ICP-MS based analytical screening of phosphorylated and labelled proteins

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Abstract

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is used already for many biological applications. Apart from qualitative analysis, this technique offers quantitative information of elements in proteins which is important in determining the state and changes of the biological system. Utilizing this advantage, two different approaches were investigated in this work to detect and quantify phosphorylated and labelled proteins using Laser Ablation (LA) ICP-MS. The first approach is based on direct detection of natural hetero-elements being present in nearly all proteins, especially $^{31}P^{+}$, and the second one involved the detection of proteins via controlled labelling by means of lanthanides using chelating complexes and staining methods.

LA-ICP-MS was used in this work for detection of phospho-proteins after separation with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transfer onto a membrane. Measuring phosphorus in proteins by this method, linearity of the calibration in the range from 20 to 380 pmol was achieved with good reproducibility and sufficient sensitivity to realize a detection limit of 1.5 pmol. Quantification of phospho-proteins was performed with standards by 1) dotting onto membranes and 2) SDS-PAGE separation and blotting. The latter quantification procedure was applied to study the changes of the phosphoproteome induced by epidermal growth factor and hydrogen peroxide of an urothelial cell line 5637.

In the second part of the work, a procedure was developed to simultaneously detect and quantify multiple proteins in a mixture via labelling techniques using LA-ICP-MS in Western blots. Different labelling procedures were investigated using bi-functional chelating agents namely, 2-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and diethylenetriaminepentaacetic acid dianhydride (DTPA). The assay, using DOTA and iodination for labelling of proteins and antibodies, was optimised for ICP-MS detection and were combined to investigate different enzymes of Cytochromes P450 concomitantly. By this method a generic approach for detecting different proteins simultaneously could be established.

Zusammenfassung

Die induktiv gekoppelte Plasma-Massenspektrometrie (ICP-MS) wird bereits für viele biologische Anwendungen eingesetzt. Diese Methode liefert qualitative und quantitative elementspezifische Informationen, die zur Bestimmung des Zustands und der Veränderung eines biologischen Systems wichtig sind. Diese Fähigkeit wurde in dieser Arbeit genutzt, um zwei unterschiedliche Methoden zu untersuchen, die dazu dienen sollen, phosphorylierte und markierte Proteine durch Laserabtrags- (LA) ICP-MS zu bestimmen. Die erste Anwendung basiert auf der direkten Detektion natürlich vorkommender Hetero-Elemente in Proteinen, wie z.B. $^{31}P^{+}$ in phosphorylierten Proteinen. Die zweite Anwendung beinhaltet den Nachweis von Proteinen durch kontrolliertes Markieren mithilfe der Lanthanide unter Einsatz von Chelat-Komplexen und Färbe-Methoden.

Die Laserabtrags-ICP-MS wurde zum Nachweis von Phosphor-Proteinen nach Trennung mittels Natrium Dodecyl Sulfat Polyacrylamid Gel Elektrophorese (SDS-PAGE) eingesetzt. Mit dieser Methode konnte eine Linearität im Bereich von 20 bis 380 pmol Phosphor in Proteinen mit guter Reproduzierbarkeit erreicht werden und außerdem eine ausreichende Empfindlichkeit um eine Nachweisgrenze von 1,5 pmol zu verwirklichen. Eine Quantifizierung von phosphorylierten Proteinen wurde durch zwei unterschiedliche Methoden versucht: Auftrag von Phosphorstandards auf die zu messende Membran und durch Verwendung eines Standardproteins während der Trennung. Dieses Quantifizierungsverfahren wurde angewandt, um die Veränderungen des Phosphor-Proteoms zu untersuchen, das durch den Epidermalen Wachstumsfaktor (EGF) bzw. durch Hydrogenperoxid (H_2O_2) in der Harnblasen-Zelllinie 5637 erzeugt wurde.

In dem zweiten Teil der Arbeit wurde ein Verfahren entwickelt, um mehrere Proteine in einer Mischung gleichzeitig nachzuweisen und zu quantifizieren, und zwar durch Markierungstechniken unter Einsatz der LA-ICP-MS an Western Blots. Unterschiedliche Markierungsverfahren wurden untersucht, basierend auf der Verwendung von bi-

funktionalen Chelatbildnern, nämlich 2-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododekan-1,4,7,10-tetraessigsäure (DOTA) und Diethylen-triamin-pentaessigsäure Dianhydrid (DTPA). Die Proteine und Antikörper wurden mit DOTA und Lanthaniden markiert, jodiert und für den ICP-MS Nachweis optimiert, um unterschiedliche Enzyme von Cytochrom P450 gleichzeitig zu untersuchen. Durch diese Methode konnte ein allgemeines Verfahren zum gleichzeitigen Nachweis verschiedener Proteine ausgearbeitet werden.

