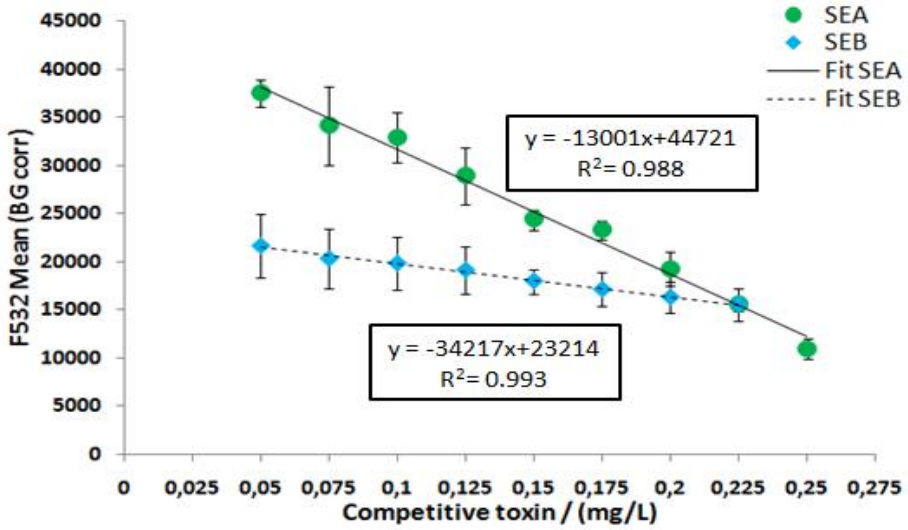
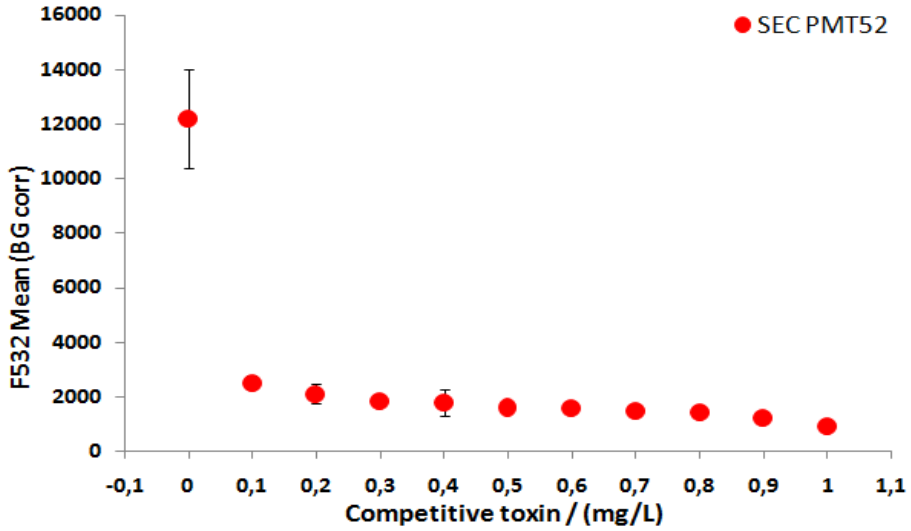


Figure 42. Graph of the first competitive trial with SEC on a hydrophobic patterned slide

Finally, the toxins SED and SEH are added to the competitive system and all five toxin types are tested at the same PMT voltage to generate on overview of calibration ranges and fluorescence intensity ranges of the different types of toxins (Figure 43). A concentration of 200 mg/L competitive SED and 25 mg/L Cy3-<SED> was used for GPTS slides. 250 mg/L and 20 mg/L were used for toxin type SEH.


SEA PMT52
SEB PMT52
F532 Mean (BG corr)
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
-0,025 0 0,025 0,05 0,075 0,1 0,125 0,15 0,175 0,2 0,225 0,25 0,275
Competitive toxin / (mg/L)


SEA
SEB
Fit SEA
Fit SEB
y = -13001x+44721
R2= 0.988
y = -34217x+23214
R2= 0.993
F532 Mean (BG corr)
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
0 0,025 0,05 0,075 0,1 0,125 0,15 0,175 0,2 0,225 0,25 0,275
Competitive toxin / (mg/L)


SEC PMT52
F532 Mean (BG corr)
16000
14000
12000
10000
8000
6000
4000
2000
0
-0,1 0 0,1 0,2 0,3 0,4 0,5 0,6 0,7 0,8 0,9 1 1,1
Competitive toxin / (mg/L)

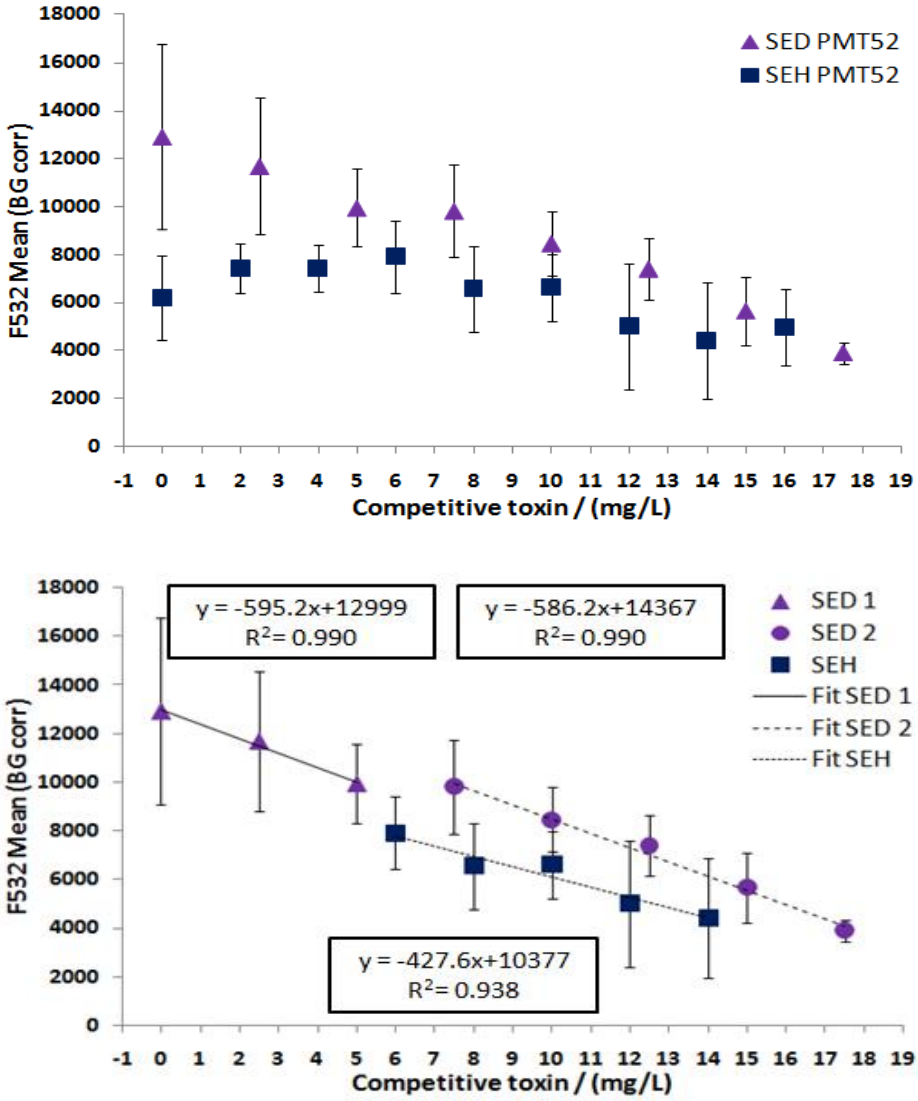


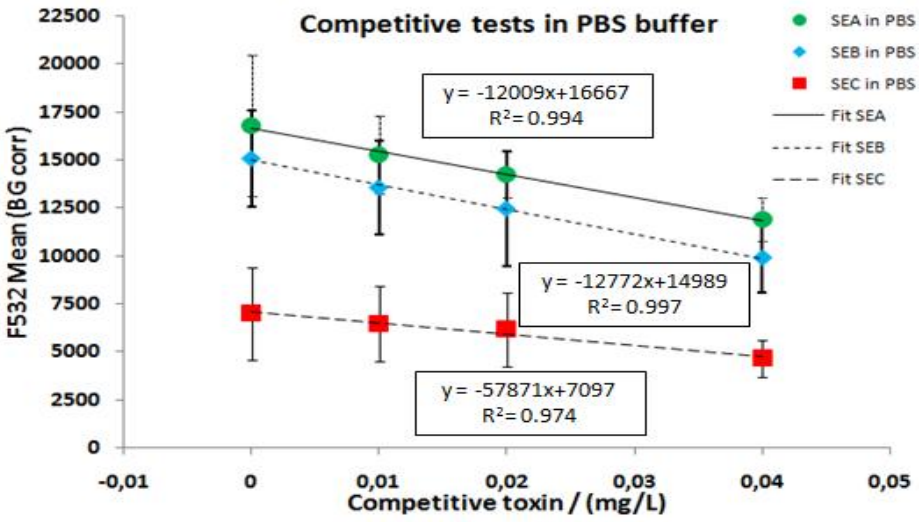
Figure 43. Comparison of all five toxin types on three slides with one PMT voltage (PMT52)

All five toxin types were tested at a PMT voltage of 52. This corresponds to the need of a voltage in the mid-region of the bandwidth. For SEA and SEB, the competitive range of 0-0.25 mg/L toxin was tested. The SEA curve is decreasing linearly for concentrations beginning at 50 ng/mL (0.05 mg/L), up to 250 ng/mL (0.25 mg/L), with R^2=0.988. For SEB, the curve is linearly decreasing from 50-225 ng/mL with R^2=0.993. The slope of the SEA curve is decreasing significantly whereas the SEB curve decreases slightly. It is not possible to verify the calibration regions of interest from 0.1-10 ng/mL with this array type. In general, a linear region comprises minimum one decade of concentration. In addition, a minimum signal decrease of 10% has to be reached within the defined range. Only the second criteria can be

fulfilled for the SEA and SEB curves. Finally, this array type can be used for highly contaminated food samples, but do not fulfill the BIOTRACER criteria.

For SEC, a range of 0.1-1 mg/L seems to be linear with slight decrease, but the interesting region of 0-0.1 mg/L is also not defined clearly. Fortunately, the curve of the region 0.1-1 mg/L is corresponding to the linear range demands. The range of 0-0.1 mg/L could not be monitored in all experiments of this array type due to extremely differences in the characteristics of the curve. Linearity is expected within this range, but could not be monitored precisely. Unfortunately, the fluorescence intensity is in the lower region at PMT52. SED and SEH were applied in the range of 0-17.5 mg/L (SED) and 0-16 mg/L (SEH) to get an overview of the characteristics of the GPTS system. Unfortunately, the fluorescence intensity for SEH is only in a mid range at medium PMT voltage of 52 whereas the intensity for SED is optimal. The SED curve is decreasing over the progression of 0-17.5 mg/L, the SEH curve starts to decrease at 6 mg/L. Within the region of 0-17.5 mg/L competitive SED, two potential calibration regions can be monitored. The first region reaches from 0-5 mg/L, with a R^2 of 0.99. The second is monitored from 7-16 mg/L with a R^2 of 0.99. Only the first range is attractive for measurements within the BIOTRACER requirements. The concrete characteristics were shown in further experiments with PBS and milk. For SEH, a region with potential linear decrease was setted from 6-14 mg/L with a R^2 of 0.938. This low R^2 value indicates the uncertainty of this region and points the need of a lower range for calibration.

The competition: milk and PBS standards on Patterned Epoxy Slides



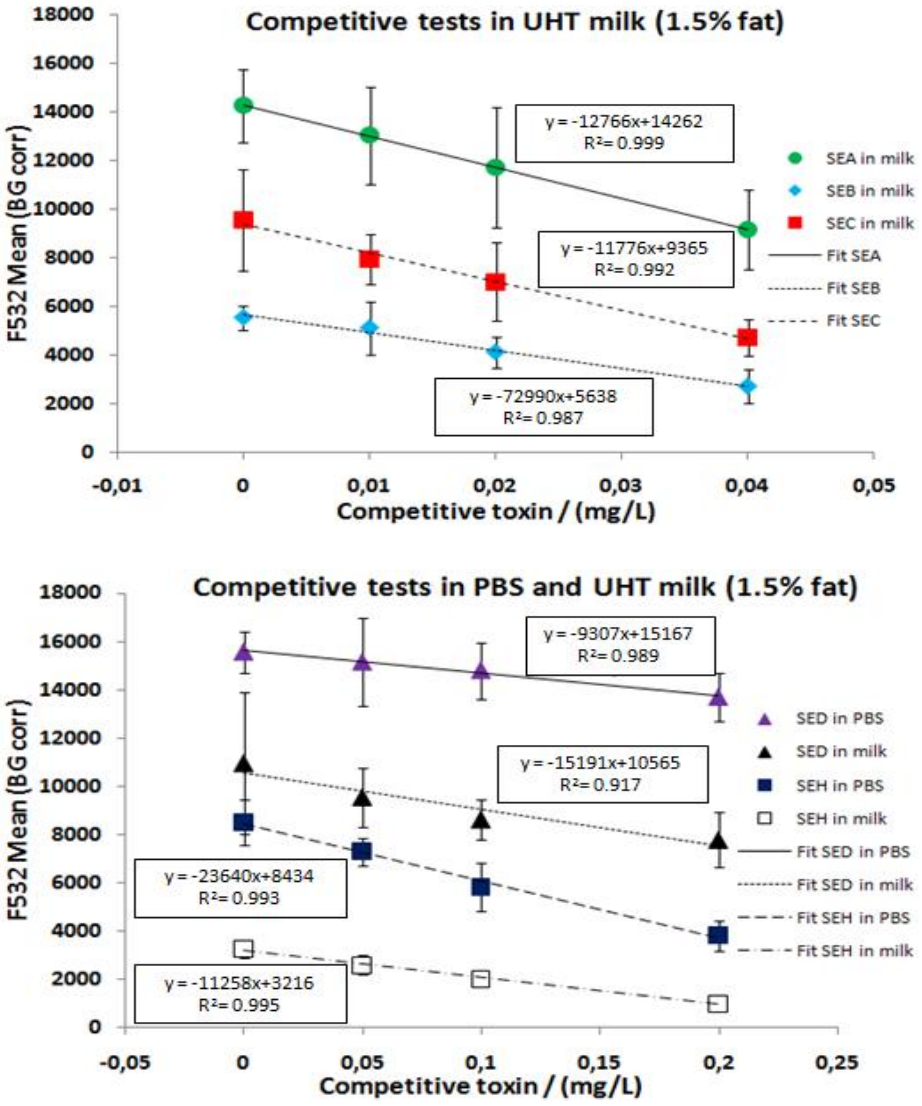


Figure 44. Competitive experiments for SEA-SED/SEH in PBS buffer and UHT milk 1.5% fat

The toxin concentration ranges of 0-0.04 mg/L (SEA-SEC) and 0-0.2 mg/L (SED and SEH) are mixed in PBS buffer and undiluted UHT milk (1.5% fat content) for a competition test (Figure 44). The milk charges can be considered as artificially contaminated milk samples. The toxins are pre-incubated with the UHT milk to guarantee their natural adaption into the milk matrix. Antibody additions are made similarly for all solvent types, PBS buffer and milk. In general, the fluorescence intensities for toxins bound to labeled antibodies in milk are lower than those for PBS buffer. All fluorescence intensities can be detected at a PMT voltage in the mid-range and belong to the same dimension. For SEC, the low R^2 values in milk and PBS indicate the beginning of the linear range between 20-40 ng/mL. For SEA and SEB, the high R^2 values near 1 suggest linearity over the whole range, but the characteristics of the milk

experiments are monitoring other tendencies. Like for SEC, the beginning of the linear range seems to be between 20-40 ng/mL or at concentrations above 40 ng/mL. For SED and SEH, the linear ranges are starting between 100-200 ng/mL toxin content or above 200ng/mL.This is rather indicated by the low R^2 for SED in PBS and milk. This estimation method cannot be applied for SEH, as R^2 is too near to 1.

The criteria of detecting 0.1-10 ng/mL sample cannot be fulfilled at this stage of development. Furthermore, the criterium of covering minimum one concentration decade is not proceeded. Undiluted milk samples induce no detection problems concerning background fluorescence or smearing of the array surface. Pipetting of 1 µL milk spots on the array is not as easy as with PBS, due to the lipophilic character of the sample. Detection of toxins in large sample volumes requires large amounts of labeled antibody and was not applicable within this project budget. Another problem of the primary system is the existence of five types of labeled antibodies with five different dye-to-protein ratios and therefore different basic fluorescence intensities of the labeled solutions. Furthermore, discrepancies in fluorescence intensities on the array are expected due to the disparing association constants (K_A) of the antibodies and their differing D/P ratio. This requires fluorescence normalization for all antibody types on the array and makes array analysis very complex. Generally, fluorescence intensities of the five toxin arrays differ too much as well as the standard deviations are too high (around 15-30 %). Positively, the signal-to-noise ratios are high and the background intensities are low.

5.2. Alternative System on Patterned Slides: Secondary Antibodies as Detection Elements

Within this chapter, the development of an alternative array system is described. Toxin layer and primary antibody layer concentrations are kept constant. Only a new layer with a secondary, Cy3-labeled antibody is added to avoid the problems caused by five separate labeled <SEX> with differing D/P and F/P. All primary antibodies are used unlabeled from now on.

5.2.1. The Detection Unit: Labeled Secondary Antibody Layer Tests

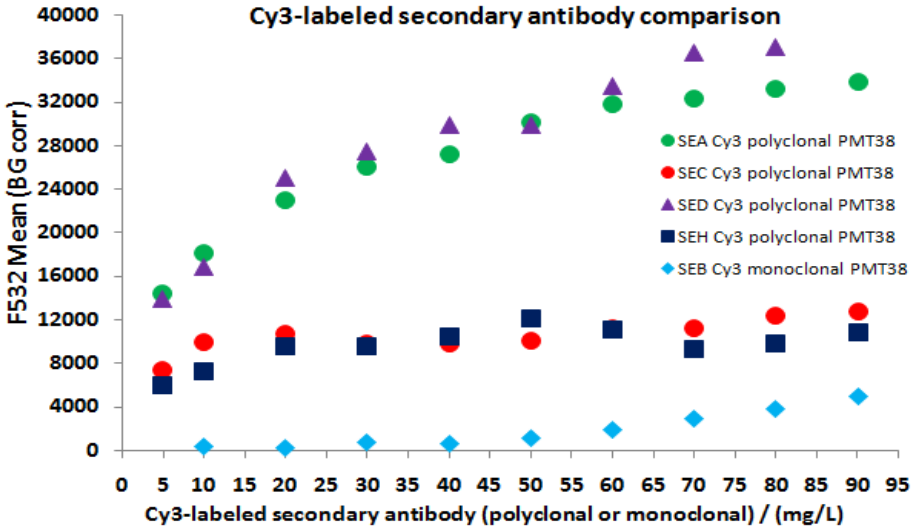
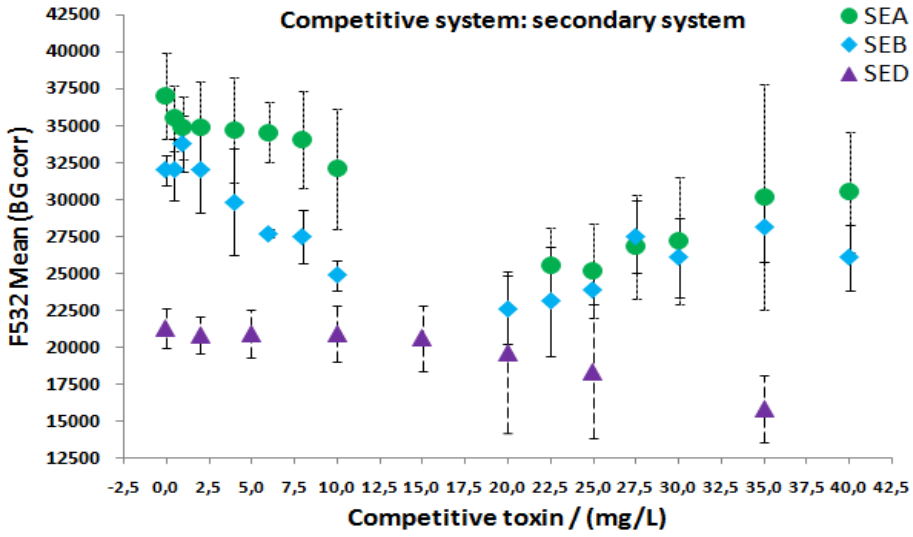


Figure 45. Concentration tests for secondary Cy3-labeled goat-<SEX>

The need of fluorescence normalization due to five different efficient antibodies was eliminated via secondary, Cy3-labeled polyclonal antibody (Figure 45 and Table 13). The basic concentration pairs (toxin SEX and <SEX>) are kept constant for all five toxins types. For a comparison, the SEB pair was alternatively detected with monoclonal primary <SEB> and secondary Cy3-antibody. All polyclonal variants exhibit adequate fluorescence intensities at a moderate PMT voltage. Except the monoclonal variant offers low fluorescence and requires high concentrations of secondary antibody for acceptable intensity. 50 mg/L polyclonal secondary antibody is considered to be sufficient to obtain moderate intensity values at PMT38 for this new array type.

5.2.2. Competitive Assay Development: Linear Detection Ranges for Enterotoxins

As next step, the new secondary system was tested within a competitive assay (Figure 46). 0-35 ng/mL toxin samples in PBS were prepared for SEA, SEB and SED. 50 mg/L secondary antibody was applied as detection element with moderate PMT voltage. This resulted in excellent fluorescence intensities and close intensity ranges for all three tested toxin types. Linear regions can be defined as 7.5-22.5 ng/mL for SEA, 2.5-10 ng/mL for SEB and 15-35 ng/mL for SED. Low R^2 values for SEA and SEB indicate the high uncertainties, only the SEH curve possesses a R^2 near 1. Except the SEB curve was within the requirement of detecting 0.1-10 ng/mL toxin in the sample. The criterium of covering minimum one concentration decade is not achieved for any array.