Abbreviations

1D	One-Dimensional
2D GE	Two-Dimensional Gel Electrophoresis
2D DIGE	Two-Dimensional Differential Gel Electrophoresis
3-MC	3-Methylcholanthrene
Ab	Antibody
APS	Ammoniumperoxodisulphate
Ar	Argon
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Cer	Cerium
cps	counts per second
CYP	Cytochromes P450
DIGE	
DNA	Deoxy-ribonucleic acid
DTPA	Diethylenetriaminepentaacetic acid dianhydride
DTT	Dithio-DL-threitol
ECL	Electrochemiluminescence
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme Linked Immunosorbent Assay
EROD	Ethoxyresorufin-O-deethylase
ESI-MS	Electro Spray Ionization-Mass spectrometry
Eu	Europium
FCP	Folin-Ciocalteu's Phenol
FCS	Fetal calf serum
h	hour(s)
He	Helium
HEPES	(4-(2-hydroxethyl)-1-piperazineethanesulfonic acid)
Ho	Holmium
HR-ICP-MS	High Resolution – Inductively Coupled Plasma –Mass Spectrometry
I	Iodine
ICP-QMS	Inductively Coupled Plasma – Quadrupole Mass Spectrometry
ICP-SF-MS	Inductively Coupled Plasma – Sector Field – Mass Spectrometry
IgG	Immunoglobulin
kDa	kilo Daltons

LA-ICP-MS	Laser Ablation – Inductively Coupled Plasma – Mass Spectrometry
LC	Liquid Chromatography
LOD	Limits of detection
LR	Low Resolution
MALDI TOF-MS	Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry
MCN	Microconcentric nebulizer
min	minute(s)
MR	Mass Resolution
MW	Molecular weight
NC	Nitrocellulose
P	Phosphorus
PAH	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffer saline
PE	Polyethylene
PFA	Perfluoroalkoxy
PMT	Photon Multiplier Tube
ppb	parts per billion
p-SCN-Bn-DOTA (referred as DOTA in this work)	2-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RT	room temperature
s	second(s)
S	Sulphur
SDS-PAGE	Sodium Dodecyl Sodium Sulphate Polyacrylamide Gel Electrophoresis
SEM	Secondary Electron Multiplier
SILAC	Stable isotope labelling by amino acids in cell culture
Tb	Terbium
TEMED	Tetramethylethylendiamine
TXRF	Total Reflection X-ray Fluorescence

Chapter 1

Introduction

1.1 Inductively Coupled Plasma Mass Spectrometry

Inductively Coupled Plasma Mass Spectrometry is a highly sensitive mass spectrometry technique that is used for elemental analysis. It was developed in the late 1980s by Houk *et al.*[1] and Gray.[2] The instrumentation is capable of multielemental analysis often at the parts per trillion levels in solutions. It is widely used in the fields of geochemistry, environmental, life and forensic sciences and archaeology.[3] It is also the method of choice in the speciation analysis of certain metals such chromium and arsenic.

In ICP-MS samples are introduced to an argon plasma torch which has very high temperatures (6000-10000 K) sufficient to vaporize, atomize, ionize and excite all elements in the periodic table.[4] The plasma is a highly ionized argon gas maintained via the high frequency electrostatic and magnetic fields generated by an induction coil in a symmetrically flowing argon gas. In the plasma all molecular information of the analyte is lost, but on the other hand the ionization process is more or less decoupled from the matrix, so that substance independent calibration procedures can be used for quantification. This ease of quantification is one of the most important features of ICP-MS and is one of the reasons for this feasibility study.

A fraction of the ions formed in the plasma is extracted into a low pressure interface containing both a sampler and a skimmer cone. The ions are then focused and transmitted to a mass analyser via an ion lens system prior to detection by either an ion counting or an analogue detector.

In recent times due to the improvements in its instrumentation ICP-MS is more and more used in the biological research for the determination of metals in the biological environment. The advantages of using ICP-MS in biological applications are 1) its ease of quantification due to compound independent elemental response, 2) high sample throughput, 3) low detection limits of many elements of the periodic table, 4) wide linear dynamic range, 5) concomitant elemental detection, 6) minimal matrix interferences, 7) high sensitivity and 8) hyphenation capabilities. Because of these advantages this

technique is already used for trace metal analysis in a wide variety of biological samples, for e.g. bones and teeth,[5] blood, hair and urine,[6] tablets[7] etc. The benefit of ICP-MS is that it is useful for detecting elements at very low levels in complex sample mixtures. Utilizing these features, this technique looks promising in new application areas for instance in biological research, and in particular in proteomics.

Being a metal detector, ICP-MS can be used to detect and analyse proteins via their hetero-element compositions, and for instance ICP-MS is already used for qualitative detection of metalloproteins present in a biological environment.[8] However, the interactions of the metals with the proteins are always not strong, they can be lost during the sample preparation steps and it cannot be used efficiently for experiments where quantification is needed. Alternatively hetero-element tagged proteins (naturally present or labelled) where elements are covalently bound to the proteins can be utilized for indirect quantification of proteins using ICP-MS. Thus inorganic MS is extremely suitable for calibration and quantification of proteins since it shows high sensitivity for detection of elements and wide dynamic detection range.[9] Since it detects only the amount of element present in the proteins simple standards can be used for the quantification of proteins avoiding other complex methods. Considering these aspect ICP-MS can be applied as one of the complementary tools in the field of proteomics related research, especially in quantitative proteomics.

1.2 Definition and Methods in Proteomics

The term `Proteomics' describes the study of the proteome of an organism (or cell) expressed by its genome.[10] It represents the large scale study of proteins, their structures, functions and also their post translational modification such as phosphorylation. The proteome is a highly dynamic system which changes in response to various intra and extracellular environmental signals which can lead to different signalling pathways. The protein composition present in a system varies qualitatively and quantitatively. In addition to the qualitative detection, quantification of proteins is important to determine the biological states of the system.[11] For instance in medicine, quantification of proteins can be used to compare 2 different systems such as healthy and diseased patients which can lead to biomarker discovery.[12] There exist different methods

to evaluate these qualitative and quantitative processes. Some of them will be discussed in the following paragraphs. Many of these methods are tedious and time consuming and do not always give reproducible results. New developments are necessary in this area in addition to the improvements in the presently available methods. There is also lack of comparable and complementary methods which can reinstate the findings.

As mentioned before, the basic aim of the proteomic methods is to identify, detect and quantify the whole proteins of a cell, tissue or organism. Depending on the complexity of the protein samples and the requirement different combinations of methods can be chosen. Initially proteins are separated by using gel electrophoresis, high performance liquid chromatography (HPLC) or capillary electrophoresis. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional gel electrophoresis (2D GE) are the traditional gel separation techniques and size exclusion, reverse phase, ion exchange etc. are the established HPLC based methods.

The most powerful tool for protein separation is 2D GE which can separate proteins in 2 dimensions, first by isoelectric focusing and second by SDS-PAGE. The protein spots can be picked and subjected to tryptic digestion and the resulted peptides are identified using organic MS and databank research.[13] This combination of methods requires large amounts of proteins and it is a laborious process and has drawbacks such as low resolution.[14] In another way, these 2D gels can be stained for visualization of the proteins with different techniques such as silver nitrate staining, fluorescent reagents, Coomassie blue etc. With the specific gel stains up to 4 different patterns can be compared and up and down regulated proteins can be identified. For example by comparing the total protein stained gels with the phosphorus stained gels the phosphorylation degree could be determined.[15] But the sensitivity and detection limit of these methods rely on specific gel stains.

Alternatively after separation by 1D or 2D GE, these proteins can be visualized by radioactivity or luminescence methods. Traditional methods such as radioactive labelling involve hazardous material handling and the experiments can be performed only at specialized labs. The proteins can then be identified after proteolytic digestion and sequenced using Edman degradation.[16] But this method also requires abundant protein samples.