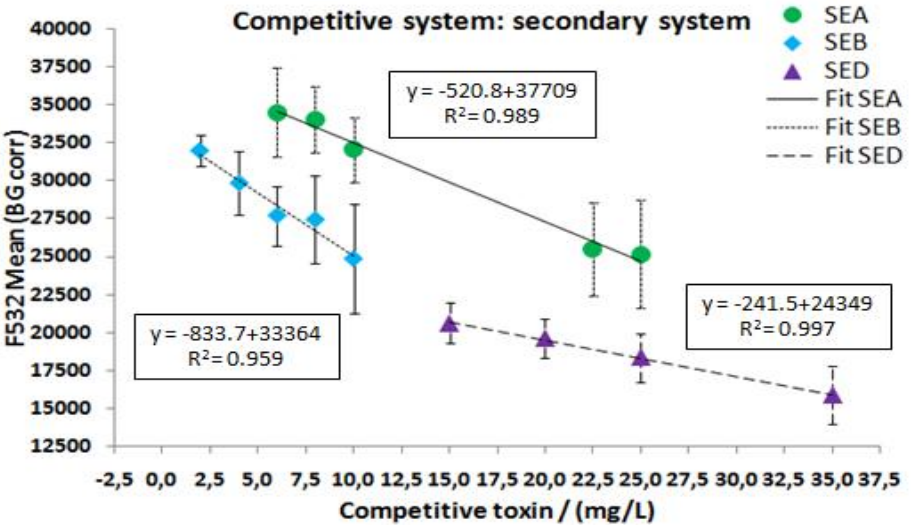


Figure 46.

Competitive assay for the secondary system (50 mg/L Cy3-labeled secondary antibody)

A defined array improvement in fluorescence intensity and linear regions is monitored by the use of polyclonal secondary Cy3-labeled antibody. Unfortunately, the systems for individual toxins differ again, so a complete new array system is constructed with the use of secondary Cy3-labeled antibody.

5.2.3. Advanced Secondary Systems

Antigen Layer Tests on Epoxy Slides

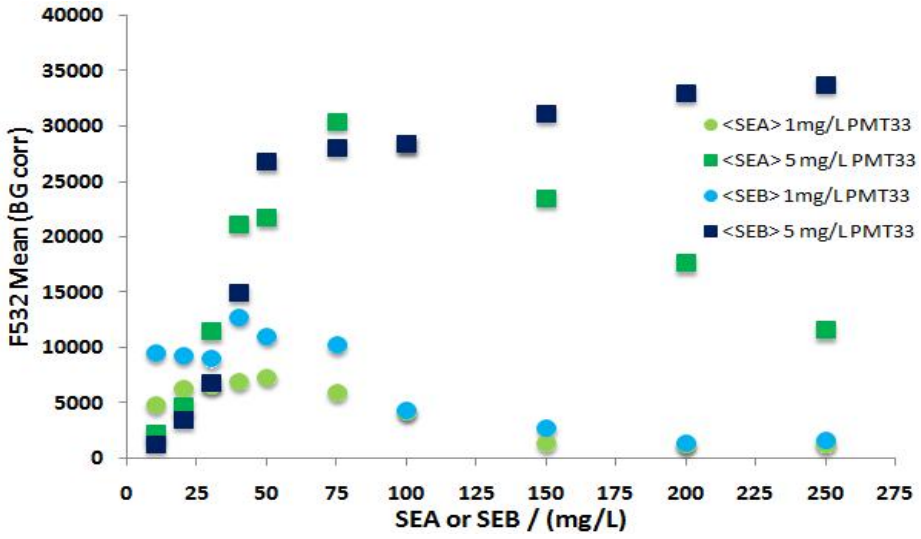


Figure 47. Antigen layer test with the new secondary system on epoxy slides

First <SEX> (A-C) antibody concentrations of 1 and 5 mg/L were applied to build an overview test system of the GPTS surface (Figure 47 and Table 13). The slides were coated with a concentration of 10-250 mg/L toxin. This new system is based on polyclonal primary unlabeled antibodies and a secondary Cy3-labeled antibody (5mg/L) and includes a completely new development of all layers. Herein, a concentration of 50-75 mg/L seems to be adequate for SEA and SEB to form a layer where fluorescence intensity is near the saturation region at a moderate PMT value of 33. The signal-to-noise ratio is optimal on GPTS slides, the background is very low at 562 nm emission wavelength. By trend, a primary antibody concentration of 5 mg/L is more adviced than 1 mg/L with regard to fluorescence intensities.

Table 13. Signal-to-Background data for Figure 47

ß(SEA)/(mg/L) (<SEA> 1 mg/L)	10	20	30	40	50	75	100	150	200	250
Signal-to-Background ratio	92.7	121.1	124.7	132.7	140.2	113.7	79.9	27.2	24.7	24.1
ß(SEA)/(mg/L) (<SEA> 5 mg/L)	**10**	**20**	**30**	**40**	**50**	**75**	**100**	**150**	**200**	**250**
Signal-to-Background ratio	29.2	62.1	150.6	277	258.3	398.5	371.9	308.1	230.8	151.9
ß(SEB)/(mg/L) (<SEB> 1mg/L)	**10**	**20**	**30**	**40**	**50**	**75**	**100**	**150**	**200**	**250**
Signal-to-Background ratio	328.6	321.2	308.6	440.5	380.5	352.9	150.7	94.7	46.4	55.6
ß(SEB)/(mg/L) (<SEB> 5 mg/L)	**10**	**20**	**30**	**40**	**50**	**75**	**100**	**150**	**200**	**250**
Signal-to-Background ratio	28.7	76.6	148.3	326.7	583.6	611.3	618.9	678.8	719.2	735.7

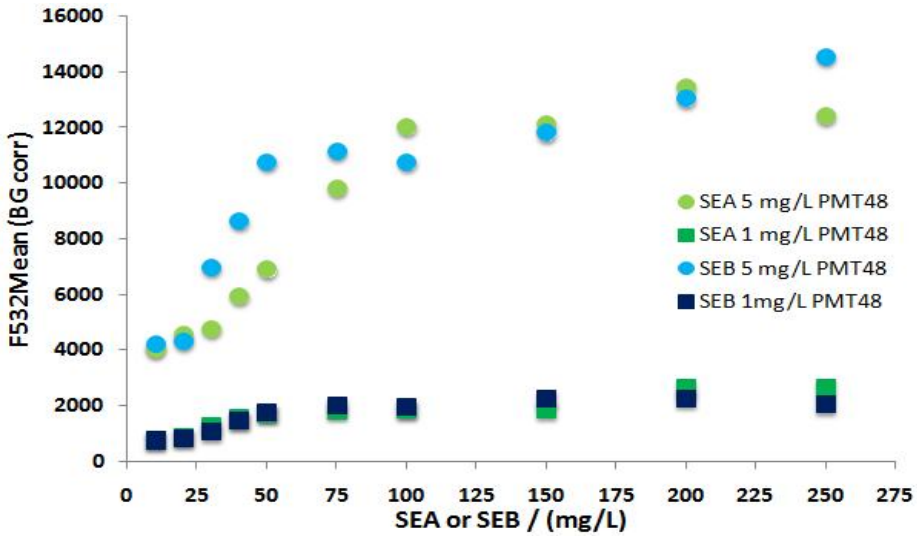


Figure 48. Antigen layer test with a comparable primary system on epoxy slides

Table 14. Signal-to-Background data for Figure 48

ß(SEA)/(mg/L) (<SEA> 1 mg/L)	10	20	30	40	50	75	100	150	200	250
Signal-to-Background ratio	1.97	2.24	3.19	3.84	4.30	4.56	4.62	4.65	6.54	6.64
ß(SEA)/(mg/L) (<SEA> 5 mg/L)	**10**	**20**	**30**	**40**	**50**	**75**	**100**	**150**	**200**	**250**
Signal-to-Background ratio	5.02	5.64	5.94	7.40	8.58	12.22	14.95	15.04	16.74	15.43
ß(SEB)/(mg/L) (<SEB> 1mg/L)	**10**	**20**	**30**	**40**	**50**	**75**	**100**	**150**	**200**	**250**
Signal-to-Background ratio	5.31	5.87	7.5	10.27	12.35	14.02	13.71	15.81	15.97	14.47
ß(SEB)/(mg/L) (<SEB> 5 mg/L)	**10**	**20**	**30**	**40**	**50**	**75**	**100**	**150**	**200**	**250**
Signal-to-Background ratio	43.63	45.03	72.71	90.10	112.2	116.2	112.1	123	135.9	151.7

Cy3-<SEA> and Cy3-<SEB> concentrations of 1 and 5 mg/L were applied to build an alternative primary overview test system of the GPTS surface (Figure 48 and Table 14). This comparison system is based on polyclonal primary Cy3-labeled antibodies and includes a completely new development of all layers. Herein, a concentration of 75 mg/L seems to be adequate for SEA and SEB to form a layer where fluorescence intensity is near the saturation region. For SEC, 100 mg/L are suggested. The signal-to-noise ratio is optimal on GPTS slides and the background is very low at 562 nm emission wavelength. Again, a primary antibody concentration of 5 mg/L is more adviced than 1 mg/L with regard to fluorescence intensities.

Primary Antibody Layer Tests

The ambitious goal is to find appropriate antibody concentrations for <SEA>-<SEC>. As a third layer, Cy3-labeled secondary antibody was used with a concentration of 5 mg/L.

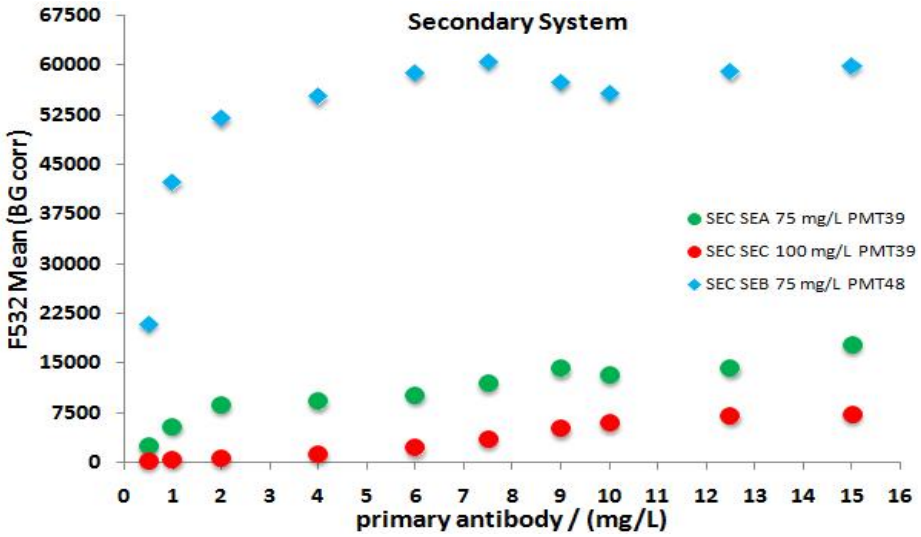


Figure 49. Primary antibody layer tests for <SEA>-<SEC> (Cy3-sec. Ab: 5 mg/L) on epoxy slides with the secondary system (SEC)

Table 15. Signal-to-Background data for Figure 49

ß(<SEA>)/(mg/L)	**0.5**	**1**	**2**	**4**	**6**	**7.5**	**9**	**10**	**12.5**	**15**
Signal-to-Background ratio	29.6	65.1	103.5	109.4	121	142.1	168.1	157.3	169.4	212
ß(<SEB>)/(mg/L)	**0.5**	**1**	**2**	**4**	**6**	**7.5**	**9**	**10**	**12.5**	**15**
Signal-to-Background ratio	66.8	128.7	212.5	283.1	283.9	287.2	289.1	256.4	239.2	244.5
ß(<SEC>)/(mg/L)	**0.5**	**1**	**2**	**4**	**6**	**7.5**	**9**	**10**	**12.5**	**15**
Signal-to-Background ratio	4.2	7	8.9	18.1	30.8	49.8	72.2	83.7	99	102.3

Within the new secondary antibody system, 2 mg/L <SEA>-<SEC> provide proper fluorescence intensity and signal-to-background values at a PMT value of 39 for the given conditions (Figure 49 and Table 15).

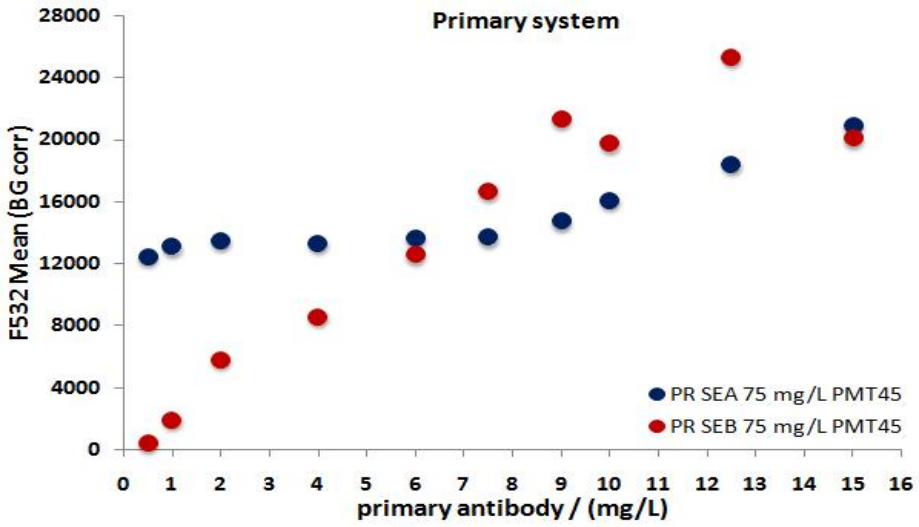


Figure 50. Primary antibody layer tests for Cy3-<SEA> and Cy3-<SEB> on epoxy slides

Table 16. Signal-to-Background data for Figure 50

ß(<SEA>)/(mg/L)	**0.5**	**1**	**2**	**4**	**6**	**7.5**	**9**	**10**	**12.5**	**15**
Signal-to-Background ratio	1.16	1.22	1.25	1.24	1.27	1.27	1.37	1.50	1.71	1.95
ß(<SEB>)/(mg/L)	**0.5**	**1**	**2**	**4**	**6**	**7.5**	**9**	**10**	**12.5**	**15**
Signal-to-Background ratio	1.97	8.02	21.91	34.42	49.77	67.01	81.48	76.57	93.53	76.95

4 mg/L Cy3-<SEA> and Cy3-<SEB> provide proper fluorescence intensity values at an optimal PMT voltage of 45 (Figure 50 and Table 16). Furthermore, the concentration is below the satisfaction region and therefore with the optimal range. Compared to the new secondary system, the fluorescence intensity is lower and an increased antibody concentration is needed. This is unprofitable with regard to linear ranges for which the antibody concentration should be as low as possible to generate an acceptable competitive range for toxins. This means the complete range of antibody concentration in its toxin-bound variant, starting from 0 mg/L to the used concentration, e.g. 4 mg/L, should create the linear region.

Competitive Array Test for SEA

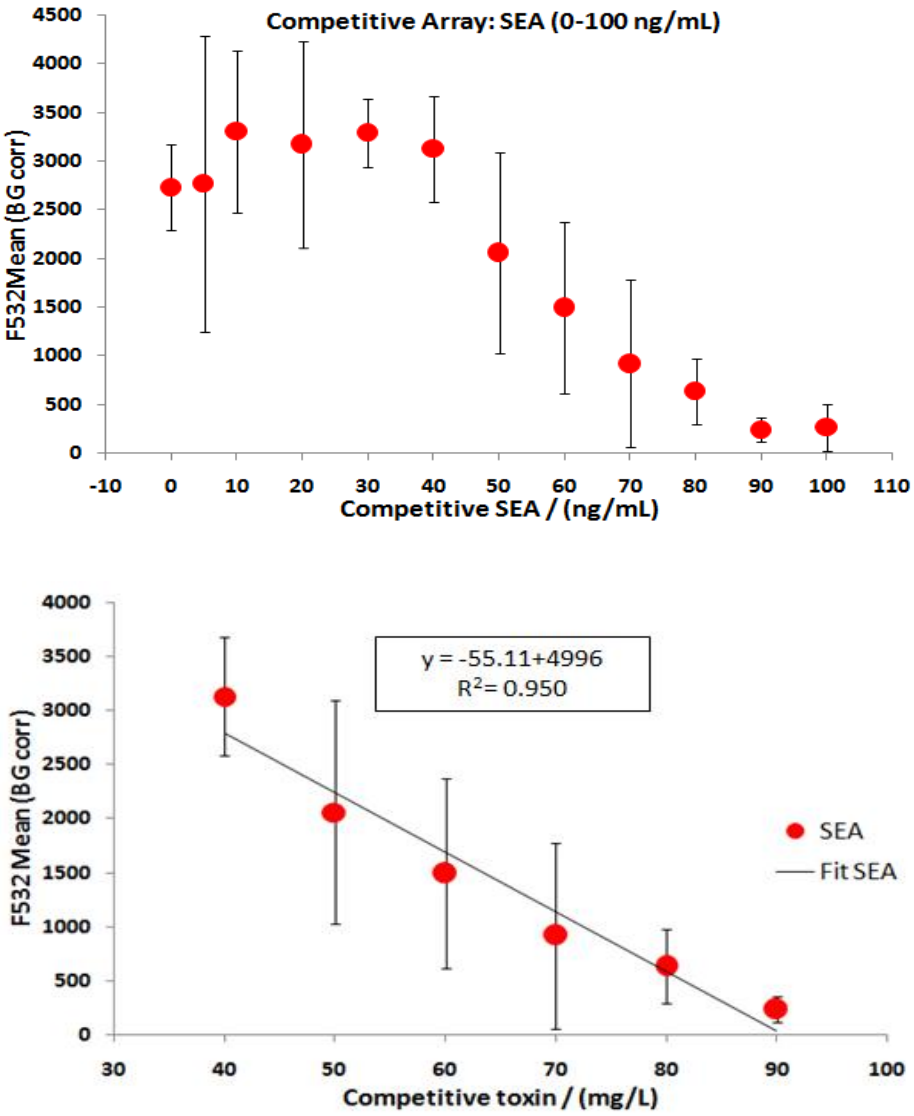


Figure 51. Results of the competitive array test with the new secondary system for SEA

Finally, the new secondary system was tested within a competitive assay (Figure 51). 5 mg/L secondary antibody was applied as detection element and a moderate PMT voltage was applied. This resulted in moderate fluorescence intensities. The fluorescence intensities have large standard deviations and therefore they are not acceptable. Furthermore, a linear region can be monitored from 40-90ng/mL with a low R^2 of 0.95. For this new system, the criteria of detecting 0.1-10 ng/mL sample cannot be fulfilled at this stage of development either. Concentrations below 40 ng/mL cannot be calculated by linear regression.

Furthermore, the criterium of covering minimum one concentration decade is not proceeded.

Table 17. Overview of LODs and linear ranges for all tested competitive systems

System	Toxin type	LOD (estimated)	Linear range / (ng/mL)
Primary (PBS)	SEA	5	10-40
	SEB	5	10-40
	SEC	8	10-40
	SED	25	50-200
	SEH	40	50-200
Primary (Milk)	SEA	5	10-40
	SEB	7	10-40
	SEC	5	10-40
	SED	25	50-200
	SEH	40	50-200
Secondary (1^{st} configuration)	SEA	5	5-25
	SEB	1	1.25-10
	SED	14.5	15-35
Secondary (Advanced)	SEA	36	40-90

An overview of LODs and linear ranges of all tested systems is presented in Table 17. With Biotracer requirements of detecting 0.1-10 ng toxin/mL sample, only the 1^{st} secondary configuration matches the needs. All other systems are not sensitive enough and provide a list of changellenges that are still to solve.

5.3. Cross-reaction Tests

5.3.1. Primary Detection System

Polyclonal Primary Antibody Systems: Cross-Reaction Array with Fluorescence Normalization

SEA, SEB, SEC, SED, SEH and their primary fluorescence-labeled antibodies were spotted on one array for direct competition. Every toxin and antibody type was combined. Detection occurs via Cy3-label on the primary antibody. Fluorescence normalization is necessary due to the application of 5 Cy3-labeled primary antibodies with differing D/, F/P and fluorescence intensity within the same concentration. Normalization is done via separate normalization array and equations.

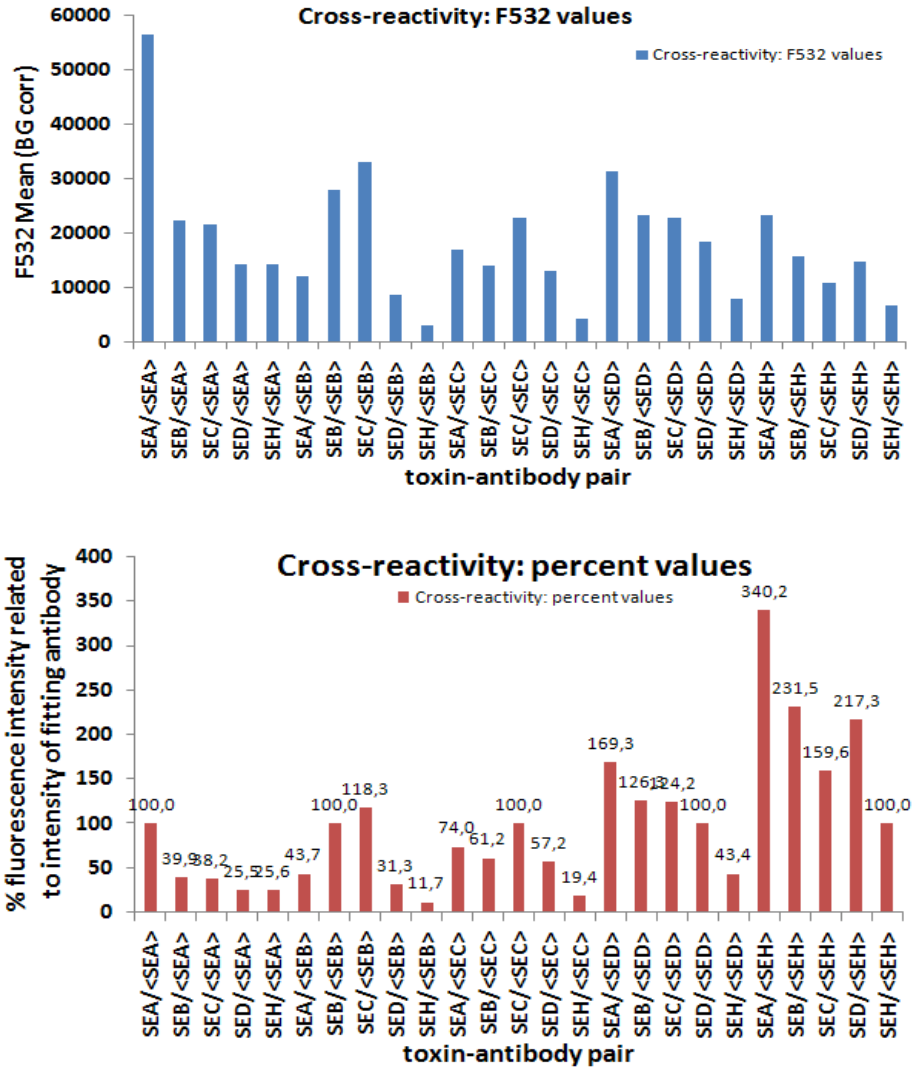


Figure 52. Results of the cross-reaction test of the primary system

The cross-reaction assay was carried out with the following parameters: SEA-SEH: each 50 mg/L, <SEA>-<SEH>: each 25 mg/L with a blocking time of 0.75 h. All 25 possible combinations of antibody and toxin on the surface were applied. The results are presented in Figure 52. The matching combination pair is calculated to 100% percent, all others are

adjusted. Only <SEA> is highly specific for SEA, which is monitored by low percentage values of the other combinations. <SEB> reacts nearly similar with SEA and SEB. <SEC>-<SEH> are highly cross-reacting with other types. As presented in 5.3.2, high specifity cannot be monitored for all matching combinations. Therefore it can be assumed that problems caused by five labeling procedures and their clean-up yield in this high cross-reactivity structure. The primary system shows less specifity of different types and provides non-optimal conditions for array construction.