As mentioned earlier organic mass spectrometry such as Electrospray Ionization Mass Spectrometry (ESI-MS) and Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) can help to identify proteins after 2D separations.[13]

For introduction of protein samples in the MS liquid chromatography methods are often used. Molecular mass of the proteins can be estimated because of its soft-ionization nature. Peptide mass fingerprinting is the combination of separation techniques with organic MS based methods. With the help of databank search it is used to identify the proteins. It has many advantages such as it works over wide mass ranges. But the disadvantage of peptide mass fingerprinting is that it is less accurate for non purified proteins and cannot handle complex protein mixtures.[17] It has little success for identification of proteins which are not in the database and hence it is limited to certain protein sequences.

The quantification of proteins is also an important parameter needed in the clinical proteomics field, e.g. in biomarker discovery and diagnostics of various diseases. ESI-MS and MALDI–TOF-MS are increasingly used in combination with isotopic labelling methods for relative quantification of proteins.[18] Proteins can be labelled via chemical, enzymatic and metabolic methods. Their advantages are discussed in chapter 4. Alternatively for naturally presented trace metals in proteins the labelling step can be avoided by directly detecting by ICP-MS. For example using ICP-MS $^{31}P^{+}$ can be directly detected and quantified in proteins where it is covalently bound to it. Some of the drawbacks of using organic mass spectrometry for quantification of phospho-proteins are discussed in section 3.1.1.

1.3 Previous Work

At the “Institute of Analytical Sciences - ISAS” in the past 20 years ICP-MS was used for trace metal analysis and speciation studies. For the past 10 years hetero-elements have been investigated to be used for detection of bio-molecules. First examples are presented for DNA analysis where phosphorus of the DNA backbone was used as a natural occurring tag for quantification of DNA-adducts formed with styrene oxide[19] and melphalan.[20]

The concept of analysing protein phosphorylation using capillary LC coupled with ICP-MS for $^{31}P^{+}$ detection was demonstrated by Wind *et al*.[22] The sample used was a complex mixture of synthetic phosphopeptides and a set of tryptic digests of three phospho-proteins. The identification of phosphopeptides was performed using capillary LC interfaced alternatively to ICP-MS and ESI-MS. With the ICP-MS a detection limit of approximately 0.1 pmol could be achieved using this method. With this method using $^{31}P^{+}$ detection high selectivity could be achieved and the signal intensity obtained was directly proportional to the molar amount of $^{31}P^{+}$ in the capillary LC eluate. Thus it should be studied in more detail how feasible this new approach is for phosphoproteomics studies.

After phosphopeptide identification, the use of ICP was extended to phosphorus detection of phospho-proteins after SDS-PAGE separation and blotting onto membranes.[21] Laser ablation was used as the hyphenation technique for ablation of the membrane material and the dry sample is then transported to the ICP-MS. The LA cell used in this study had a length of 100 mm and a width of 10 mm into which the membranes were fixed. So each lane of the membranes had to be cut in order to fit to the geometry of the cell and only small fractions of the whole membrane can be used for the detection at the same time. For the next sample the membranes had to be exchanged and it is a very time consuming step. Apart from these limitations this method was well suited for the detection of a mixture of myoglobin, α-casein and the subunits of fibrinogen. Normalizing the $^{31}P^{+}$ detection signal from a single laser ablation trace by the total amount of phospho-protein applied to the gel, a detection limit of 5 pmol of phosphorus could be estimated.

Due to the success of these first experiments a new laser ablation cell was constructed in which the whole membrane can be analysed in one run. And it was the main aim of this study to explore the capabilities of this new cell and to develop novel applications in the area of proteomics.

1.4 Aim of the Study

The use of LA-ICP-MS in proteomics can be exploited in 3 different ways, 1) by way of naturally tagged proteins, e.g. phosphorus, sulphur etc., 2) by staining with metal-

containing stains and 3) through bio-conjugation, i.e. labelling of proteins with elements via chelating agents, thereby proteins can be detected and quantified. This work is aimed at improving ICP-MS based applications in proteomics research and can be broadly classified into three topics.

1) Phosphoproteomics

The first objective of the work is to develop a generic approach for phosphoproteomics allowing the analysis of phospho-proteins using LA-ICP-MS and gel electrophoresis. As mentioned before, in a previous work of Wind *et al.*[22] it was already demonstrated that linear relationship between the $^{31}P^{+}$ intensity and the amount of a standard phospho-protein exists, but it was not sure if this could be elaborated to a new concept for phosphoproteomics. Thus it was one aim of this study to develop methods for quantification of the phosphorylation status of proteins, and test them at hand of a model system, the human urothelial carcinoma cell line 5637.