5.3.2. Secondary Detection System

Monoclonal Antibodies in Secondary Systems: Cross-Reaction Performance

SEA, SEB, SEC, SED, SEH and monoclonal <SEB> were spotted on one array for direct competition. Detection occurs via Cy3-labeled secondary goat-<mouse>. The assay was carried out with the following parameters: SEA-SEH: 50/50/100/250/200 mg/L, <SEB>: each 5 mg/L and secondary Cy3-antibody: 100 mg/L with a blocking time of 1h. The results are presented in Figure 53.

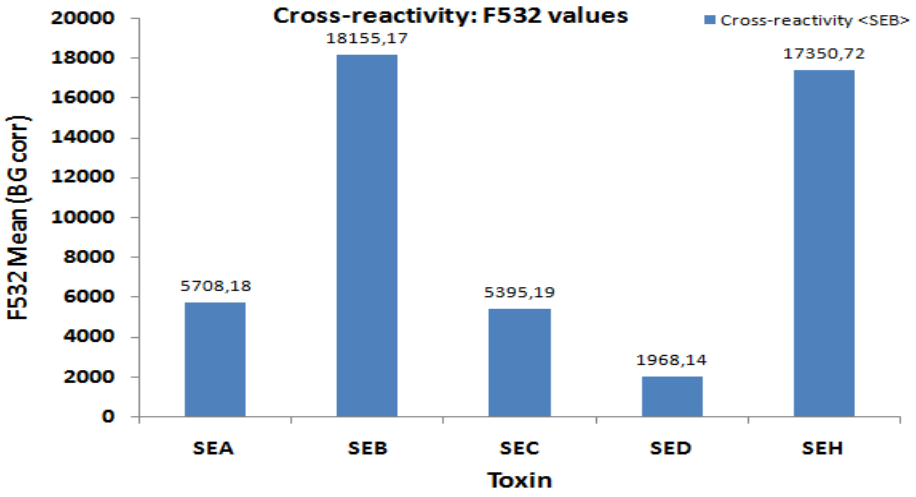


Figure 53. Results of the cross-reaction test of the monoclonal system

This monoclonal system shows specifity for SEB, but also for SEH. This system does not show specifity for SEB in samples that contain SEB and SEH and therefore provides non-optimal conditions for array construction.

Advanced Secondary System

SEA, SEB, SEC and their primary antibodies were spotted in all combinations on one array for direct competition. Detection occurs via Cy3-labeled secondary goat-<rabbit>. The assay was carried out using the following parameters: SEA-SEC: each 75 mg/L, <SEA>-<SEC>: each 4 mg/L and secondary Cy3-antibody: 5 mg/L with a blocking time of 1h.

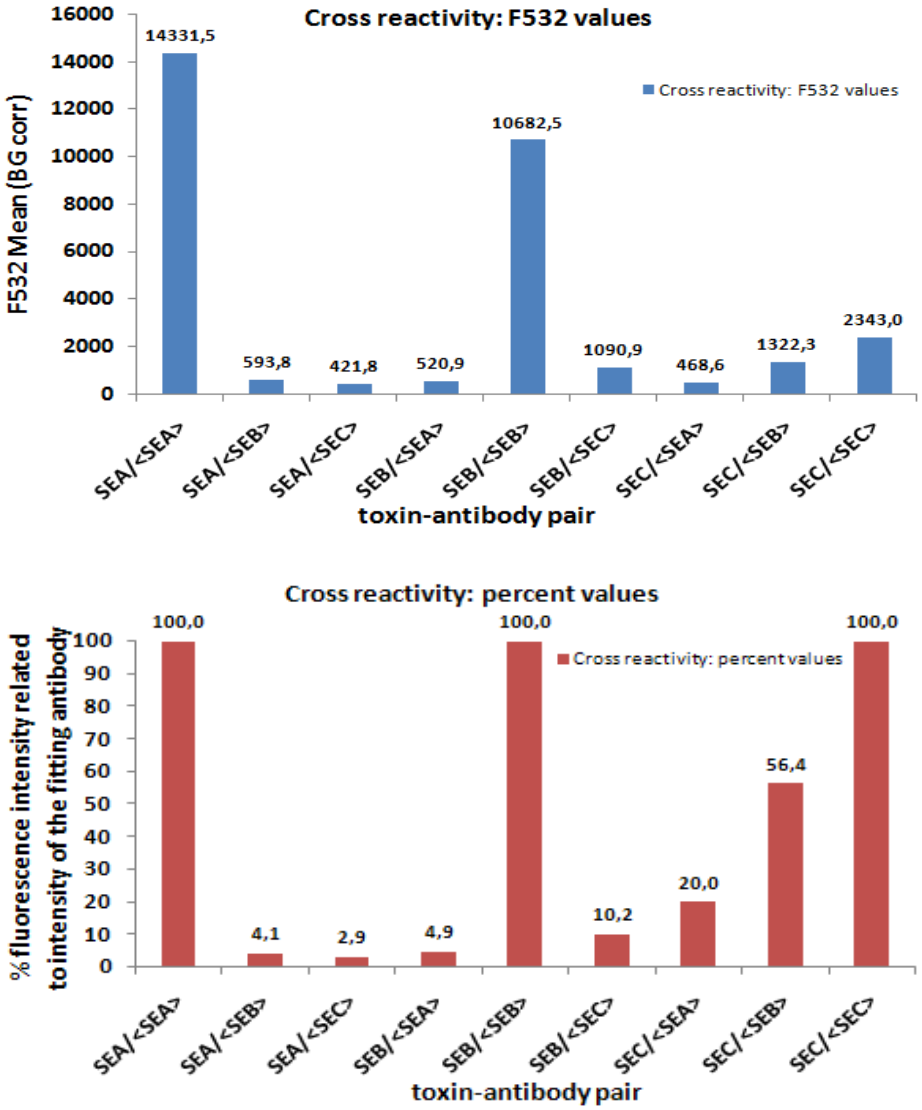


Figure 54. Results of the cross-reaction test of the new system

The results are presented in Figure 54. The matching combination pair is calculated to 100% percent, all others are adjusted. All antibodies are highly specific for their toxin, which is monitored by low percentage values of the other combinations. This new secondary type of assay with low cross-reaction reactivity of different types of toxins provides best conditions

for array construction. The low selectivity and high cross-reactivity of the primary system is not induced from the antibody and the toxin itself. Quenching problems of the Cy3 dye or strerical hindrance of antiboidy and toxin due to the dye presence within the primary antibody incubation step can be the reason for the results in the primary incubation test.

5.4. Buffer Tests

5.4.1. Blocking Buffer

After the spotting of enterotoxin(s) on the array surface, there are free binding sites left. To cover the free binding areas and to prevent unspecific binding, the slides are immersed in blocking solution. Two different blocking types are used, protein and protein-free blockers, whereas Nexterion Blocker and Pierce Blocking Buffer belong to the second category. Casein Blocker and bovine serum albumin (BSA)-containing solutions are protein buffers. Protein-free blocking buffers were also applied due to the risk of high background fluorescence when using protein blocking buffers. Different blocking buffer types were spotted on one array for direct competition. Nexterion Blocking Buffer, Pierce Blocking Buffer, 5% BSA in 1x PBS pH 7.4 and 5% BSA in Pierce Blocking Buffer were compared. The assay was carried out on an SEB-Array (SEB: 50 mg/L, <SEB>: 2 mg/L and secondary Cy3-antibody: 5 mg/L) with a blocking time of 1h.

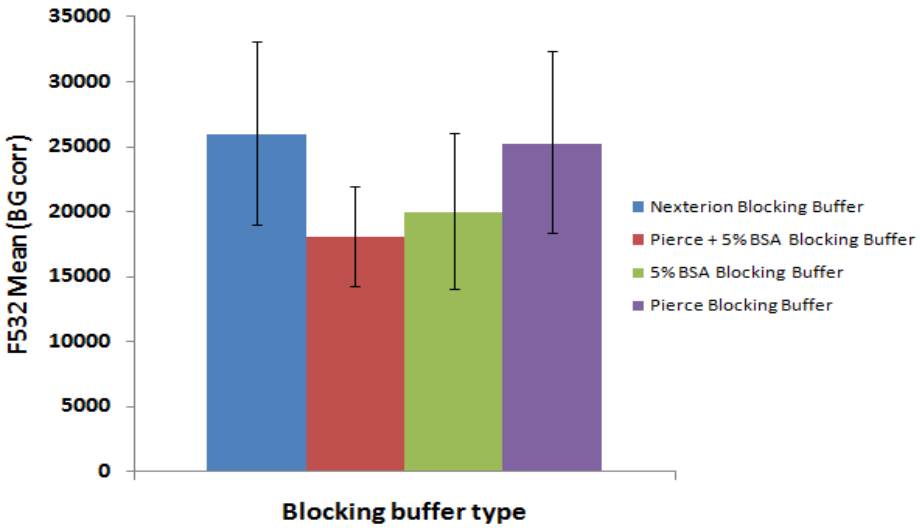


Figure 55. Results of the Blocking Buffer Test

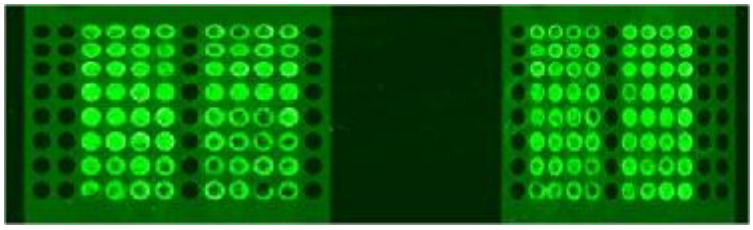


Figure 56: Effect of different blocking buffers on the fluorescence intensity of microarray spots: Pierce Buffer, 5% BSA in PBS, 5% BSA in Pierce Buffer and Nexterion Blocking Buffer (from left to right)

The results are presented in Figure 55 and 56. Pierce Buffer without BSA effects higher fluorescence intensity than its variant with BSA content and the classic BSA blocking buffer itself. This leads to the assumption that Pierce Buffer is blocking only fragmentary and unspecific binding of labeled antibodies is happening. Nexterion Buffer reacts in similar ways like classic BSA blocking buffer. BSA blocking buffer buffer and Nexterion Buffer are the best choices. Due to its relatively high price, Nexterion Buffer is the second choice and the homemade 5% BSA buffer is used now.

5.4.2. Spotting Buffer

Different types of spotting buffer were tested to prevent high background fluorescence and low immobilization rate of the toxins due to buffer evaporation effects (Figure 58). Furthermore, three spotting buffer types were prepared on one array for direct competition. 1 x PBS pH 7.4, 1x PBST pH 7.4 (0.5% Tween20) and Nexterion Spotting LE were compared. The assay was carried out on an SEB-Array (SEB: 50 mg/L, <SEB>: 2 mg/L and secondary Cy3-antibody: 5 mg/L) with a blocking time of 1h. The results are presented in Figure 57 and 58. Due to the relative low fluorescence intensities of Nexterion Spotting LE and 1 x PBST pH 7.4 buffer, 1 x PBS pH 7.4 buffer was chosen as spotting buffer. Furthermore, 1 x PBS pH 7.4 effects relatively uniform distribution of fluorescence intensity within the spot and good inter-spot equivalence.

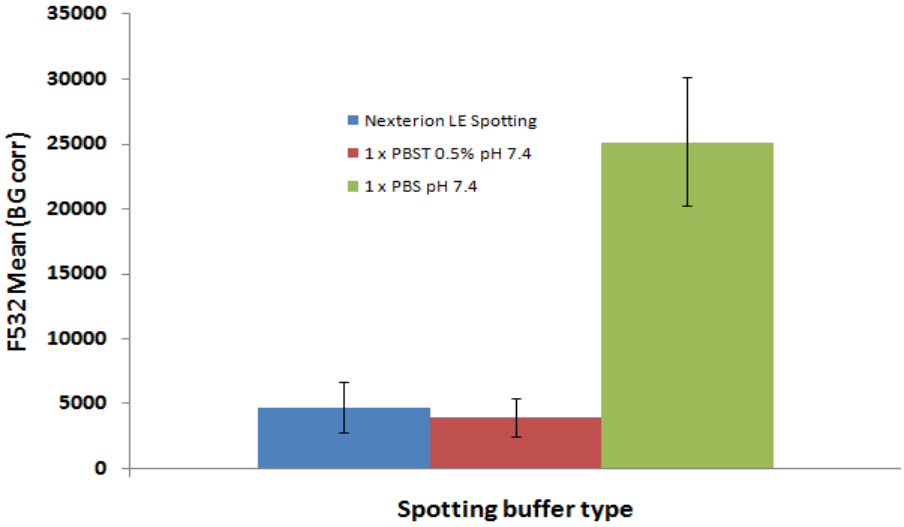


Figure 57. Results of the Spotting Buffer Test

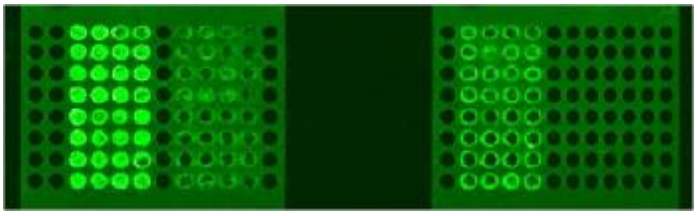


Figure 58. Effect of different spotting buffers on the fluorescence intensity of microarray spots: 1 x PBS pH 7.4, 1 x PBST pH 7.4 and Nexterion Spotting Buffer (from left to right)

5.5. Primary System: Raw Milk as Sample Application

In this chapter, raw milk samples from Switzerland are tested with the primary system due to the requirements of the BIOTRACER project.

Calibration Measurements for the Detection of Toxins in Raw Milk Samples

The calibration curves for SEA, SEB, SEC and SEA/SEB/SEC in PBS and uncontaminated raw milk from Switzerland are presented in Figure 59.

The results of tested raw milk samples are presented in Figure 60 and 61.

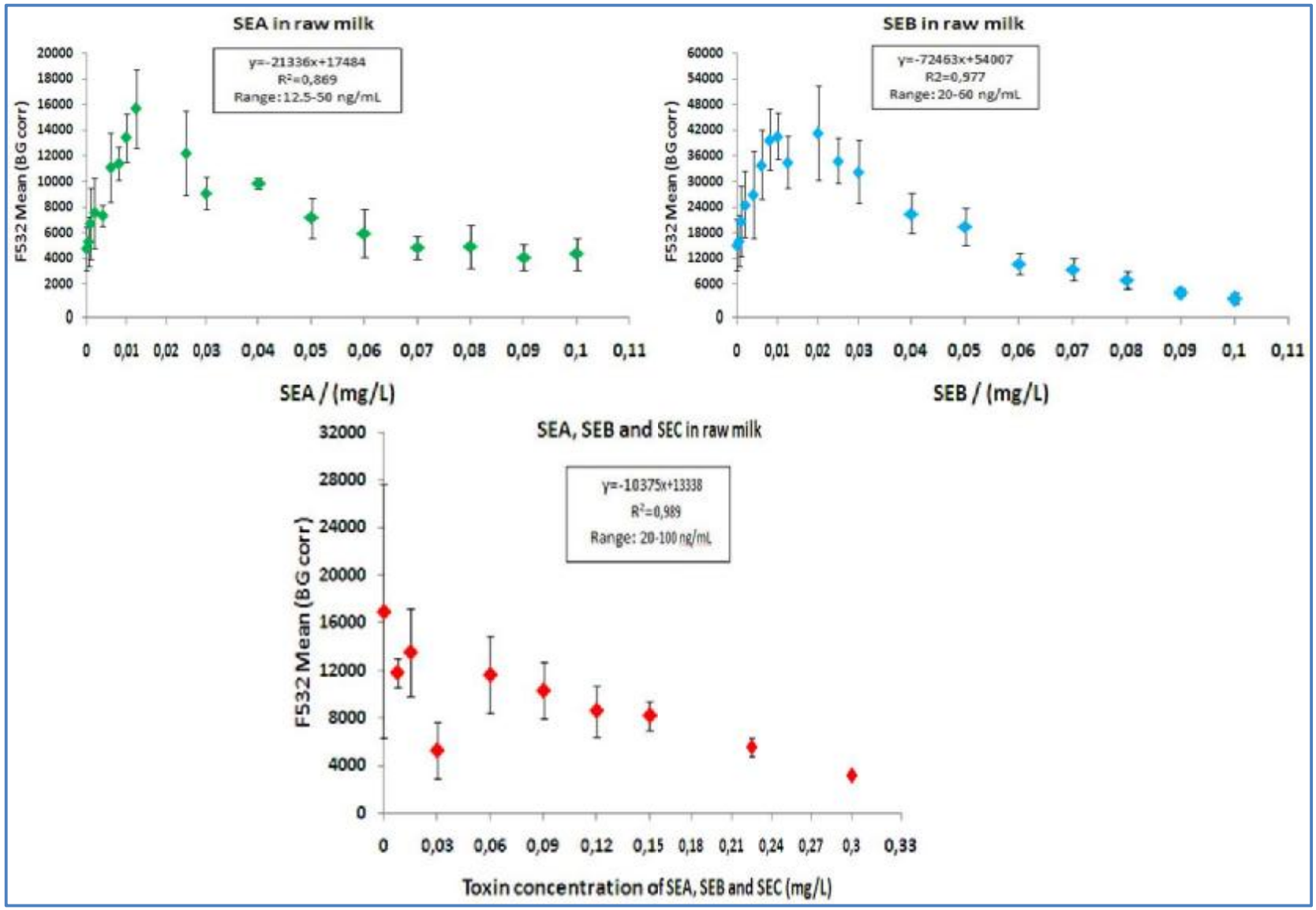

SEA in raw milk
y=-21336x+17484
R²=0,869
Range: 12.5-50 ng/mL
F532 Mean (BG corr)
SEA / (mg/L)
SEB in raw milk
y=-72463x+54007
R2=0,977
Range: 20-60 ng/mL
F532 Mean (BG corr)
SEB / (mg/L)
SEA, SEB and SEC in raw milk
y=-10375x+13338
R²=0,989
Range: 20-100 ng/mL
F532 Mean (BG corr)
Toxin concentration of SEA, SEB and SEC (mg/L)

Figure 59. Calibration curves for the detection of toxins in raw milk sample

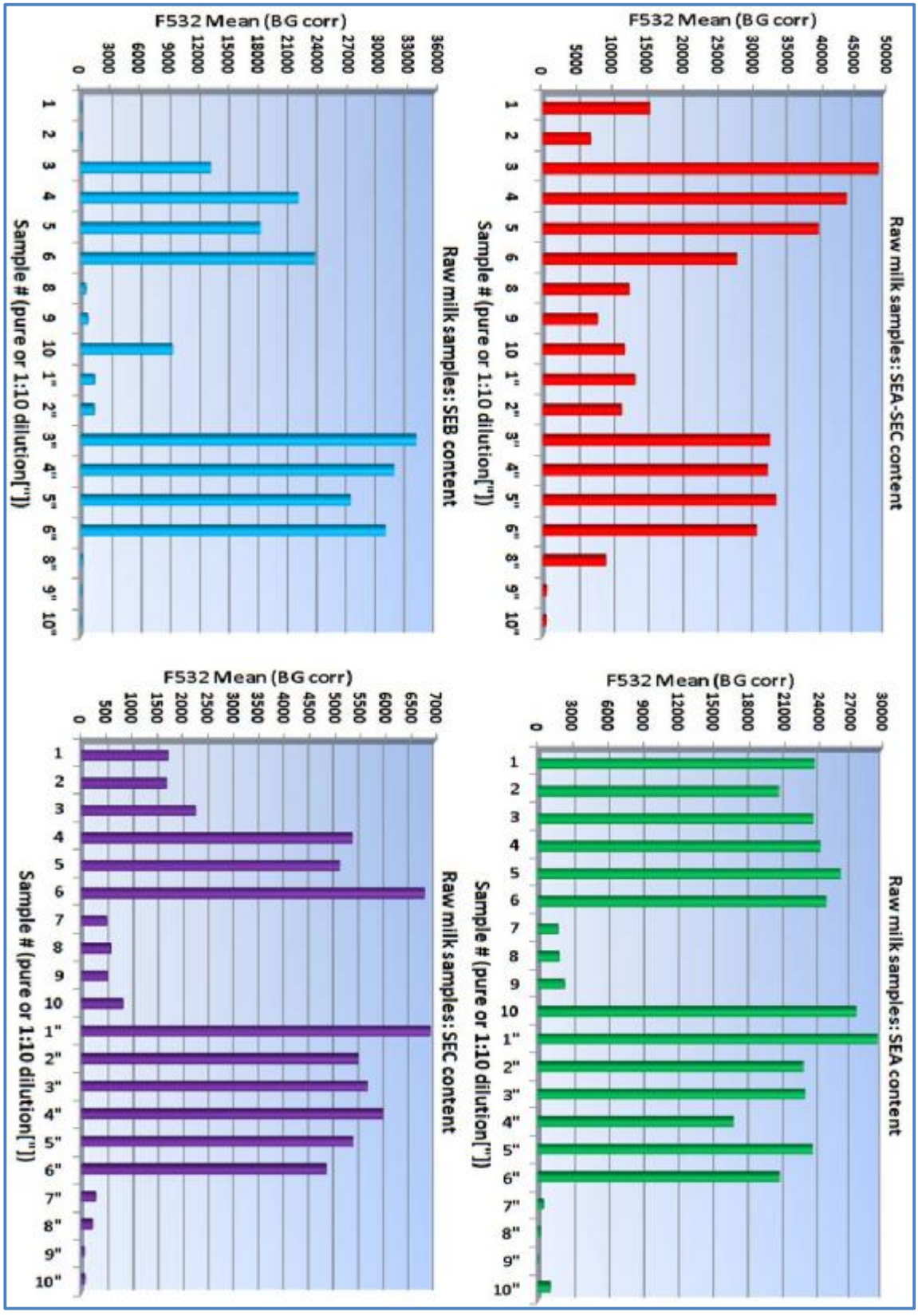


Figure 60.
Results for all tested raw milk samples and toxin types (SEA, SEB, SEC or SEA/SEB/SEC)

Sample name	ID	ABC	ABC (1:10)	A	A (1:10)	B	B (1:10)	C	C (1:10)
Tankmilk 12, natural raw	1	neg		neg		neg		neg	
Tankmilk 1 spiked B 4ng/ml centrif., V=1 ml	2	63.2 ng/ml		neg		neg		neg	
Tankmilk 4 centrif., V=15 ml	3	neg		neg		neg		neg	
Tankmilk 6 centrif., V=15 ml	4	neg		neg		pos		neg	
Tankmilk 9 centrif., V=15 ml	5	neg		neg		pos		neg	
Tankmilk 12 Centrif., V=15 ml	6	neg		neg		pos		neg	
Tankmilk 12 spiked B 4ng/ml, V=1 ml	8	9.8 ng/ml		73.5 ng/mL		pos		pos	
Tankmilk 1 spiked B 4ng/ml centrif., V=15 ml	9	53.8 ng/ml		71.7 ng/mL		pos		pos	
Tankmilk 12 spiked A 4ng/ml, centrif., V=1 ml	10	15.8 ng/ml		neg		pos		pos	

Figure 61. Calculated results of the raw milk array experiment

Detection of Toxins in Raw Milk Samples from Switzerland (5% raw fat content)

The calibration curve for the overall toxin concentration of SEA-SEC is calculated without any problems. All other calibration curves have parabola-like curves with 2 x-values per y-value. A well-defined correlation of concentration and sample values is not possible therefore. A possible explication might be the self-quenching of bound labeled primary antibody in the milk matrix. Concentrations below 15 ng/mL (SEA) and 10 ng/mL (SEB) monitor this limit. The only guideline for contamination that is given, are the microbial determinations of the samples which allow a systematically definition. Only Tankmilk 12 exhibits a low concentration of S. aureus Enterotoxin-expressing strains, all other samples do not contain toxin-expressing strains. So, only Milk 12 is able to build enterotoxins within the given terms.

The results of the raw milk experiment are presented in Figure 60 and 61. All values for the 1:10 dilutions have to be measured again with 1:10 milk in PBS standard curves, due to the different consistency of pure milk and the 1:10 mixture. As only Tankmilk 12 contains the correct strains for toxin production, only samples 1 and 6 could be contaminated naturally. All other samples which are prepared with Tankmilk 12 are contaminated artificially in excess. Tankmilk 1 is completely free of *Staphylococcus* strains and therefore used as blank and spiking milk. So, samples 2, 8 and 9 are artificially contaminated and contain SEA or SEB.

For the gross sum of SEA/SEB/SEC, all samples were analyzed correctly with regard to the status "contaminated/uncontaminated", only the value itself is often not correct. Sample 1 and 3-6 are analyzed correctly as negative due to the prediction of the microbial pre-examination. Sample 2 and 8-10 are analyzed correctly as contaminated samples. After precalculations due to spiking volumes and concentrations (SEA and SEB, samples 2 and 8-10), Sample 2 and 10 should contain around 5.33 ng/mL, due to the compression rate after centrifugation. Sample 8 should contain 4 ng/mL, because it is not treated after spiking. Sample 9 should possess a value of 46.2 ng/mL after centrifugation. The false results of sample 2, 8 and 10 might be caused by the linear range which is starting at higher concentrations. The concentration in sample 9 is 16% above the spiked concentration. This result is within a conventional frame of standard deviation. For SEA, 6 from 9 samples were analyzed correctly with regard to the status "contaminated/uncontaminated". Sample 1-6 are analyzed correctly as negative due to the prediction of the microbial pre-examination. Sample 10 was not recognized as an SEA-containing sample (false-negative) whereas samples 8 and 9 were analyzed misleadingly as such types (false-positive). Unfortunately, sample 10 could not be calculated. For SEB, 4 from 9 samples were analyzed correctly with regard to the status "contaminated/uncontaminated". Samples 1, 3, 8 and 9 were analyzed correctly. Unfortunately, sample 8 and 9 cannot be calculated. Sample 2 was not recognized as SEB-containing analysis. Samples 4-6 and 10 should not contain any SEB, they are recognized as false-positives! For SEC, 6 from 9 samples were analyzed correctly with regard to the status "contaminated /uncontaminated". Samples 1-6 are analyzed correctly, samples 8-10 are false-positives.

False-positive and false-negative results are induced by the cross-reaction of antibodies and toxins, due to the structural and genetic similarity.

The experiment could not be repeated a second time due to the time and sample limit during the stay at the Veterinary University of Vienna.

5.6. Comparison with the miniVIDAS system for Sample Applications

In this chapter, the miniVIDAS system is tested with SEA-spiked raw milk cheese samples and SET2. The method uses an enzyme-linked fluorescent assay with polyclonal anti-enterotoxin antibodies. The Vidas SET2 is a rapid and fully automated kit detecting, without differentiation, the SEA to SEE, using a cone coated with antibodies specific for SEA, SEB, SECs, SED and SEE. An immune complex is formed between (i) the coated antibodies, (ii) the toxins in the concentrated extract and (iii) the anti-SE antibodies conjugated with alkaline phosphatase. All reagents are included in the wells of the strip used. Briefly, 0.5 mL of the concentrated protein extract or 0.5 mL of the controls (positive or negative) are distributed in the strip and incubated in the automate miniVIDAS. Two fluorescence measurements (sample, blank) are performed for each test by the automate. The ratio (relative fluorescence value) between these two measurements is interpreted to declare or not a sample as positive. The miniVIDAS method has a sensitivity of at least 0.5 ng/g food. Staphylococcal enterotoxin detection from Raclette Cheese and Alpine Cheese was performed after an extraction step of the VIDAS SET2 instruction. The theoretically calculated results are shown in Table 18.

Table 18.
Calculated results after all extraction steps for Raclette Cheese(R) and Alpine Cheese (A)

Sample #	ß(SEA) in TRIS / (ng/mL)
R1	0,0
R2	0,0
R3	12,6
R4	14,0
R5	22,9
R6	25,2
R7	27,4
R8	14,8
R9	25,2
R10	37,3
A1	153,7
A2	165,8

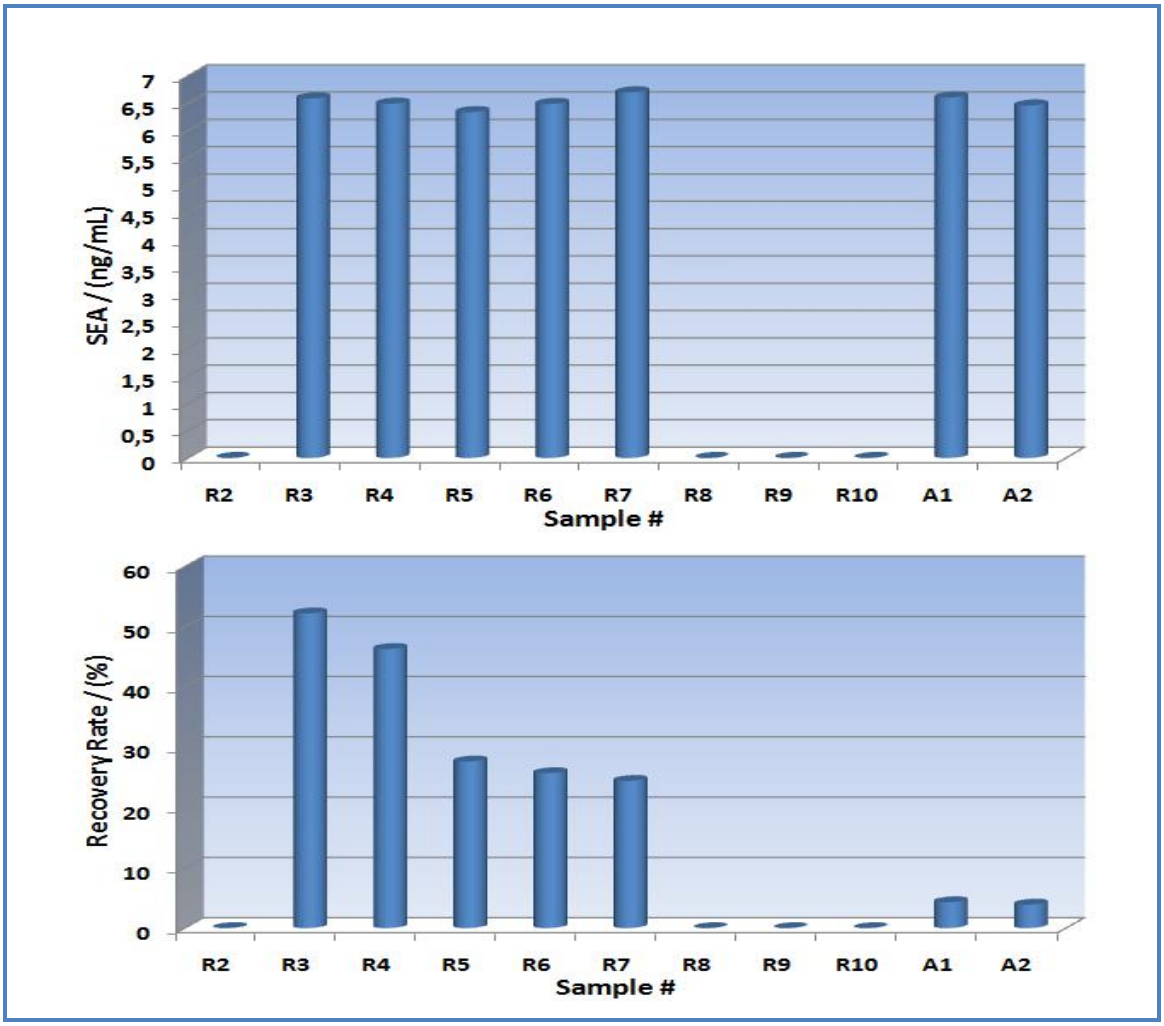


Figure 62. Results of the miniVIDAS test with cheese samples

The effective results are presented in Figure 62. Within this trial, all cheese samples, except R1 and 2 which are blanks, are spiked with 25 ng SEA/g cheese. This seems to be a very high contamination rate, but it is used here for testing the loss of toxin during the extraction. The ISO licensed method miniVIDAS with SET2 is used as determination method to guarantee real values with minimal standard deviation. SET2 is based on an ELISA KIT which measures the gross toxin content of SEA-SEE in the sample. Only sample R2 has reacted as calculated (no SEA spiking), all other samples are tested much lower than the SEA content should be. The fact can be monitored with the received recovery rates of SEA, which are much lower than expected. This allows the conclusion of toxin loss during the numerous extraction and clean-up steps when using cheese samples. Positively, it can be stated, that the toxin can be extracted generally. But unfortunately, a minimum of 48% is lost during the way. This

extraction method seems to be not applicable for low toxin contents due to the high loss of toxin. For improvement of the recovery rate, three possibilities are attractive: increased number of repetitions for every extraction step, decreased extraction volumes or application of an alternative up-concentration method. The use of Millipore Filter Units (10kDa) instead of TCA-Precipitation might keep the activity of the protein and enables increased recovery.

6. Surface Plasmon Resonance Experiments including Validation

In this chapter, an alternative method for the detection of Staphylococcal Enterotoxins was tested. Surface Plasmon Resonance was the basic method for this approach.

6.1. Toxin Immobilization Control for the used SAM SPR chips

High quality SPR chips require reproducible toxin immobilization on the SAM-modified gold layers. In Figure 63, the time-dependent immobilization of SEA on a 16-Mercapto-hexadecanoic acid monolayer-modified gold chip is presented. The immobilization of toxin on the monolayer is performed via EDC coupling mechanism. Furthermore, it can be monitored (via increasing refractive index n) that EDC not hydrolyzed and so SEA is bound on the layer.

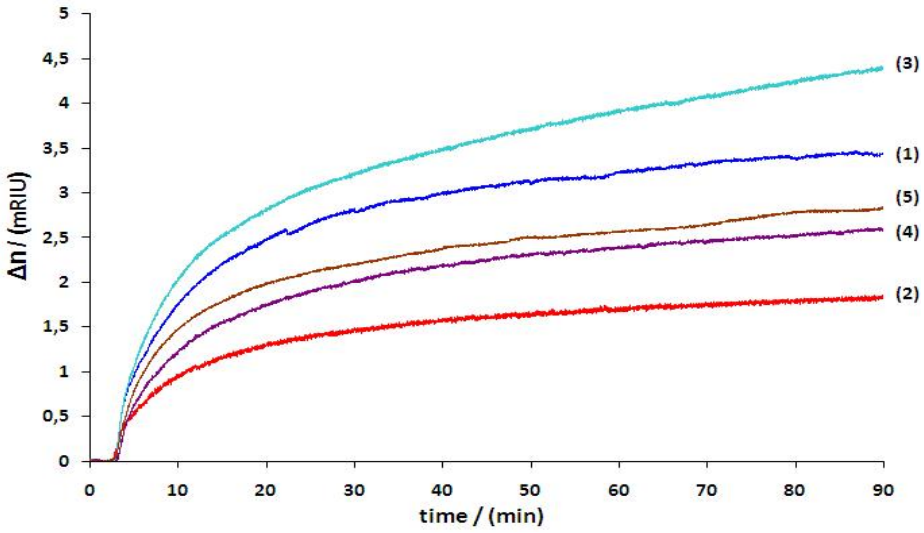


Figure 63. Immobilization of SEA on SAM-modified SPR chips via EDC coupling mechanism: Change in the refractive index for EDC-immobilized SEA (1-5) on 16-Mercaptohexadecanoic acid monolayer-modified gold SPR chips

The assignment of the chip surface with SEA over time is detectable due to the slowly increasing refractive index. This increase is caused by increasing assignment of the surface but not by changes in the refractive index of the sample solution, which could be monitored by a branch in the progression. The immobilization was successful with this ratio of components and could be reproduced. Variations in Δn are caused by changing reactivity of EDC and the variying quality of the SAM on the different chips.

The results for SEB are analogue and satisfying as well as the ones for SEA (Figure 64).

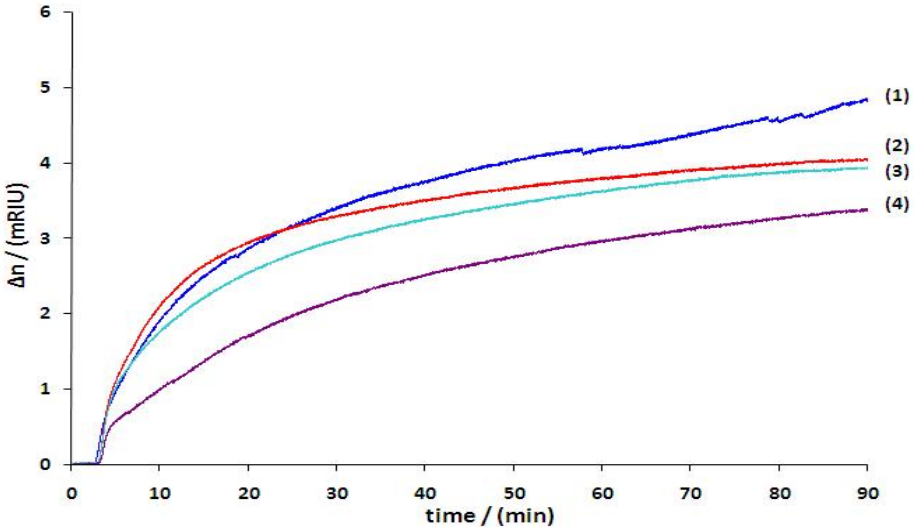


Figure 64. Immobilization of SEB on SAM-modified SPR chips via EDC coupling mechanism: Change in the refractive index for EDC-immobilized SEB (1-5) on 16-Mercaptohexadecanoic acid monolayer-modified gold SPR chips

A proposition of the efficiency of surface coating (occupiancy rate) could be estimated by the Langmuir isotherm (Figure 65):

$$q = \frac{q_{\max} \cdot K_b \cdot c}{1 + K_b \cdot c}$$

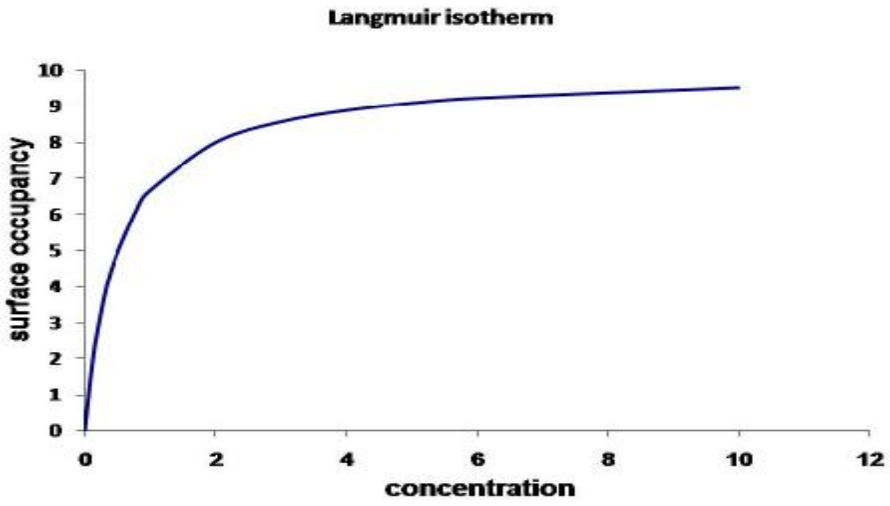


Figure 65. Calculated Langmuir isotherm for θ_{max}=10, K_b=2

SEB3 and **SEB4** are additionally blocked with BSA Blocker to prevent unspecific binding after toxin immobilization. After immobilization (and blocking), the chips are washed with 140 mM NaCl solution and the results are compared in Table 19.

Table 19. Change in Δn after the corresponding step compared to the baseline of 140 mM NaCl solution

Trial #	Δn / (mRIU) after immobilization	Δn / (mRIU) after blocking	Δn / (mRIU) after washing
SEA 1	3.44 (100%)	-	3.1 (90.1%)
SEA 2	1.85 (100%)	-	1.51 (81.6%)
SEA 3	4.40 (100%)	-	4.16 (94.5%)
SEA 4	2.59 (100%)	3.18 (123%)	2.75 (106%)
SEA 5	2.83 (100%)	3.45 (122%)	3.11 (110%)
SEB 1	4.86 (100%)	-	4.69 (96.5%)
SEB 2	4.04 (100%)	-	3.72 (92.1%)
SEB 3	3.95 (100%)	4.37 (111%)	3.92 (99.2%)
SEB 4	3.39 (100%)	3.66 (108%)	3.21 (94.7%)

A significant increase in the refractive index is measurable, compared to the baseline of the 140 mM NaCl solution. Due to this, immobilization with EDC seems very effective and reproducible. The complete immobilization process is presented in Figure 66.

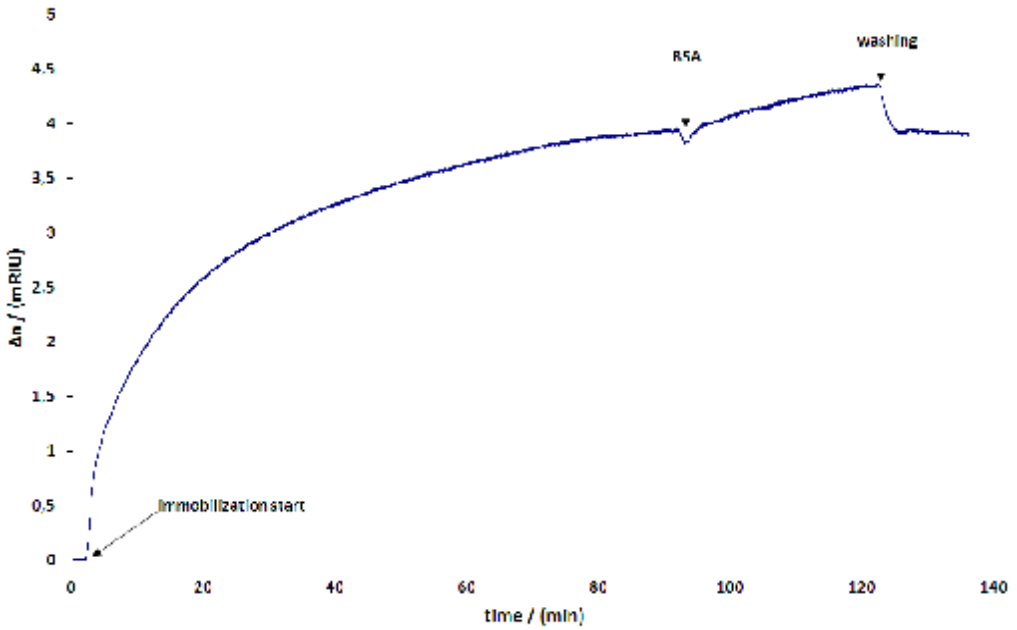


Figure 66. SEA immobilization (50 mg/L) with BSA-blocking and PBS buffer washing step

6.2. Determination of K_A(anti-SEA/SEA) and K_A(anti-SEB/SEB)

A high affinity constant is the key factor for an antibody-antigen-system to be used in a sensor system. Herein, the affinity constants of the 2 antibodies, <SEA> and <SEB>, are measured twice to guarantee specific and preferably complete binding of the analyte. The constant is determined twice for every species. Therefore, four different antibody concentrations/ system are measured against PBS buffer.