2) Labelling and staining of proteins

The second objective is to try different staining and labelling methods in order to simultaneously detect and quantify the target protein which does not contain an LA-ICP-MS detectable element. In this part it is necessary to investigate different labelling strategies using chelating complexes such as 2-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (p-SCN-Bn-DOTA, referred as DOTA in this work) and eventually the diethylenetriaminepentaacetic acid dianhydride (DTPA). For this purpose the procedures for labelling of proteins via p-SCN-Bn-DOTA have to be optimised for detection by LA-ICP-MS.

3) Labelling of antibodies

The third objective is to detect and quantify multiple antigens after SDS-PAGE and Western blotting using differentially labelled monoclonal antibodies and by employing LA-ICP-MS as the detection method. A standard procedure for labelling of antibodies using DOTA and iodination described in literature will be applied too. Antigen samples (groups of structurally similar enzymes) and purified monoclonal antibodies were provided by PD. Dr. Peter Roos from IfADo, Dortmund and this work was done in close collaboration.

Finally it should be mentioned that at the beginning of this thesis work, ISAS was just starting its bioactivities and thus the expertise and experience with biochemical work flows in proteomics was not available. In particular there was little experience with protein separations based on SDS-PAGE and detection of proteins by conventional staining and detection assays. Therefore it was one of the main goals of this thesis to build up the infrastructure needed and to test different procedures described in textbooks and literature and to optimise some of these procedures for ICP-MS detection strategies.

Chapter 2

Principle, Instrumentation and Experimental

2.1 Principle

2.1.1 Inductively Coupled Plasma Mass Spectrometry

ICP-MS is a highly developed analytical technique used for elemental determinations. It combines the high temperature argon plasma with the mass spectrometer. The ions that are produced by the plasma source are separated and detected by the mass spectrometer. The majority of ICP-MS instruments utilise quadrupole based mass analysers. But the resolution that is offered by these mass analysers is not sufficient for analysing elements which are prone to argon plasma, solvent or sample based spectral interferences. For instance detection of phosphorus with its only isotope at mass 31 is hindered by various polyatomic interferences such as $^{15}N^{16}O^{+}$, $^{13}C^{18}O^{+}$, $^{12}C^{18}O^{1}H^{+}$, $^{14}N^{16}O^{1}H^{+}$ and $^{15}N^{16}O^{+}$. To overcome the problem of these interferences high resolution sector field mass spectrometer was developed and this was used mainly in this work.

Inductively Coupled Plasma Sector Field Mass Spectrometry (ICP-SF-MS) uses a magnetic sector as mass analyser which can be operated in a high mass resolving mode to overcome limitations by spectral interferences. In this mode it can separate masses with much smaller mass differences often needed to identify an isotope of an analyte element with high accuracy. ICP-SF-MS instruments can either be operated in a low mass resolution mode with high sensitivity or in a high mass resolution mode with moderate sensitivity.

A typical setup of a reverse design Nier-Johnson HR-ICP-MS is shown in Figure 2.1 and consists of the components: 1) Nebulizer and spray chamber, 2) Inductively coupled plasma, 3) Interface, 4) Lens system, 5) Magnetic and electrostatic sector and 6) Detector.