Figure 67 and 68 monitor the behavior of the SEA/<SEA> system.

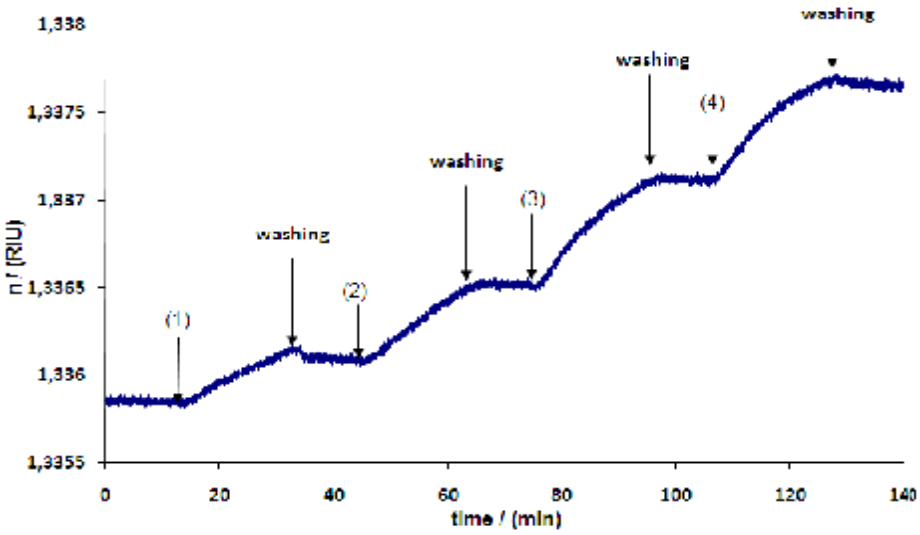


Figure 67.
Change in the refractive index during the interaction of <SEA> with the immobilized SEA on the chip surface. (ß (<SEA>): (1) 0.75 mg/L, (2) 1.5 mg/L, (3) 3 mg/L, (4) 6 mg/L)

The change in the refractive index corresponds approximately to Langmuir characteristics in the time frame from 0 to 20 minutes. Using the highest antibody concentration, the saturation region of the Langmuir isotherm is nearly reached, and therefore, the signal is not increasing at all.

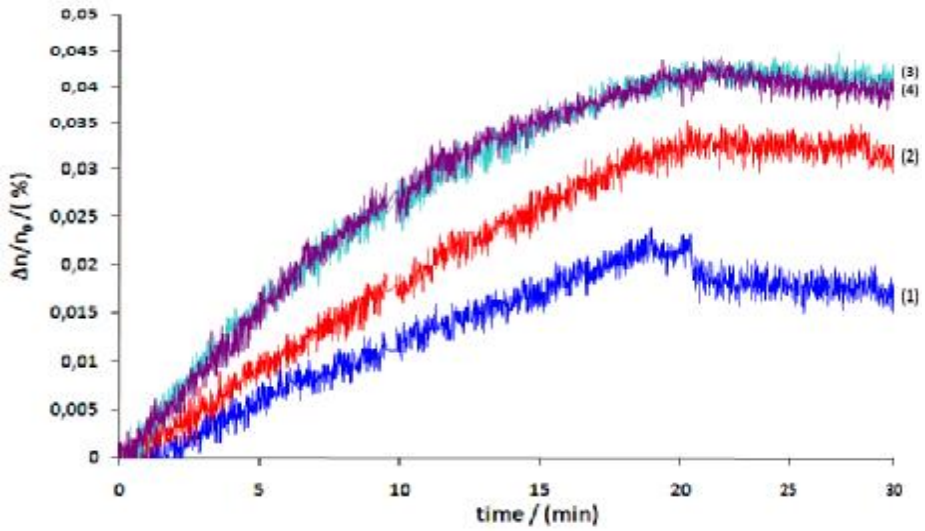


Figure 68. Relative change in the refractive index for different <SEA> concentrations: Immobilization (0-20 min) and washing with PBS buffer (20-30 min) (ß (<SEA>): (1) 0.75 mg/L, (2) 1.5 mg/L, (3) 3 mg/L, (4) 6 mg/L)

The SEB-<SEB> interaction is characteristically the same (Figure 69 and 70).

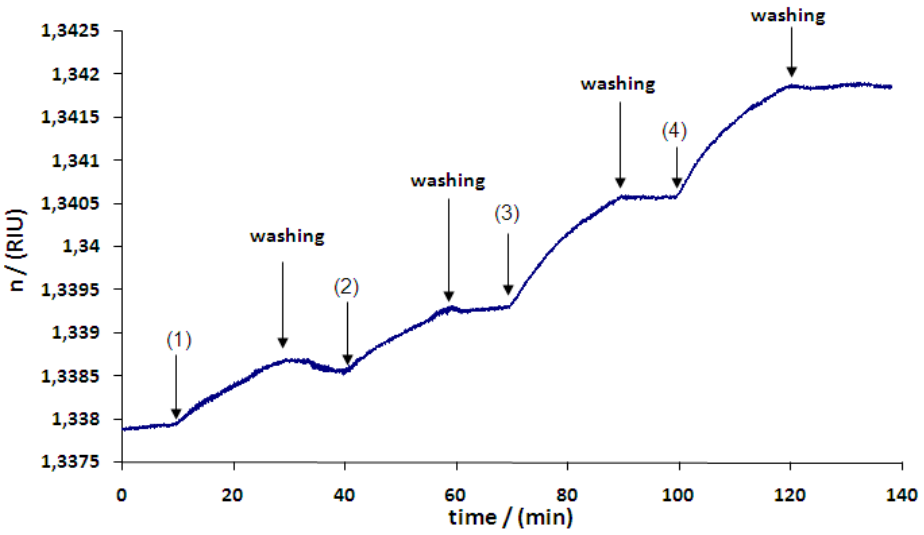


Figure 69.
Change in the refractive index during the interaction of <SEB> with the immobilized SEB on the chip surface. (ß (<SEB>): (1) 0.75 mg/L, (2) 1.5 mg/L, (3) 3 mg/L, (4) 6 mg/L)

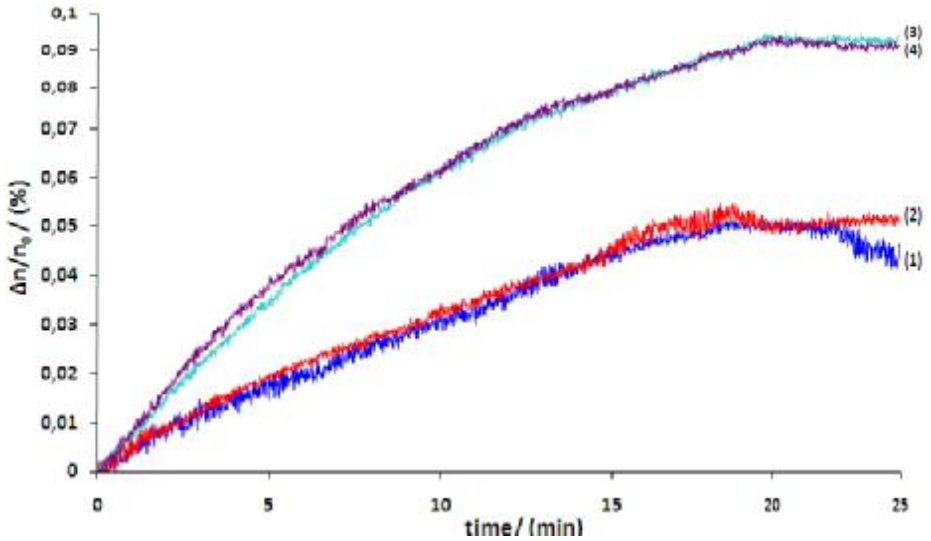


Figure 70: Relative change in the refractive index for different <SEB> concentrations: Immobilization (0-20 min) and washing with PBS buffer (20-30 min) (ß (<SEB>): (1) 0.75 mg/L, (2) 1.5 mg/L, (3) 3 mg/L, (4) 6 mg/L)

The values are plotted double-reciprocal and K_As are calculated from the linear slopes with the Langmuir equation (Figure 71). The molecular mass of the antibody is calculated with 150800 g/mol.

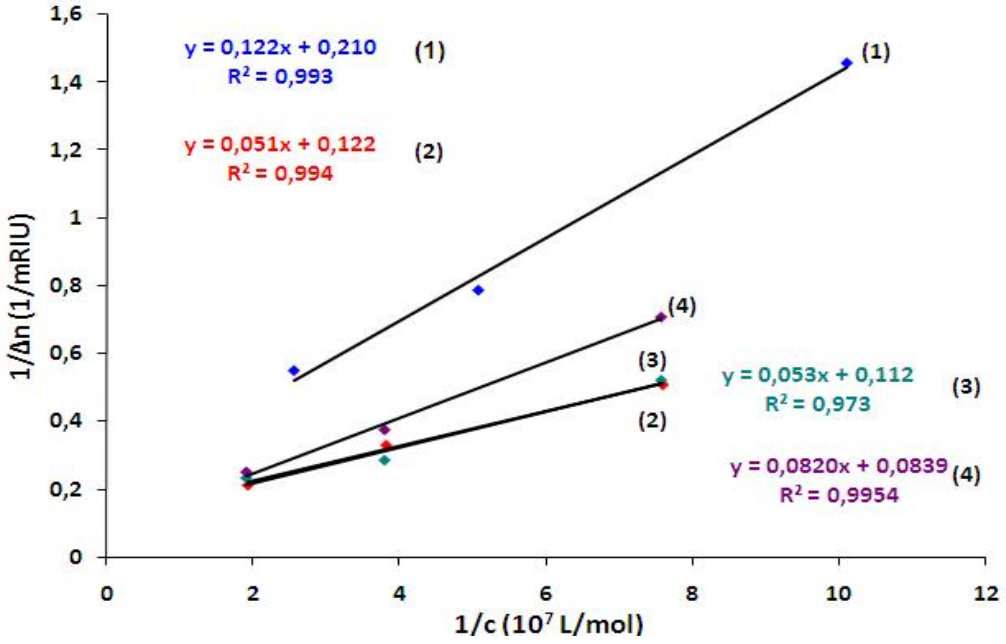


Figure 71.
Double-reciprocal plot of Δn against c(SEA1) (1), c(SEA2) (2), c(SEB1) (3), c(SEB2) (4)

K_A was estimated according to the Langmuir model to: 2.1 10^{+7} L/mol for <SEA> and 1.6 10^{+7} L/mol for <SEB>. Unspecific binding cannot be prevented because it is not guaranted that all binding places are occupiued after 20 minutes of toxin immobilization. Therefore, a blocking substance, BSA, is used. The blocking step was efficient and successful and kept the change in n in the left slope of the angular-dependent SPR signal after antibody addition.

6.3. Determination of Linear Concentration Range and LOD for SEA and SEB in UHT Milk

Both antibody-toxin pairs exhibit good affinity values and can be applied in a sensor system. Firstly, a calibration curve for SEA and SEB in milk is measured. Therefore, UHT milk (3.5 % fat) and PBS are mixed in a ratio of 1:1. Toxin concentrations of 25, 50, 100 and 200 ng/ml in milk-PBS mixture are proceeded to keep the refractive index in an applicable range. 2 mg/L of the appropriate antibody is added to every sample and the sample-antibody mixture is incubated for 1 hour following application to the SPR system. The antibody concentration was chosen due to its sensitivity the calibration range. The results are presented in Figure 72 and 73.

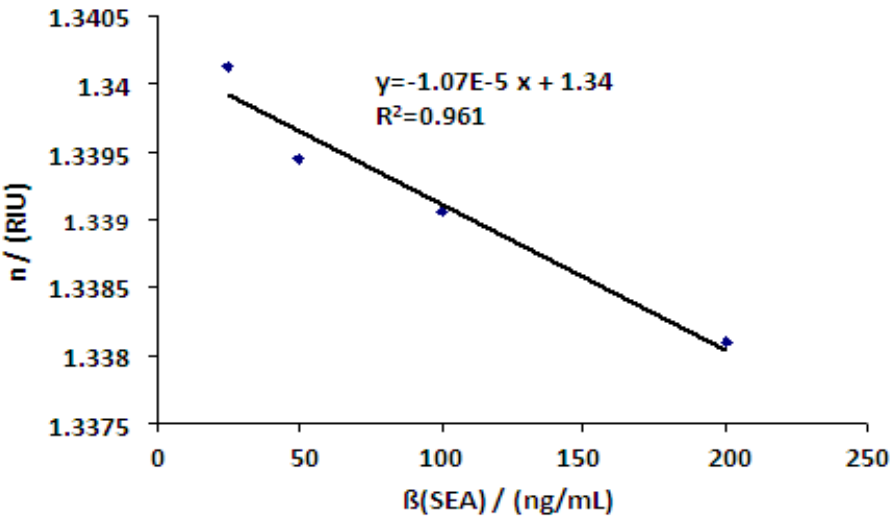


Figure 72. Calibration curve for SEA in UHT milk/PBS (1:1)

Within this experiments, a competitive assay type is used. At 0 ng/mL, the mass of deposited antibody on the chip is at its maximum and therefore, the n-value too. The n-value is

decreasing with increasing toxin and decreasing antibody concentrations. Figure 72 does not include the value for 0ng/mL due to its low precision.

The limit of detection (LOD) is for SEA is calculated after LOD = $y_B + 3\sigma_B$. Herein, $3\sigma_B$ is subtracted from the base value, due to the decreasing character of the calibration curve for increasing toxin concentrations. The LOD (SEA) is calculated to 6.9 ng/mL.

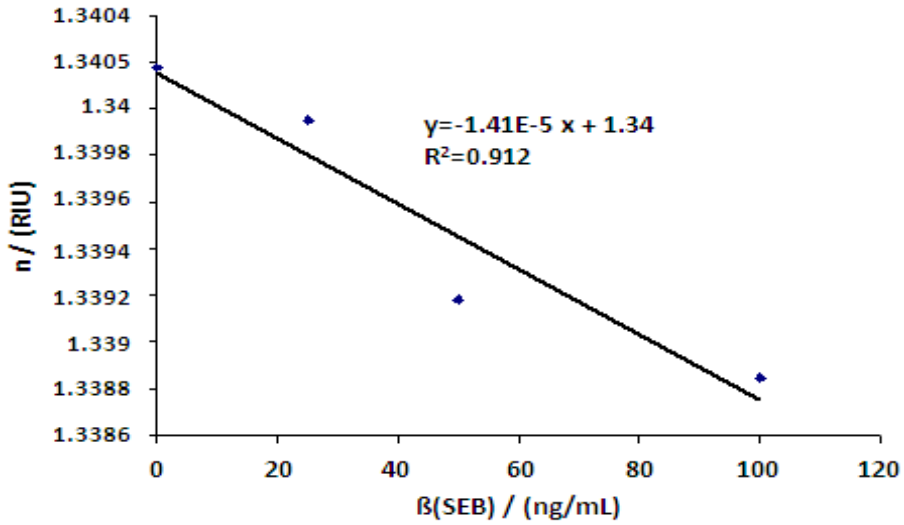


Figure 73: Calibration curve for SEB in UHT milk/PBS (1:1)

ß (SEB) = 200 ng/mL was not considered because it was lower than the PBS value. LOD (SEB) is calculated to 1.7 ng/mL. The competitive assay type was chosen due to the not reproducible immobilization of the antibody as capture layer, its baseline underlies strong changes. In addition, the small molecular size of the toxins caused only very small changes in the refractive index when binding to the much bigger antibody.

LODs for SEA and SEB in UHT milk were calculated to 6.9 and 1.7 ng/mL. BIOTRACER requirements (linear range of 0.1-10 ng/mL) could be nearly fulfilled for SEB and partly fulfilled for SEA by means of this SPR chip arrangement.

Analogue LOD values for buffer and milk samples were described by Homola et al. **[D1, D2]**.

Improvement of LODs and linear range is achievable with variation of antibody-sample incubation time, choice of blocking agent and slight modifications in milk sample preparation. A possible modification in sample preparation could be done with a defattening step or up-concentration via filtration membrane. This SPR chip system is the preferable

analysis method for milk samples after further development steps and is able to reach the requirements of the BIOTRACER project completely. In addition, a multi-field SPR chip decreases to total analysis time for a larger number of samples.

Advantages of SPR are online-monitoring of the signal in real time, the ability of achieving low LODs with low material input due to circle-flow incubation, label-free detection, easy background correction and maintenance of the biological activity of biological material due to permanent buffer flow. Disadvantages of SPR are expensive gold chips, prolonged analysis time on comparison to the array technique and missing high throughput possibilities concerning the number of samples. Advantages of microarrays are cheap chip material, shorter reaction times compared to SPR and the ability of scanning large numbers of analyses parallely. Disadvantages of the microarray system are the large number of washing and drying steps, high intra- and interspot standard deviations, high material input, inactivation of biological material due to drying phases, different chip surface preparation due to 2D structure and extreme dependence from the quality of biological material and dye.

7. Experimental Part

7.1. Materials and Methods

7.1.1. Materials, Instrumentation and Software

Chemicals and solvents were purchased from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany) and Carl Roth (Karlsruhe, Germany) unless stated otherwise.

Other materials and suppliers are listed as follows (Table 20).

Table 20. Materials and Suppliers

Supplier	Material
Toxin Technology (Sarasota, FL, USA)	Primary Antibodies against Enterotoxins
Acris Antibodies GmbH (Herford, Germany)	Primary Antibodies against Enterotoxins
Rockland Immunochemicals, Inc (Gilbertsville, PA,USA)	Secondary Cy3-labeled Antibody against rabbit
Qiagen (Venlo, Netherland)	Antibody against His_6, Secondary Peroxidase-labeled Antibody, Substrate for Peroxidase, Alexa532-labeled Antibody against His_6
Sigma-Aldrich (Steinheim, Germany)	PBS tablets, Casein 10x Blocker, GPTS, APTES, MPTS, Tween 20, Thiols for SPR
Carl Roth (Karlsruhe, Germany)	Roti Nanoquant (5x), BSA
Pierce (Rockford, IL, USA)	Melon Gel Spin Purification Kit, Nab Protein A Plus Spin Kit, Zeba Empty Spin Columns, Protein-free Blocking buffer, Disuccinimidyl suberate (DSS), BCA Assay Reagents
Schott Nexterion (, Germany)	Nexterion Block E, Nexterion Epoxy slides
ABCR (Karlsruhe, Germany)	Aldehyde Silane
Millipore (Billerica,MA, USA)	Amicon Ultra Filter Units 10 kDa (0.5 mL and 15 mL)
Whatman (Sanford, USA)	Fast Slides, Micro Caster
Thermo Fisher Scientific (Portsmouth, NH, USA)	Microarray slides with hydrophobic inc
GE Healthcare (Munich, Germany)	Cy3/Cy5 NHS ester, Sephadex G-25/G-15
Active Motif (Tegernheim, Germany)	Chromeo546 NHS ester
Biomérieux Germany (Nuertingen, Germany)	VIDAS SET2 Kit
Biochrom (Berlin, Germany)	50 ml Falcon tubes
Apollo Scientific Limited (Stockport, UK)	N-(5-Amino-1-carboxypentyl)iminodiacetic acid (NTA)
Greiner Bio One (Kremsmünster, Austria)	96 and 386 well plates (clear and black, round and F-Bottom)

Toxins and antibodies were obtained as lyophilized crystalline powders and diluted with Millipore water to a final concentration of 2000 mg/L (SEA, SEB, SEC), 1000 mg/L (all antibodies) and 500 mg/L (SED, SEH). The structure and spectral data of Chromeo 546 were published in **[E1].**

Instrumentation

Absorption spectra and data in PMMA cuvettes (1 x 1 x 3 cm) were acquired on a Cary 50 Bio UV-visible spectrophotometer from Varian (Palo Alto, CA, USA). Fluorescence spectra and data were measured on an Aminco Bowman AB2 luminescence spectrometer (SimAminco, Rochester, NY, USA) equipped with a 150-W continuous wave xenon lamp as the excitation source. Standard quartz cuvettes were used for all experiments. Absorption data and fluorescence emission intensities of solutions in microplates were measured on a Tecan GENios Plus microplate reader (Zurich, Switzerland) with ten flashes, respectively.

Microarrays were read out with different sources: A Tecan LS 200 (Zurich, Switzerland) was used at the University Hospital of Regensburg, an Affymetrix 428 Arrayscanner (Santa Clara, CA, USA) at the University of Regensburg and an Axon GenePix 4000B Arrayscanner at the Austrian Research Center Seibersdorf (Dr. Claudia Preininger, Austria). The Tecan LS 200 apparatus works with two lasers, at 633 nm and at 532 nm. The dichroic filters were set to 575 ± 50 nm and 692 ± 45 nm. The Affymetrix 428 works with two lasers, at 532 and 635 nm. The filters were set to 570 ± 10 nm and 670 ± 10 nm. The Axon GenePix 4000B works with lasers at 532 and 635 nm. The filters were set to 575 ± 17,5 and 670 ± 20.

Contact angles measurements were done with a tensiometer from Erma (Tokyo, Japan). A Schott Blueline pH Meter was used for adjusting the pH of the buffers. A Lab dancer vortexer from VWR International (Darmstadt, Germany) and a shaker - Akku-Schüttler (KM - 2 Akku) - from Edmund Bühler GmbH (Heching, Germany) was used. The protein purification was carried out with an Eppendorf centrifuge 5415 R with 45° fixed angled rotor (FA 45-24-11, up to 16.000 rcf(g)/13100 rpm). For spotting the Erie microarrays an Eppendorf Research pro Electronic Pipette (0.5 - 10 µL) was used, whereas for the FAST slides the MicroCaster hand spotting device from Whatman (Sanford, ME, USA) was applied. A µ-box from was used as humidity chamber for all incubations. Furthermore, all cleaning and blocking steps were

carried out in 50 mL falcon tubes from Biochrom (Berlin, Germany). The Biosuplar 6 from Mivitec GmbH (Sinzing, Germany) was used for all SPR measurements.

Several microarray formats were used: 96 well (1.5 mm diameter) glass microarrays with hydrophobic patterned wells from Thermo Fisher Scientific (Portsmouth, NH, USA), blank glass slides from Menzel-Gläser (Braunschweig, Germany), Nexterion Epoxy slides from Schott (Jena, Germany) and the FAST slides from Whatman (Sanford, ME, USA).

Software

The data were analyzed with GenePix Pro 6.0 (Molecular Devices, Union City, CA, USA), Origin (Microcal, Northampton, MA, USA) and Office 2003/2007 (Microsoft, Redmond, USA).

7.1.2. Gel Filtration and Affinity Chromatography

Labeled antibody was separated from unlabeled dye by gel permeation chromatography using Sephadex G-25 or G-15 from GE Healthcare as stationary phase (placed in a 10 ml Zeba Spin Column) and 1 x phosphate-buffered saline (PBS) buffer 10 mM pH 7.4 as eluent. Spin columns for Affinity Chromatography from Pierce (Melon Gel Spin Purification Kit and Nab Plus Spin Kit) were used as alternative clean-up method.

7.2. Buffer Preparation

In this chapter, the receipts and preparation guidances of all applied buffers were provided.

1 x PBS/PBST buffer

1 x PBS from PBS tablets

5 tablets are dissolved in 1 L Millipore water and yield 10 mM phosphate buffer, 2.7 mM KCl and 137 mM NaCl, pH7.4 at 25°C.

1 x PBS from salts

8 g NaCl (137 mM), 0,2 g KCl (2.68 mM), 3.63 g $Na_2HPO_4 \bullet 10\ H_2O$ (11.3 mM) and 0.24 g KH_2PO_4 (1.76 mM) are diluted in 1 L Millipore water. The pH is adjusted to 7.4.

PBST:

PBST is a 1 x PBS buffer, pH 7.4 with 0.5 % (w/w) Tween 20.

Bicarbonate buffer (BCB) (basic protocol for pH 8.3)

2.1 g of $NaHCO_3$ are diluted in 25 mL Millipore water and the pH is adjusted to 8.3 with 1 M NaOH. Storage is best at 4° C.

Pierce Protein-free Blocking buffer with Tween 20

Add 0.5% (w/w) Tween 20 to Pierce Protein-free Blocking buffer to yield a final volume of 1 L. Store at 4° C.

Casein Blocking buffer for Nitrocellulose slides

100 mL of 10 x Casein Blocking buffer concentrate (Sigma-Aldrich) are diluted 10 fold. Store at 4° C for 4 weeks.

BSA Blocking buffer

5 g BSA are diluted in 1 x PBS buffer pH 7.4 to a final volume of 100 mL and shaked slowly to prevent foam. BSA buffer should always be freshly prepared prior to spotting on microarrays.

7.3. Surface Modification for *Staphylococcus aureus* Enterotoxin Arrays

Chapter 7.3 is related to the basic construction steps of the developed *S. aureus* Enterotoxin Array. Surface cleaning of the glass slides is performed prior to the silanization process. Afterwards, the toxin layer is immobilized on the surface-treated microarray slides following blocking of the free binding sites. As a final step, the target molecules are bound on the array, which is now ready for read-out.

7.3.1. Surface Cleaning

Untreated slides are cleaned in a 1:1:5-ratio solution of NH_3 (20%), H_2O_2 (30%) and Millipore water for 10 minutes at 80° C. The temperature must be reached before adding the slides to the purification medium. Afterwards, slides are extempted from residues by shaking in Millipore water two times and subsequent drying in an Argon steam. The cleaning solution can be reused up to 3 times. The resulting surface quality was tested with contact angle measurement before and after the procedure. This protocol was adapted from the Diploma thesis of Michaela Chwala (2007, **[E2]**).

7.3.2. Silanization

Silanization process:

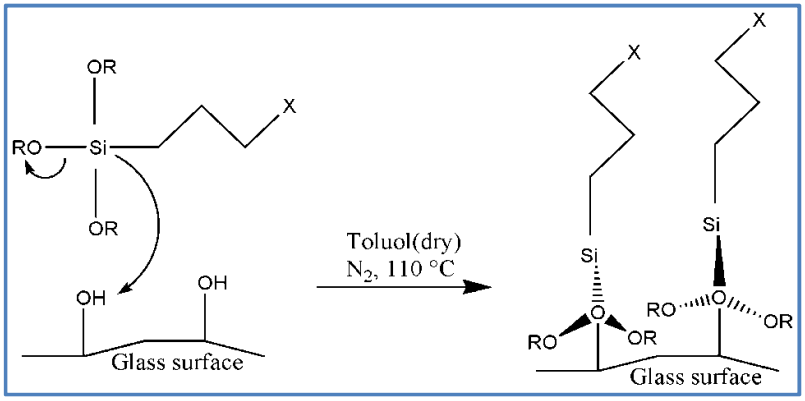


Figure 74. Silanization process scheme for glass slides (reproduced from **[B17]**)

Basic silanization protocol:

Cleaned slides are silanized under reflux in a 2% (v/v) solution of silane in 200 mL dry toluene for 3 h at 115° C (Figure 74). Schlenk flasks are used for this step to guarantee nitrogen atmosphere. To remove white silane residues, the slides are swilled in toluene and shaked in ethanol (pA) for 5 minutes. Afterwards, the slides are dried in an Argon steam. If silane residues are still visible at the glass surface, slides must be cleaned again in ethanol with ultrasonification for 1 minute. The resulting surface quality was tested with contact angle measurement.

Several silanization parameters **[D3]** where tested as presented in Table 21.

Table 21. Applied silanization methods and parameters for glass slides

Method #	Silanization agent	Reaction time [h]	Solvent-Silane mixture	Comments	Reference
A	GPTS	3	no solvent, 100% silane	RT, no N_2 atmosphere, with coverslip, EtOH	**[E5], [E6]**
B	GPTS	1	2,5 % in EtOH /13 mM acetic acid	RT, no N_2 atmosphere, cleaning in EtOH, silanization in 50 ml Falcon	**[E4]**
C	APTES	2	no solvent, 100% silane	RT, no N_2 atmosphere, with coverslip, EtOH	**[E5], [E6]**
D	APTES	1	5% in 95/5 (v/v) ETOH/H_2O	RT, no N_2 atmosphere, cleaning in 95/5 (v/v) EtOH/H_2O, silanization in 50 ml Falcon	**[E4]**
E	MPTS	1	1% in EtOH /18 mM acetic acid	RT, no N_2 atmosphere, cleaning in EtOH/18 mM Acetic acid, silanization in 50 ml Falcon	**[E4]**
F	Aldehyde silane	2	no solvent, 100% silane	RT, no N_2 atmosphere, with coverslip, EtOH	**[E5], [E6]**
G	Aldehyde silane	1	2% in EtOH	RT, no N_2 atmosphere, cleaning in EtOH, silanization in 50 ml Falcon	**[E4]**
H	APTES	3	3% in toluene	under reflux, N_2 atmosphere Schlenk flask	**[E7-E9]**
R	**GPTS**	**3**	**2% in toluene**	**under reflux, N_2 atmosphere, Schlenk flask**	**[E1], [E7-E9]**

Method #R was now chosen as standard silanization process for all homemade Epoxy slides with hydrophobic teflon pattern.

7.3.3. Toxin Immobilization on the Array Surface

3 different slide types were applied:

1. Homemade silane slides with hydrophobic Teflon inc (2 x 96 wells for 1µL solution)
2. Plain glass slides with Epoxy silane-coating (Schott Nexterion Slide E)
3. 3D Nitrocellulose slides (Fast Slides from Whatman)

Each slide type requires a special protein spotting technique:

1. The homemade slides were spotted with an Electronic Pipette from Eppendorf (0.5-10 µL volume). 1 µL solution is pipetted in each cavity.
2. Plain glass slides were spotted with an Electronic Pipette from Eppendorf (0.5-10 µL volume) as well. 0.5 µL solution is pipetted per spot.
3. Nitrocellulose slides were treated with the MicroCaster (Whatman), a hand-arraying system especially for Fast slides. Fabrication of this layer is made following the MicroCaster protocol **[E10].**

Basic spotting protocol:

The toxin layer has to be spotted rapidly to prevent dessication and smearing effects on the array surface. Following spotting, the array is incubated in a humidity chamber for 1 h at room temperature. Afterwards, 2-3 clean-up runs in PBST and one in Millipore water, each for 5 minutes are applied. The Argon steam-dried slides can be stored up to one year and are best to use after 3 months of storage under vacuum at room temperature.

The basic spotting protocol is used unless stated otherwise.

7.3.4. Blocking of Free Silane Binding Sites

3 different blocking modes were applied:

1. Blocking with coverslip (BSA-PBS blocking buffer)
2. Blocking in 50 mL Falcon tube (Casein Blocker and Protein-free blocking buffer)
3. Fast Frame for Fast Slides (all buffers)

Blocking protocol:

BSA-PBS blocking buffer slides were drained with 1 mL of buffer solution on the surface and covered with glass coverslips and put on a rocking plate with gentle agitation. Fast Slides were covered with the Fast Frame and 700 µL buffer per field was filled in. They were put on the rocking plate as well. All other slides were put in a 50 mL Falcon tube, filled with 40 mL of blocking solution and retained on a rocking plate. Following incubation, the slides are shaken 2-3 times in PBST and once in Millipore water. Each run lasts 5 minutes. All slides,

except the Fast Slides which require nitrogen treating, are dried in an argon steam. Table 22 depicts the used buffers and their characteristics.

Table 22. Blocking protocols for different microarray types

Blocking buffer	Buffer preparation	Blocking parameters
Protein-free Blocking buffer	Add 5g/L Tween 20	1-2 h at RT
BSA-PBS buffer	Add 5 g/L BSA to 1x PBS pH 7.4	1-2 h at RT
Casein Blocker (only for Fast Slides)	Dilute 1:10 with Millipore water	1 h at 37°C
Block E (for Nexterion Slide E)	Dilute 1:4 with Millipore water	15 min at 50°C

7.3.5. Target Incubation

Preparation of Standards:

Toxins stock solutions are solved in PBS buffer or either in UHT/raw milk. If milk is used, 1h at room temperature and soft shaking is suggested to enable toxin incorporation into the matrix.

Antibody Addition:

Prior to incubation on the array, the pre-concentrated antibody solution is pipetted to the standard or sample and incubated with moderate agitation for 1 h at room temperature.

Incubation on the Array:

If labeled primary antibodies (primary system) against the toxins are utilized, only the first incubation step is necessary. For use with a secondary labeled antibody (secondary system), the second step is done as well. According to the three slide types, different incubation methods are applied according to Table 23.

Table 23. Target incubation protocols for different slide types

Microarray format	Primary system		Secondary system	
	Incubation Method	Incubation Condition	Incubation Method	Incubation Condition
Teflon inc array	Pipet 1mL of target solution in each cavity	1.5 h, dark, humidity chamber	Pipet 1mL of target solution in each cavity	1 h, dark, humidity chamber
Plain glass slide	Pipet 100 µL of target solution onto the array and cover with second slide	1.5 h, dark, humidity chamber	-	-
Nitrocellulose slides: FAST™ slides	Fast Frame: Affix the frame on the slide and fill in 700 µL (1-pad slide) or 100 µL (16-pad slide) of target solution	1.5 h, dark, Fast Frame with plastic seal	-	-

After every systems application, the arrays are cleaned by shaking in PBST (2-3 times) and water (once), each for 5 minutes. Afterwards, they are dried in an Argon steam.

7.4. Surface Modification for SPR measurements

Chapter 7.4 describes the basic construction steps of the developed *S. aureus* Enterotoxin Surface Plasmon Resonance Chip. Surface cleaning of the SPR Chips is performed prior to the chip modification with self-assembled monolayers. Calibration of the chips with NaCl solutions is considered as the next step. Afterwards, the toxin layer is immobilized via EDC on the surface-treated SPR chips following blocking of the free binding sites. As a final step, the target molecules are bound on the SPR chip.

7.4.1. Cleaning Procedure and Preparation of SAM

SAMS are formed spontaneously on gold and other metal surfaces via alkanthiol coating. The major advantage of the gold surface is given by its stability against most oxidating substances. Immediately before use, the chips are cleaned in Ethanol in an ultrasonic bath for 20s. Prior to functionalization, the gold surface of the SPR chip is cleaned in Piranha solution, a 1:3-mixture of H_2O_2 (30%) and concentrated H_2SO_4. Afterwards, the chips are

cleaned with ultra-pure water and dried. 16-Mercaptohexadecanoic acid forms a self-assembling monolayer when incubated as a 20 µM solution (in Ethanol) over night. Following SAM attachment, the chips are cleaned in Ethanol again and dried.

7.4.2 Preparation of Working Solutions

Sodium chloride stock solution and dilutions

The stock solution is a 600 mM NaCl solution in ultra-pure water. The three NaCl solutions (100, 200 and 400 mM) are prepared via dilution of the stock solution with ultra-pure water. A 140 mM NaCl solution is prepared from stock for the working buffer and the standards/sample dilution.

Physiological, phosphate-buffered sodium chloride solution (1xPBS pH 7.4)

The physiological PBS solution (140 mM NaCl, 25mM NaH_2PO_4, pH 7.4) is made from NaH_2PO_4*$2H_20$ via dilution with the 140 mM NaCl solution. The pH is adjusted to 7.4 with 1 M HCl.

EDC-Toxin solution

2.5 mg EDC, 1.95 mL 140 mM NaCl solution and 50 µL toxin stock solution (2 mg/mL, SEA or SEB) are mixed in an Eppendorf Cup. This solution can`t be stored and must always be mixed freshly due to the hydrolysis of the EDC.

BSA Blocking solution

A 2 mg/mL solution is prepared from ultra-pure BSA and ultra-pure water.

7.4.3. Calibration of the Instrument

The reflected beam is transferred into an electrical signal by use of a photomultiplier tube. The signal change is proportional to the change of the refraction index in the biological layer above the gold film. First of all, solutions with known change in reflectance have to be measured to transfer the electric signal into a physical value.

For this purpose, sodium chloride with concentrations of 100, 200 and 400 mM were used and brought into the measuring cell after the following scheme: The cell is filled with destilled water and awaited until the signal is constant. Now the sodium chloride with the lowest concentration is applied and the signal change is monitored. If the signal seems to be stable (10 minutes are applied here), the cell is flushed with destilled water until the signal reaches its origin value. Afterwards, the higher concentrations are applied in the same manner. Furthermore, the received signal is plotted against the sodium chloride concentrations (mM). Supplementary, the refractivites of the NaCl solutions are measured with an Abbé refractometer. Now, the signal is plotted against the refraction index. Following calibration, a baseline with the 140 mM NaCl solution is made in the same manner.

7.4.4. Immobilization of Toxin on the Array and Blocking

Herein, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide is used to activate carboxy- and amino functions. The reaction is proceeded in 2 steps, activation and immobilization (Figure 75). Afterwards baseline application, the ECD-toxin solution is applied to the system and pumped in circle for 90 minutes. After this procedure, the BSA Blocker is pumped around in the system for 30 minutes, followed by a washing step with the 140 mM NaCl solution until the signal is constant. The new baseline is done with the PBS buffer.

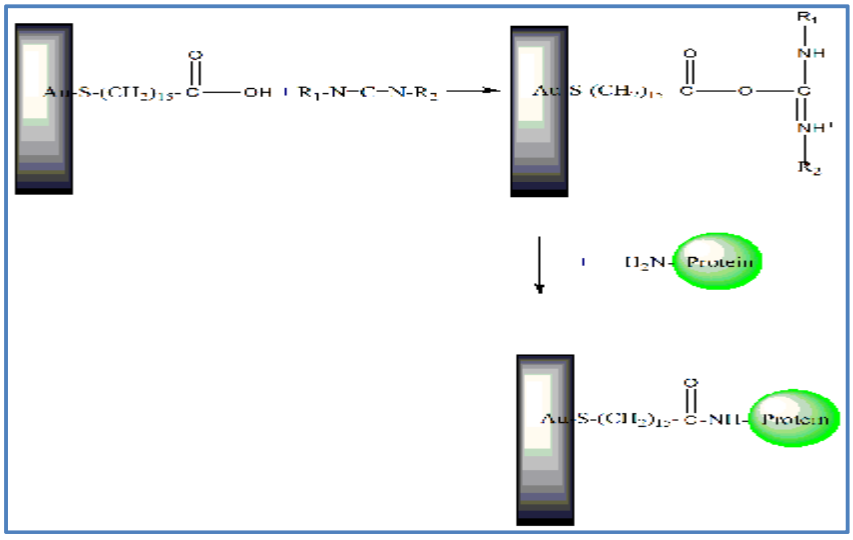


Figure 75. Protein immobilization with EDC (partially reproduced from **[B7]**)

7.4.5. Determination of the Binding Constants of <SEA> and <SEB>

A calibration curve is made separately for every antibody type. For every curve, several dilutions of the Cy3-labeled <SEA> (or Cy3-labeled <SEB>) are made in PBS buffer within the region of 0.75-8 mg/L. Beginning with the lowest antibody concentration, every solution is applied to the system for 20 minutes followed by a washing step with PBS buffer. The reference channel is measured with PBS.

7.4.6. Measurement of the Real Samples

To prepare a milk sample, 1 mL UHT milk 3.5% fat and 1 mL toxin solution (SEA or SEB in PBS) are mixed and stored at 4° C for 1 h. The toxin concentrations are ranging between 25 and 200 ng/mL. Following incubation, the labeled antibody is pipetted into the solution for a final concentration of 2 mg/L. The toxin-antibody complex in milk is incubated at 4° C for 1 h and brought to room temperature for measurement. First, the sample with the highest toxin concentration is applied, followed by lower concentrations to guarantee increased binding of increased antibody concentrations at lower toxin concentrations. The samples are pumped in the system for 20 minutes, followed by a washing step with PBS for 10 minutes. During sample measurement, the reference channel is filled with a mixture of milk and PBS (1:1). For washing, PBS buffer is used as well as for the measurement channel.

7.5. Labeling and Purification

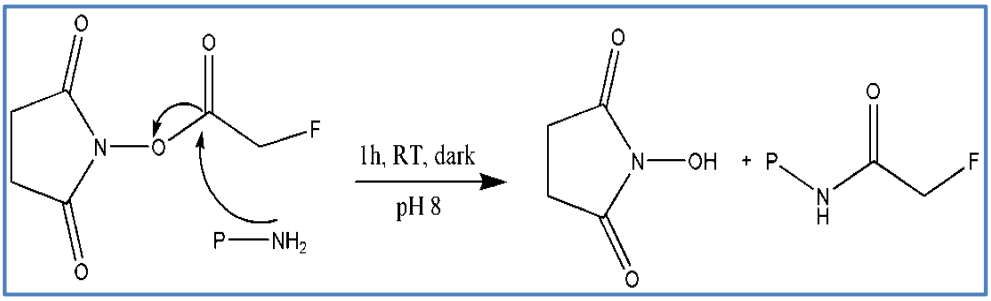


Figure 76. Labeling for molecules with amino groups (P: protein, F: fluorophore)**[B7]**

Within this chapter, the applied labeling (Figure 76), purification and determination techniques for the specific antibodies are described. The first part gives an overview of the general labeling procedure for <SEX>. The second part deals with the clean-up of the labeled antibodies followed by a description of quantitative protein determination methods. The calculation of the Dye-to-Protein and Fluorescence-to-Protein ratio represents the final component.

7.5.1. Fluorescent Labeling of GST- and *Staphylococcus aureus* Enterotoxin Antibodies

Cy3-NHS ester and Chromeo546-NHS ester are applied as labeling agents **[E11, E12].** <GST> and <SEX> (X: A, B, C, D and H) are labeled with the Cy3-NHS ester from GE Healthcare (Figure 77, Table 24). Alternative labeling experiments were proceeded with the Chromeo 546-NHS ester from Active Motif (Figure 78, Table 25).