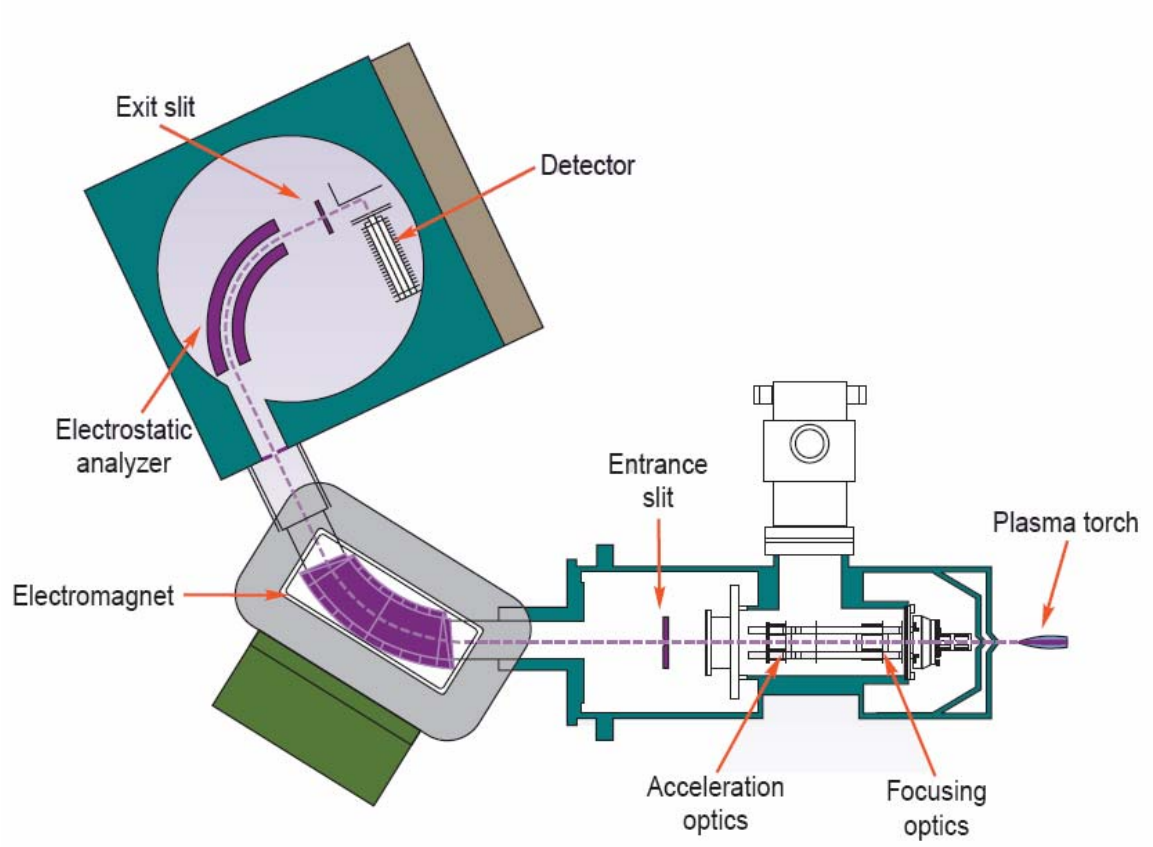


Figure 2.1: Schematic representation of a reverse design Nier-Johnson high-resolution ICP-MS.[23]

2.1.1.1 Nebulizer and Spray Chamber

Samples are introduced into the ICP-MS either as solids or liquids. The liquid samples are introduced into the plasma via nebulizers as a fine aerosol by assistance of a peristaltic pump.[24] Nebulizers are used to produce aerosol droplets of less than 10 µm diameter. Solid samples are generally introduced via laser ablation which generates particles which are introduced into the Ar plasma. Some pneumatic nebulizers generate droplets of diameters greater than 10 µm in addition to small droplets. These droplets can affect the plasma discharge conditions or are not fully evaporated so that they have to be removed by use of a spray chamber.

It depends on the analytical requirement which types of nebulizers are used, such as concentric and cross flow designed nebulizers. For example for good sensitivity and stability concentric design can be employed. The other types of nebulizers are microflow, microconcentric, ultrasonic, membrane desolvation, electrothermal vaporization etc.

Microconcentric nebulizer (MCN) and cinnabar spray chamber were used in this work for sample introduction in HR-ICP-MS. MCN is a highly efficient nebulizer for limited sample volumes and the matrix effects are reduced by using it with a cyclonic spray chamber. This spray chamber operates by centrifugal forces and droplets are discriminated according to their size by means of a vortex produced by the tangential flow of the sample aerosol and argon gas inside the chamber. Meinhard type concentric nebulizer and Scott double pass spray chamber were used for liquid sample introduction into a quadrupole based ICP-MS. The spray chamber selects the small droplets by directing the aerosol into a central tube, where they enter into the sample injector of the plasma torch.[24] The larger droplets emerge from the tube and by gravity, exit the spray chamber via a drain tube.

2.1.1.2 Inductively Coupled Plasma

These small droplets reach the inductively coupled high temperature argon plasma (6000 to 8000 K). This plasma is capable of atomizing and ionizing all analytes and it generates mainly positively charged ions. The plasma is formed in a stream of argon gas flowing through a quartz torch, which consists of three concentric quartz tubes. An induction coil is connected to a radio frequency generator and is used for plasma generation.