Table 24. Properties of Cy3-NHS ester (adapted from **[E11]**)

Dye	Cy3
M / [g/mol]	765,95
ε / [M^{-1} cm^{-1}]	250000
λ_{abs}(max) / [nm]	549
λ_{em}(max) / [nm]	562

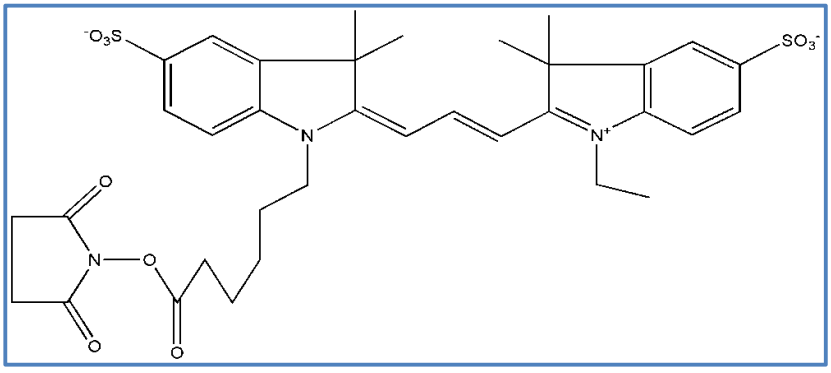


Figure 77. Structure of Cy3-NHS ester (adapted from **[E11]**)

Table 25. Properties of Chromeo-NHS ester (adapted from **[E12]**)

Dye	Chromeo
M / [g/mol]	703,82
ε / [M^{-1} cm^{-1}]	98800
λ_{abs}(max) / [nm]	545
λ_{em}(max) / [nm]	561

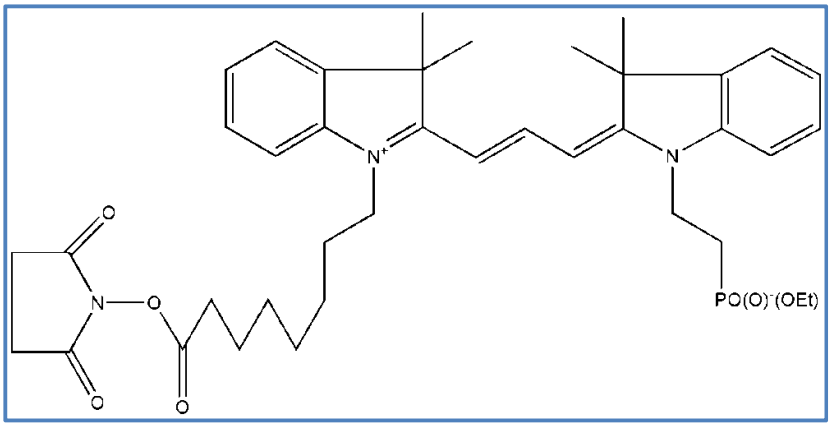


Figure 78. Structure of Chromeo 546 NHS ester (adapted from **[E12]**)

The volume of fluorescent dye which is speifcially used per labeling batch (MR, concentrations of dye/protein is calculated by the following equations:

$$n(dye) = n(protein) \cdot MR$$

$$\frac{V(dye) \cdot b(dywe)}{M(dye)} = \frac{ß(protein) \cdot V(protein)}{M(protein)} \cdot MR$$

$$V(dye)[mL] = \frac{ß(protein) \cdot V(protein) \cdot M(dye)}{ß(Dye) \cdot M(protein)} \cdot MR$$

Table 26. Labeling trials

Trial #	pH (BCB buffer)	Reaction time (h)/ Stopping time (h)	MR	Dye	Protein type	Comment
1	8.3	1/0.5	1, 3, 5, 8, 10, 12, 15, 18, 20	Cy3	<GST>	MR test
2	8.3	1/0.25	20 (x3)	Cy3	<GST>	3 clean-up methods
3	8.3	1/0.25	15 (x6)	Cy3	<GST>	5 clean-up methods + reference
4	8.3	1/0.5	10, 12, 15	Cy3	<SEA>	MR test
5	7.5 7.8 8.3 8.7 8.0	19/1,25 19/1.25 1.75/0.5 1.75/0.5 19/1.75	12	Cy3	<SEB>	pH + time test
6	7.5 8.7	19/1.25 1.75/0.5	12	Cy3	<SEA>	pH + time test, 1 clean-up method
7	7.5 8.0	4/0.5	12	Cy3	<SEA>, <SEB>	pH test, 1 time + clean-up method
8	8.0	3/0.5	12	Cy3	<SEA>, <SEB>	1 time + clean-up method + pH
9	8.0	1/0.25	12	Chromeo 546	<HSA>, <bovIgG>	
10	8.0	2/0.25 2/0.25	12	Chromeo 546 Cy3	<SEA>, <SEB>	Dye contest
11	8.0	3/0.5	12	Cy3	<SEA>, <SEB>	Acris
11	8.0	3/0.5	12	Cy3	<SEA>, <SEB>, <SEC>, <SED>, <SEH>	Toxin Tech

Basic labeling protocol:

The antibody or protein solutions are re-dissolved or diluted to a protein content of 1 mg/mL. The dye is reconstituted in DMSO to a final concentration of 10 mg/mL. 10% (v/v) of 1 M 10 x BCB buffer are pipette to a defined volume of the solution and is followed by addition of the calculated fluorescent dye volume. The reaction occurs in 3 h by gentle

shaking in the dark at room temperature. Stopping is done by adding 10% (v/v) of 1.5 M NH_2OH*HCl for 30 minutes. Purification is proceeded immediately via sephadex column or spin column. Several parameters where tested as presented in the following Table 26. The following parameters were defined as standards for all array-applicated labeled antibodies:

MR: 12
Reaction time: 1h
Stopping time: 30 minutes

7.5.2. Purification Techniques: Sephadex Column and Spin Columns

Sephadex G-25/G-15 medium in Zeba Column [E13]

Sephadex is delivered as dry powder and must be swollen in separation buffer prior to use. It is diluted with 4-6 mL of 1 x PBS buffer of pH 7.4 per gram for 3 h at 20° C or 1 h at 90° C under stirring conditions. 10 mL of swollen slurry are filled into the Zeba Column and washed with PBS. The sample is gently loaded onto the column resin. 1 x PBS is applied as separation buffer and elutes the labeled proteins at low flow rates and without external pressure. Otherwise, the resin bed would be destructed. The first band (deep pink) includes the Cy3-labeled protein. Fractions are collected until the solution is clear (around 8-10 mL) and pipetted into Eppendorf cups until further treatment. Store in the dark.

Millipore Amicon Ultra Filter Unit 10kDa 0.5 mL [E14]

500 µL of labeled protein solution are filled in the Amicon 10 kDa Filter Unit. Separation of unbound dye is proceeded at 1000 rpm for 10 minutes followed by 15 minutes at 2000 rpm. The labeled fraction remains in the filter unit and is carefully pipetted into an 0.65 mL Eppendorf cup and stored in the dark.

Melon Gel Spin Purification Kit [E15]

The Melon Gel IgG Purification System purifies antibodies by removing non-relevant proteins under physical pH allowing the antibody to flow through in a mild buffer suitable for storage and downstream applications.

Spin column procedure:

Melon Gel Support and Purification Buffer are equilibrated to room temperature. Furthermore, the support bottle is swirled to obtain an even suspension. 500 µL of slurry are dispensed into a Spin Column and placed in a microcentrifuge tube. The uncapped column/tube assembly is centrifuged for 1 minute at 6000 rpm (2000-6000 rcf (g)/4600-8000 rpm recommended), then the spin column is removed and flow-through is discarded. 300 µL of Purification Buffer are added and the centrifuge is pulsed for 30 seconds. This wash is repeated once. The bottom cap is now placed on the column. The labeled protein sample is mixed with 80 µL Binding buffer, added to the column and incubated for 5 minutes at room temperature with end-over-end mixing. The bottom cap is removed, the top cap is loosed and the column is re-inserted in the collection tube. Centrifuging is done for 1 minute to collect the purified antibody in the microcentrifuge tube. After a cleaning step, the gel may be regenerated three times without significant loss of selectivity.

Nab Protein A Plus Spin Kit [E16]

NAb Spin Kits are convenient for rapid, small-scale affinity purification of antibodies from a variety of sample types.

Spin column procedure:

Column and buffers are equilibrated to room temperature and the centrifuge is setted to 7300 rpm (5000 rcf (g)/7300 rpm recommended). The sample is diluted with 80 µL binding buffer. Top cap and bottom closure are removed and opened. The column is placed in an Eppendorf cup and centrifuged for 1 minute and the flow-through is discarded. The column is equilibrated by adding 400 µL of binding buffer and mixing briefly. Following centrifuging and discarding of the flow-through, the column is capped and the sample applied and incubated at room temperature for 10 minutes. Caps are loosened and removed and the column is centrifuged for 1 minute. This first collection tube contains the non-bound sample components. The sample column is transferred to a new collection cup and washed with 400 µL of binding buffer. Prior to a 1 minute centrifugation, the resin is briefly mixed with the sample. Two additional washes are made. 40 µL of neutralization buffer is added to a collection cup and the spin column is placed in it. After 1 minute of centrifugation, the sample is slided in the cup and neutralized.

The immobilized protein column may be used up to 10 times without significant loss in binding capacity.

7.5.3. Quantitative Protein Determination in Solution

Following removal of unbound fluorescence dye from the sample, the protein content has to be determined. At the beginning of labeling experiments 4 different methods are used, whereas three are photometric ones and one is fluorometric. Photometric approaches are based on intrinsic absorbance at 280nm, Bradford assay or BCA assay. Fluorescence intensity measurements are applied at 562 nm emission wavelength. Final determinations are always performed with the Bradford assay and fluorescence intensity measurements.

Pierce BCA (2, 2' – bicinchoninic acid) Assay

Chemicals:

1. BCA Reagent A (1% Na_2BCA in carbonate buffer, pH 11.25) and B (4% $CuSO_4 \cdot 5H_2O$ solution)
2. ultra-pure BSA stock solution (2 mg/mL)

BCA reagent: Mix Reagent A and B at a ratio of 50:1. The final solution has to be deep green.

Assay protocol:

BSA standard solutions are diluted from stock (2 mg/mL, Table 27).

Table 27. BSA standards

Standard / (mg/L)	20	80	100	300	600	800	1000	1500	2000
BSA stock / (mL)	1	4	5	15	30	40	50	75	100
PBS / (mL)	99	96	95	85	70	60	50	25	0

For Melon Gel samples, 10 µL of eluate is mixed with 90 µL PBS (1:10 dilution) and applicated as described below. Sephadex column samples are used without dilution. 100 µL of sample (standard or analyte) are pipette into a 2 mL reaction tube. 2 mL of BCA reagent are added and vortexed. The samples are incubated for 30 minutes at 37° C following cool-down to room temperature. Photometric determination is proceeded at λ = 562 nm with ultra-pure water as blank value. The assay scheme is presented in Figure 79.

Reaction scheme:

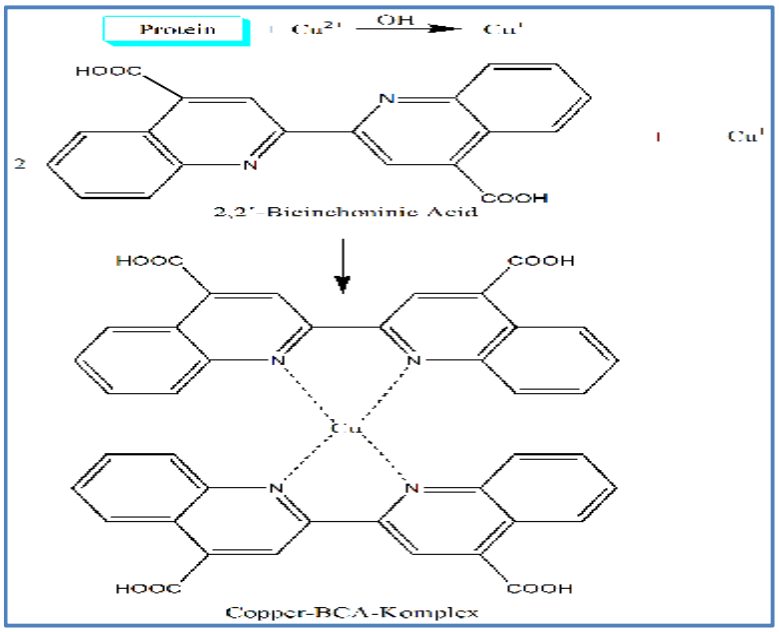


Figure 79. BCA assay scheme (adapted from **[E17]**)

<u>Bradford Assay</u>

Chemicals:

1. Homemade Bradford reagent:
   Coomassie Brilliant Blue G250, Phosphoric acid and Ethanol.
2. Roti Nanoquant (5-fold)
3. ultra-pure BSA stock solution (2 mg/mL)

Coomassie solution:

1. Homemade:

   25 mg Coomassie Brilliant Blue are diluted in 12.5 mL Ethanol and 75 mL of a phosphoric acid-water solution (1:3) are added. The stock solution is diluted 1:5 with ultra-pure water to obtain the ready-to-use reagent.

2. Roti Nanoquant:

   Ready-to-use as Coomassie stock solution. The stock solution is diluted 1:5 with ultra-pure water to obtain the ready-to-use reagent.

Assay protocol for microtiter plates:

BSA standard solutions are diluted from stock (2 mg/mL, Table 28):

Table 28. BSA standards

Standard / (mg/L)	**1,25**	**2,5**	**5**	**7,5**	**10**
BSA stock / (µl)	9	18,5	37,5	56	75
PBS / (µl)	141	131,5	112,5	94	75
Well complete / (µl)	150	150	150	150	150

BSA-Standards are pipetted in the standard wells according to Table 18. 1 µL of unlabeled antibody and 149 µL of PBS are pipette in a separate well for used as protein reference. For determination of the analyte, 2 µL of each sample and 148 µL of PBS are prepared in the corresponding sample analysis wells. Each standard, reference and analyte is prepared 4-fold on the plate. 150 mL of Coomassie solution is added to each well. The read-out is proceeded at 590 nm after 10-60 minutes reaction time with PBS-Coommassie as blank.

The reaction scheme is presented in Figure 80.

Reaction scheme:

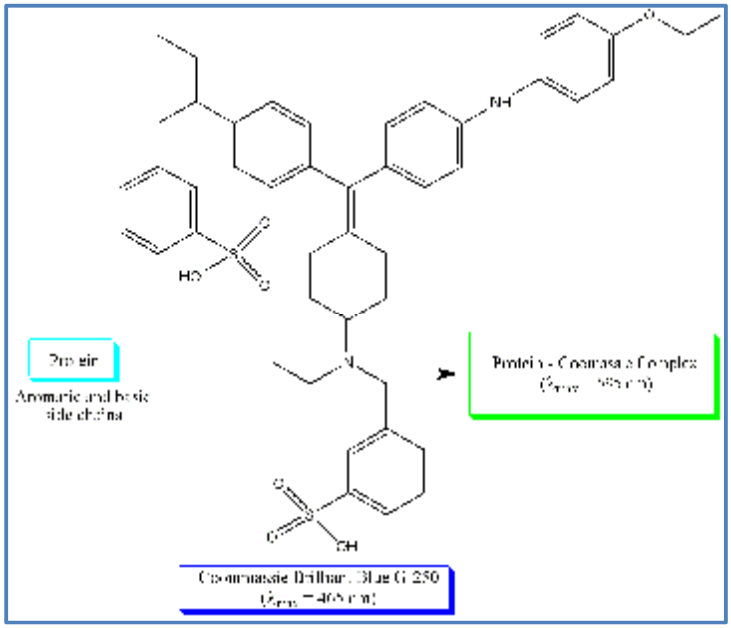


Figure 80. Bradford reaction scheme (adapted from **[E17]**)

7.5.4. Determination of the Dye-to-Protein ratio and Fluorescence-to-Protein ratio

Dye-to-protein ratio:

First, the absorbance has to be measured in order to calculate the Dye-to-Protein ratio. Ideally, the absorbance values reside in a region of 0.1.

The concentration is calculated using the Lambert-Beer equation:

$$A = \varepsilon * c' * d$$

$$c(Dye)' = \frac{A}{\varepsilon * d}$$

$$c(Dye) = DF * c(Dye)'$$

A: absorbance; ε: extinction coefficient; d: cuvette length (1cm); DF: dilution factor

The protein concentration is measured applying the BCA or Bradford assay. Bradford assay was setted as standard due to its robustness and compatibility to detergents. Most of the protein samples react dissimilarly with the reagents. So, a reference of the protein, the pure

protein, with a known concentration has to be codeterminated. Afterwards, absorbance values of the analyte measurements have to be corrected. In protein assays, mass concentration ß is widely used. To obtain the molar concentration c, the following equation 1 is used:

$$c(Protein) = \frac{\beta(Protein)}{M(Protein)}$$

The Dye-to-Protein ratio is calculated after equation 2:

$$\frac{D}{P} = \frac{c(Dye)}{c(Protein)}$$

With respect to fluorescence intensity and self-quenching, best results are attained with D/Ps of 1-2.

Fluorescence-to-protein ratio:

Fluorescence intensities F are measured at 549 nm excitation and 562 nm emission wavelength. The Fluorescence-to-Protein ratio is calculated according to equation 3:

$$\frac{F}{P} = \frac{F(Protein)}{c(Protein)}$$

7.6. Milk and Cheese Sample Preparation

Chapter 7.6 comprises preparation methods for raw milk and raw milk cheese samples.

7.6.1. Preparation of Raw Milk

Milk: Raw milk samples from Switzerland

Sample preparation protocol:

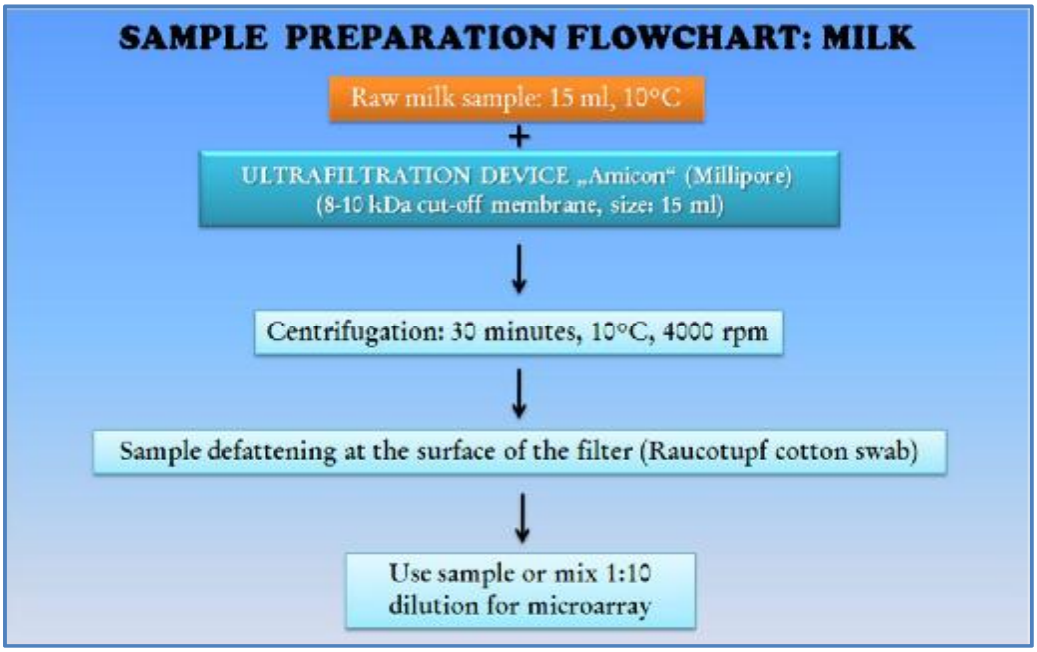


Figure 81. Sample preparation Flowchart for raw milk

15 mL of raw milk sample are filled in an Amicon Ultrafiltration device (10 kDa cut-off, 15 mL) and cooled to 10° C. The spin device is centrifuged for 30 minutes at 10° C and 4000 rpm (Figure 81). The solution remaining in the filter unit is the concentrated sample which is directly used for all microarray experiments. Preparation data is presented in Table 29.

Table 29. Sample preparation data

Sample #	Sample name	Spiking / (ng/ml)	Weight before centrifugation / (g)	Weight after centrifugation / (g)	V before centrifugation / (mL)	V after centrifugatio / (mL)
blank	Tankmilk 1 blank (centrif., 15 ml) Neg. tested	-	14,670	11,700	15	11,750
1	Tankmilk 12 (TM12)	-	1,985	-	-	-
2	Tankmilk 1 (spiked B 4ng/ml centrif., V=1 ml) (TM1 sp ZV1)	4 (SEB)	1,006	0,210	1	0,250
3	Tankmilk 4 (centrif., V=15 ml) (TM4 ZV15)	-	15,120	11,753	15	11,750
4	Tankmilk 6 (centrif., V=15 ml) (TM6 ZV15)	-	15,170	11,700	15	11,750

Sample #	Sample name	Spiking / (ng/ml)	Weight before centrifugation / (g)	Weight after centrifugation / (g)	V before centrifugation / (mL)	V after centrifugation / (mL)
5	Tankmilk 9 (centrif., V=15 ml) (TM9 ZV15)	-	14,670	11,200	15	11,250
6	Tankmilk 12 (centrif., V=15 ml) (TM12 ZV15)	-	15,180	11,397	15	11,500
8	Tankmilk 12 (spiked B 4ng/ml, V=1 ml) (TM12sp V1)	4 (SEB)	-	-	-	-
9	Tankmilk 1 (spiked B 4ng/ml centrif., V=15 ml) (TM1 sp ZV15)	4 (SEB)	14,610	11,210	15	11,250
10	Tankmilk 12 (spiked A 4ng/ml, centrif., V=1 ml) (TM12sp ZV1)	4 (SEA)	1,036	0,275	1	0,250

7.6.2. Preparation of Raw Milk Cheese

Cheese types: Raclette Cheese and Alpine Cheese (both 50% FDM)

Sample preparation protocol:

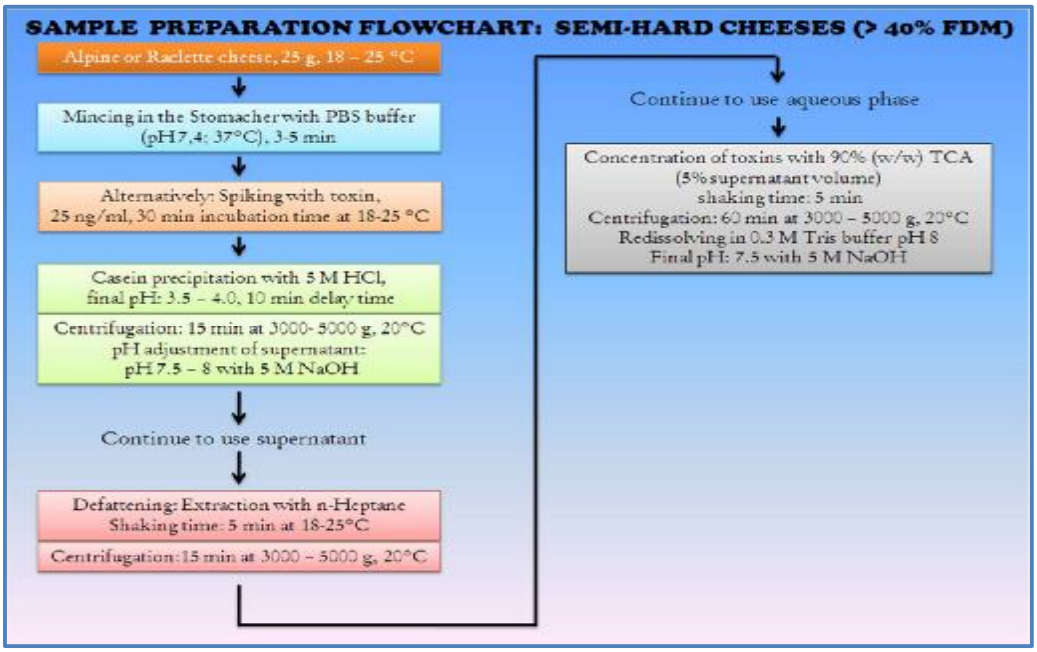


Figure 82. Sample preparation protocol for semi-hard cheeses

25 g of cheese sample and 250 mL PBS buffer (pH 7.4; T=37°C) were stomachered in a plastic bag for 5 minutes. The bag is heat-sealed and an edge is cut off to enable a clean and complete transfer of the suspension into a 500 mL Erlenmeyer flask. The suspension is incubated in a water bath for 30 minutes at 37° C with gentle agitation. Afterwards, it is transferred into a 1 L plastic beaker. The appropriate toxin spiking solution (25 ng SEA/g sample, made from stock solution 2 mg/mL) is added and shaked for another 5 minutes at 37° C in the water bath. The suspension is cooled down to room temperature outside the bath. All samples, except number 1 and 2, are spiked with SEA toxin. The milk proteins are removed by casein precipitation with concentrated HCl. The pH of the sample is measured, it should be around 6-6.5. 5M HCl is added up to a pH value of 3.5-4. Following this, proteins should immediately start to precipitate. The sample is transferred into a 500 mL centrifuge container and cooled down to 4° C. Centrifuging is done at 4° C and 4000 g for 10 minutes following 10 minutes delay time at 4° C. The upper phase is the sample, which is transferred to a beaker with the help of a serological pipette. The pH is tested and brought to pH 7.5-8 with 5 M NaOH. The solution is centrifuged again at 4° C and 4000 g for 10 minutes and reposed for another 10 minutes. The upper phase is transferred to a separation funnel and 100 mL of Heptane (15-25° C) is added. Shaking is done for 5 minutes in the funnel followed by centrifuging for 10 minutes at 4° C (4000 g). The bottom phase is the sample phase. The volume is transferred to five 50 mL Falcon tube by using a serological pipette. All samples, excluding number 1, are proceded with the heptane step. Precipitating of the toxins is done by adding 5% of the volume of 90% (w/v) TCA solution (2.5 mL per Falcon tube). Shaking is done for 30 minutes at 18-25° C. The sample is centrifuged for 30 minutes at 4000 g and 4° C. The supernatant is centrifuged for another 30 minutes. Re-union of the precipitates is done followed by solving them in 0.3 M Tris buffer pH 8(8-10 % Volume of the primary solution volume). The pH is brought to 7.5-8 with 4 M NaOH. Now, the solution is ready to use in experiments (Figure 82).

Samples R1-10 are originated from Raclette Cheese, the samples A1 and A2 from Alpine Cheese. Preparation data is presented in Table 30 and 31.

Table 30. Sample clean-up data

Sample #	m(Sample) / (g)	m(PBS) / (g)	Stomacher / (min)	Water Bath / (min)	Cool down to RT / (min)	SEA?	pH before casein precipitation; centrifugation after HCl / (min)
R1	25,22	253,8	5	30	45	no	3,8-4; 5
R2	25,13	256,8	5	30	45	no	3,8-4; 15
R3	25,12	252,4	5	30	45	yes	3,8-4; 20
R4	25,19	251,5	5	30	45	yes	3,8-4; 20
R5	25,39	251,3	5	60	45	yes	3,8-4; 10
R6	24,91	251,1	5	60	45	yes	3,8-4; 10
R7	26,51	256,7	5	30	45	yes	3,8-4; 10
R8	24,24	249,7	5	30	45	yes	3,8-4; 10
R9	24,11	251,3	5	30	45	yes	3,8-4; 10
R10	31,77	336,7	5	30	45	yes	3,8-4; 10
A1	25,47	42,25	3	-	30	yes	3,8-4; 30
A2	26,09	41,65	3	-	30	yes	3,8-4; 30

Table 31. Sample precipitation data

Sample #	# of cycles (Heptane); Centrifugation / (min)	TCA Precipitation: centrifugation / (min)	SEA level / (ng/g)	V(sample) after TRIS addition / (mL)	ß(SEA) in TRIS / (ng/mL)
R1	0; 0	15+23;	0	50	0
R2	3; 10	30;	0	20	0
R3	1; 10	2x30;	25,08	50	12,6
R4	1; 10	2x30;	25,01	45	14
R5	5 min shaking; 10	90 min shaking; 2x30;	24,81	27,5	22,9
R6	5 min shaking; 10	90 min shaking; 2x30;	25,29	25	25,2
R7	5 min shaking; 10	30 min shaking; 2x30;	23,76	23	27,4
R8	5 min shaking; 10	30 min shaking; 2x30;	25,99	42,5	14,8
R9	5 min shaking; 10	30 min shaking; 2x30;	26,13	25	25,2
R10	5 min shaking; 10	30 min shaking; 2x30;	26,44	22,5	37,3
A1	-	30 min delay time; 30	24,73	4,1	153,65

For tests with the VIDAS System, 0.5 mL of the sample was applied to the VIDAS SET2 reaction stick.

8. Summary and Outlook

8.1. In German

Im ersten Teil der Arbeit wurde auf Basis der Vorgaben des EU-Projektes „BIOTRACER“ ein kompetitiver Mikroarray zur Bestimmung der *Staphylococcus aureus* Enterotoxine SEA-SED und SEH in Milchprodukten entwickelt. Die Fluoreszenzintensität des Farbstoffes Cy3 wurde mit Hilfe eines Arrayscanners bei 562 nm Emissionswellenlänge detektiert. Das Ziel, den kompetitiven Array im gewünschten dynamischen Bereich von 0,1-10 ng/mL für alle fünf Toxintypen zu entwickeln, konnte nicht erreicht werden. Im Rahmen dieser Arbeit stellten die relativ unspezifischen Antikörper sowie Probleme bei der Mikroarray-Beschichtung nicht lösbare Herausforderungen im gegebenen Zeit- und Finanzerungsrahmen dar.

Zunächst wurde die bestehende Reinigungsmethode für Glas Slides hinsichtlich ihrer Qualität getestet und verschiedene Silanisierungsmethoden appliziert. Mit Hilfe von Fluoreszenz- und Kontaktwinkelmessungen an den silanisierten Slides konnte die Qualität als ausreichend für den Aufbau des Arrays befunden werden. Um die Bindung zwischen Antikörper und dem immobilisierten Toxin quantifizieren zu können, wurden alle Antikörper mit dem Fluoreszenzfarbstoff Cy3 markiert. Mehrere Ansätze mit Varianz in pH, molarem Quotienten, Aufreinigungsmethode, Alternativfarbstoff und Markierungszeiten wurden durchgeführt, um ein optimales Farbstoff-zu-Protein-Verhältnis und damit die beste Fluoreszenzintensität zu erhalten. Zur Abtrennung hoher Konzentrationen ungebundenen Farbstoffes wurde das Melon Gel Kit als optimal befunden. Erste Versuche zur Belegung der Antigenschicht mit Amino-silanisierten Glasslides, Nitrocellulosemembran-basierten Fast Slides und Epoxy-silanisierten Glasslides mit hydrophober Maske ergaben gute Ergebnisse für die letzten beiden Typen. Die Aminoslides schieden für das weitere Procedere aufgrund des umständlichen Handlings und der schlechten Reproduzierbarkeit aus. Auffällig war bei den Nitrocellulose Slides das sehr niedrige Signal-zu-Rausch-Verhältnis und der oft sehr hohe Hintergrund, der das Scanning und die Auswertung der Slides erheblich erschwerte. Im nächsten Schritt sollte für die gegebene Antigenschicht des primären Systems die optimale Konzentration an markiertem Primärantikörper ermittelt werden. Dies stellte sich als diffizil heraus, da trotz gleicher Optimierungsstrategie die Antikörper extrem unterschiedliche Eigenschaften aufwiesen und daher zum Erlangen relativ ähnlicher Intensitäten sehr

unterschiedliche Konzentrationen notwendig waren. Einzig die Konzentration für <SEA> und <SEB> konnte als gleich angenommen werden, auch auf den Nitrocellulose Slides lies sich dies bestätigen. Bedingt durch die Unterschiede in Konzentration und Fluoreszenz-Intensität musste für den Array eine Fluoreszenznormierung gemacht werden, die anschließend rechnerisch in jeden Array Einzug halten musste. Im nächsten Schritt wurden verschiedene Konzentrationsreihen an kompetitiven Toxingehalten und Toxinsorten durchgeführt. Nach mehreren Versuchen ergaben sich lineare Bereiche, die allerdings ausserhalb des geforderten Bereiches liegen. Immer wieder auffällig sind im primären System bei allen Toxinarten und Slidetypen die relativ hohen Standardabweichungen zwischen 15 und 30% und die schlechte Reproduzierbarkeit der linearen Bereiche der einzelnen Toxinarten. Desweiteren konnte das niedrige Signal-zu-Rausch-Verhältnis und der oft sehr hohe Hintergrund bei den Nitrocellulose Slides nicht verbessert werden, was letztendlich zum finalen Ausschluss dieses Slide-Typs für weitere Experimente führte. Ergebnisse der Kalibrationsreihen in Milch zeigten identische Ergebnisse zu jenen in Puffer, die die Basis für den Array bilden. Als Abschluss der Experimente des primären Systems mit markierten primären Antikörpern wurde eine Applikation mit realen Proben durchgeführt. Dafür wurde unbehandelte und künstlich kontaminierte Rohmilch aus einer Probensammlung in der Schweiz verwendet. Prinzipiell, aber nur sehr eingeschränkt, funktioniert das Arraysystem auch mit der Realprobe, zeigt aber wieder die Schwächen des ungenügenden dynamischen Bereiches auf. Im Anschluss an die Messungen mit Realproben wurde das erste sekundäre System etabliert, in dem die Konzentrationen für Antigen- und Antikörperschicht beibehalten wurden, lediglich der markierte Antikörper wurde durch einen nicht markierten ersetzt und die Detektion mittels eines Cy3-gelabelten Sekundärantikörpers durchgeführt. Ziel dieses Ersatzes war die Eliminierung der Fluoreszenznormierung, da nur ein Sekundärantikörper notwendig ist. Hinsichtlich Intensität und Varianz ergaben sich gute Werte für diese System im kompetitiven Test, jedoch war der lineare Bereich wiederholt ungenügend. Als nächster Schritt wurde ein weiteres sekundäres System etabliert, jedoch mit komplett neuen Konzentrationsparametern in jeder Schicht. Dies führte wiederholt zu guten Intensitäts- und Signal-zu-Rausch-Verhältnissen und zu niedrigeren Fehlern. Jedoch ließ sich der dynamische Bereich weiterhin nicht senken. Das primäre und das sekundäre System wurden desweiteren auf die Kreuzreaktivität ihrer verwendeten Antikörper getestet. Im Falle des Primärsystems ergab sich eine hohe Kreuzreaktivität, die teilweise auch auf die

unterschiedlichen Markierungseffizienzen und Intensitäten zurückzuführen ist und auch mittels Normierung nicht zu beseitigen war. Im Sekundärsystem waren die Kreuzreaktivitäten relativ niedrig, beinahe optimal. Letztendlich ist der dynamische Bereich des Arrays noch in größeren Konzentrationsbereichen als der gewünschte linearen Bereich, jedoch ist der Grundstein gelegt und das System bereits an einer Realprobenreihe getestet.

Im zweiten Teil dieser Arbeit wurde ein Vergleichssensor zur Bestimmung von *Staphylococcus aureus* Enterotoxinen in Milch auf Basis der Oberflächenplasmonenresonanz-Spektroskopie entwickelt. Durch Aufrtragung einer selbstorganisierenden Monoschicht auf der Oberfläche eines mit Gold beschichteten Glaschips konnten die Toxine SEA und SEB reproduzierbar immobilisiert und deren Bindung mit den entsprechenden Antikörpern charakterisiert werden. Ein kompetitiver Assay aus Toxin und Antikörper, der direkt an der Realprobe appliziert wurde, brachte den Zugang zu Kalibiergeraden, mit denen es möglich war, den Toxingehalt der Milch direkt zu bestimmen. Weitere Optimierung und Untersuchung des Systems erscheint noch nötig. Bis zum jetzigen Zeitpunkt konnte noch nicht geklärt werden, wie sich veränderte Monoschichten auf Immobilisierung und unspezifische Bindungen auswirken. Beispiel dafür wäre eine Erniedrigung des Anteils der Carboxylgruppen und Ersatz derselben durch Hydroxidgruppen. Die Kreuzreaktionen verschiedener Antikörper-Toxin-Paare wurden bereits auf dem Array untersucht und im Falle eines sekundären Systems für niedrig befunden. Diese Spezifikation müsste noch auf das SPR-System übertragen werden, um in Zukunft mehrere Toxine parallel auf einem Chip vermessen zu können. Großer Optimierungsbedarf besteht jedoch hinsichtlich des Zeitfaktors. Die komplette Toxin-Untersuchung einer Milchprobe benötigt derzeit bei vollständig vorbereiteter und kalibrierter Messzelle 50 Minuten inklusive aller Waschschritte. Im Vergleich zu konventionellen Methoden wie der ELISA kann zwar erheblich Zeit eingespart werden, jedoch ist mit HPLC ein ähnlich schnelles oder schnelleres Ergebnis zu erwarten. Ansatzpunkt böte der Versuch, die Anfangssteigung der Kinetikkurven mit den Konzentrationen zu koppeln. Die Auswertung der bisherigen Ergebnisse lässt eine einfache Abhängigkeit vermuten, welche die Messzeit erheblich reduzieren würde. Kosten-optimierung kann zusätzlich vorgenommen werden. Derzeit werden alle Immobilisierungen mit einer Massenkonzentration von 50 mg/L vorgenommen. Diese könnte möglicherweise ohne erhebliche Reaktionszeitverlängerung verringert werden. Zusammenfassend ist zu sagen, dass im Zuge dieser Arbeit wichtige Grundsteine für die neu konstruierte spezifische

SPR-Sensorplattform der Enterotoxine gelegt wurden, jedoch noch eine Vielzahl an Optimierungs- und Variationsmöglichkeiten vorhanden ist. Bemerkenswert ist die Vielseitigkeit und Nachweisstärke der SPR-Spektroskopie, die durchaus mit den etablierten Methoden der Analytik und Sensorik in Konkurrenz treten kann.

8.2. In English

In the first part of the work, an array on a competitive assay for the detection of *Staphylococcus aureus* Enterotoxins SEA-SED and SEH in milk products was developed due to the regulations of the project "BIOTRACER". The fluorescence intensity of Cy3 was detected with an array scanner at 562 nm emission wavelength. The goal to design the competitive array within the desired dynamic range of 0.1-10 ng/mL for all five toxins was not achieved. The unspecifity of the available antibodies and problems with the silanization of the array depicted permanent challenges within the scope of this research.

First of all, well-known cleaning methods for glass slide were tested with regard to their surface coating quality and different silanization methods were applied. The quality was considered as acceptable for array design by means of fluorescence and contact angle measurements. All five antibody types were labeled with Cy3 to quantify the binding of antibody with immobilized toxin. Multiple labeling experiments with variation in pH, molar ratio, cleaning procedure, kind of fluorescence dye and labeling time were proceeded to achieve an optimal dye-to-protein ratio and therefore the best fluorescence intensity. The Melon Gel Kit was adjudged optimal for the removal of high concentrations of unbound fluorescence dye. First trials for antigen occupancy on amino-silanized glass slides, nitrocellulose slides and epoxy-silanized glass slides with hydrophobic pattern yielded in acceptable results for the last two slide types. Amino slides were eliminated for further experiments due to the circumstantial handling and the poor reproducibility of the intensity values. The nitrocellulose slides exhibited very poor signal-to-noise ratio and high backgrounds which complicated scanning and analysis of the slides extensively. As a next step, the optimal concentration of labeled primary antibodies should be identified for the antigen layer of a primary system. This can be considered as difficile. Despite to identical optimization strategies, the antibodies had extremely differing characteristics and therefore

very different antibody concentrations were necessary for reaching similar intensities. Only the concentrations for <SEA> and <SEB> were identical, which was proven on Nitrocellulose Slides too. Fluorescence normalization was necessary for each array because of the disparities in concentration and fluorescence intensity. Furthermore, the normalization had to be integrated in all array analyses. As a next step, different competitive concentration rows were made for all toxin types and with differing toxin concentrations. Linear concentration ranges were achieved after several trials, indeed they are not within the requested concentration ranges. Within the primary system there are significant noticeable problems for all toxin types and slide types: high standard deviations between 15 and 30%, poor reproducibility of the linear ranges of single toxin types, low signal-to-noise ratios and often high background when using Fast Slides. These problems could not be overcome, so Nitrocellulose Slides were eliminated for further measurements. The results of the calibration rows in milk were identically to those in PBS buffer, which build the base of the array. An application of real samples was made as closure for the experiments within the primary system. Therefore, untreated and artificially contaminated milk samples from Switzerland were used. In principle, real samples are applicable to the array system with limitations in insufficient linear ranges. Subsequent, a first secondary system was established. The given antigen layer and primary antibody concentrations were kept constant, only the labeled antibody was changed against an unlabeled one. Detection was carried out with a secondary, Cy3-labeled polyclonal antibody. The goal of this step was the removal of the necessity of fluorescence normalization. Within competitive tests, proper intensity and variance values were achieved, with limitation in the insufficient linear ranges. Another secondary system was established, but with completely new parameters for every layer. This leaded to acceptable intensities, signal-to-noise ratios and deviations repeatedly. However, the linear range could not be decreased either. Furthermore, both system types were tested for the cross-reactivity of their antibodies. The primary system exhibits a high cross reactivity which could be partially attributed to different labeling efficiencies and intensities that were not removable by normalization. The secondary system exhibits relatively low and almost optimal cross reactivities. Finally, the array is not ready to achieve the desired linear range, but the corner stone is made and the system was already tested with real samples.

Second part of this work was the development of a competitive SPR sensor platform for the detection of *Staphylococcus aureus* Enterotoxins in milk. Based on a self-assembling monolayer on the gold surface of a SPR chip, SEA and SEB were reproducibly immobilized and the interaction with the corresponding antibodies was characterized. A competitive assay was used to establish calibration curves in a real sample. Furthermore, it was possible to determine the toxin concentration in milk directly. Further optimization and analysis of the system seems to be necessary. At the moment, it is not explained, how modified monolayers behave with regard to immobilization and unspecific binding. A decreasing proportion of carboxyl groups and exchange of them through hydroxyl groups could be an example. The cross reaction of different antibody-toxin couples was already tested on the array and was proven to be optimal for secondary systems. The specifications have to be carried forward to the SPR system to enable multi-toxin measurements on one chip. Optimization in time management is advised. Complete toxin detection in milk samples takes 50 minutes, including all washing steps and requiring a completely prepared sensor chip. Compared to conventional methods like ELISA, SPR seems to be a time-saving method, but HPLC is comparably powerful or faster. Coupling of the starting slope of the kinetic curve with the concentration could be a starting-point. The analysis of the previous conclusion allows suppose of a simple dependency, which could reduce the measuring time. Cost-optimization could be another point. At the moment, all immobilizations are done with 50 mg/L. This might be possible with lower concentrations and without enormously prolonged reaction time.

In summary, important mile stones, such as LOD and processing time, for the newly developed specific SPR sensor platform of the Enterotoxins are taken as a base herein, but further optimization and variation is neccessary. Remarkably, SPR spectroscopy is versatile and conformatory and competes with other methods in analytics and sensor technology.

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10. List of Publications and Presentations

10.1. Publications

Simone S. Moises, Michael Schäferling; Toxin immunosensors and sensor arrays for food quality control, BioanalRev (2009) 1:73–104

10.2. Poster Presentations

Simone Moises, Michael Schäferling: Development of a competitive protein immunoassay Microarray for the fluorescent detection of the Staphylococcal enterotoxins A - D and H in dairy products. *1st Annual Biotracer General Meeting, November 7-10, 2007, Athens, Greece; 2nd Annual Biotracer General Meeting, July 4-10, 2008, Dublin, Ireland; 3rd Annual Biotracer General Meeting, June 22-26, 2009, Berlin, Germany.*

Simone Moises, Michael Schäferling: Development of a competitive protein immunoassay Microarray for the fluorescent detection of the Staphylococcal enterotoxins A - D and H in dairy products. *ISCS, September 7-11, 2008, Bologna, Italy.*

11. Curriculum Vitae

Personal Data

Surname	Moises
First Name	Simone Stefanie
Date of Birth	02.03.1982
Place of Birth	Regensburg
Nationality	German
Adress	Eisgraben 6a, 84051 Essenbach

Education

09/1988 - 07/1992	Grundschule Ampfing
09/1992 - 07/1998	Ruperti-Gymnasium Mühldorf am Inn
09/1998 - 06/2001	Hans-Leinberger-Gymnasium Landshut an der Isar
06/2001	Abitur

Studies

10/2001 - 04/2006	Chemistry (Diploma) at the University of Regensburg
05/2006 - 12/2006	Diploma work at the Institute for Analytical Chemistry, Chemo - and Biosensors, supervisor: Prof. O.S. Wolfbeis / Dr. R. Schupfner Subject: "Determination of the distribution factor α of tritiated DNA"
12/2006	Academic degree: Diplomchemikerin
01/2007 - 06/2007	Scientific employee at the Institute for Analytical Chemistry, Chemo - and Biosensors

Since 07/2007	Ph.D. work at the Institute for Analytical Chemistry, Chemo - and Biosensors (head: Prof. Wolfbeis, PD Dr. M. Schäferling) Subject: "Development of a competitive protein immunoassay Microarray for the fluorescent detection of the Staphylococcal enterotoxins A - D and H in dairy products"

Extracurricular activities

06 - 09/2001 03/2002 03/2003	Working student at E.ON GmbH, Landshut an der Isar
01 - 03/2004 10 - 12/2005 11 - 12/2006	Student research assistant at the University of Regensburg
08/2009 - 09/2010	Working student at GENEART AG, Regensburg
09/2010 -	Working student at PreSens GmbH, Regensburg

12. Affidavit

ERKLÄRUNG

Hiermit versichere ich, dass ich die vorliegende Arbeit selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Essenbach, 30.09.2010

Simone Moises