Even complex samples such as biological materials are fully atomized and ionized in the plasma without any influence on the analytical signal, thus allowing a substance independent calibration. This specific feature is most important for all calibration strategies developed in this work.

2.1.1.3 Interface

An interface in the ICP-MS is used to transfer ions generated in the argon sample at atmospheric pressure into the low pressure region. The interface consists of vacuum chamber in which a sampler cone, skimmer cone and a gate valve are arranged. The sampler and skimmer are metal cones with a small orifice of typically 1 mm diameter. The ions first pass through the sampler cone and then through the skimmer cone. These cones are used to sample the centre portion of the ion beam coming from the plasma.

Behind the sampler and the skimmer a vacuum of about 10^{-6} mbar is maintained by molecular turbo pumps.

2.1.1.4 Lens System

The ions from the ICP source are then focused into the entrance slit by the extraction lenses. The extraction lenses allow the ions to get extracted and accelerated and then focused. The focus lens is used to focus the beam of ions to the transfer lens system. The transfer lens system is then used to extract the ions from the orifice of the cones and to focus and shape the ion beam to pass the entrance slit.[25]

2.1.1.5 Magnetic and Electric Sector

For the high resolution ICP-MS two analysers are used. First one is an electromagnet and the other one is an electro static analyser (ESA). In a standard reverse Nier-Johnson geometry setup a magnetic analyser is followed by an ESA as shown in Figure 2.1. The ion beam from the plasma is accelerated in the ion optic region to 8 kV and focused into the entrance slit before it enters the magnet. Mass separation is achieved in the magnetic field. The mass separated ions then enter the ESA where they are filtered with respect to their energy and focused into the exit slit, where the detector is positioned.

The mass resolution can be changed by adjusting the width of the entrance and the exit slit in the spectrometer. For achieving different resolution, different slits with varying widths are used. For achieving low resolution wide slits are used and for high resolution narrow slits are used. The system operates at fixed resolution modes, low resolution mode $m/\triangle m \sim$ 300-400, medium resolution mode $m/\triangle m \sim$ 3,000-4,000 and a high resolution mode $m/\triangle m \sim$ 8,000-10,000.

2.1.1.6 Detector

The ion detection behind the exit slit is realized by a conversion dynode and a secondary electron multiplier (SEM). Ion passes the exit slit and hits the conversion electrode. Secondary electrons are released from the surface of the conversion dynode, which are attracted and multiplied by the SEM. The detectors can be operated in 2 modes, pulse counting and analogue mode. A pulse counting mode is used for applications where extremely low current ions are needed to be detected and it requires a

multiplier with a very high gain (~10^7). In order to achieve a higher dynamic range, detectors with dual mode are used. It is applied in this study where both pulse counting and analogue modes are operated simultaneously. Large signals up to 10^9 cps are measured using analogue mode and ion signals from a few cps up to 10^6 cps are measured using a pulse counting mode.

2.1.1.7 Quadrupole based Mass Analysers

For analysis of elements that are not prone to spectral interferences, conventional ICP-MS based on quadrupole mass analysers can be used. This ICP-MS differs mainly with the mass analyser compared to the HR-SF-ICP-MS. A quadrupole consists of four cylindrical metallic rods of the same length and diameter aligned parallel to each other (Figure 2.2). Each opposing rod pair is connected together electrically and a DC and a radio frequency (RF) voltage are applied to adjacent rods creating an alternating electric field between the rods; only ions of a selected m/z ratio are allowed to reach the detector for a given ratio of voltages, while the others are ejected from the quadrupole.

2.1.2 Aridus Sample Introduction System with Desolvation

In this work Aridus sample introduction system with desolvation was used to introduce liquid samples to the HR-SF-ICP-MS. It uses the combination of Aspire perfluoroalkoxy PFA nebulizer and a heated PFA spray chamber. The Aridus consists of a nebulizer which has adjustable outer tip and capillary which is easily replaceable. It has a sample uptake rate of 50 or 100 µl/min which enables the analysis of elements with low amount of samples typically less than 1 ml. The schematic of the Aridus system is shown in Figure 2.3. The sample vapour from the heated PFA chamber is passed to the heated fluoropolymer membrane where a counter-current of argon sweep gas is used to remove solvent vapour that permeates the membrane.

The main benefit of using the Aridus system is that solvent based interferences are reduced with membrane desolvation. A micro-autosampler can be coupled to the Aridus for automated sample analysis at low volumes.

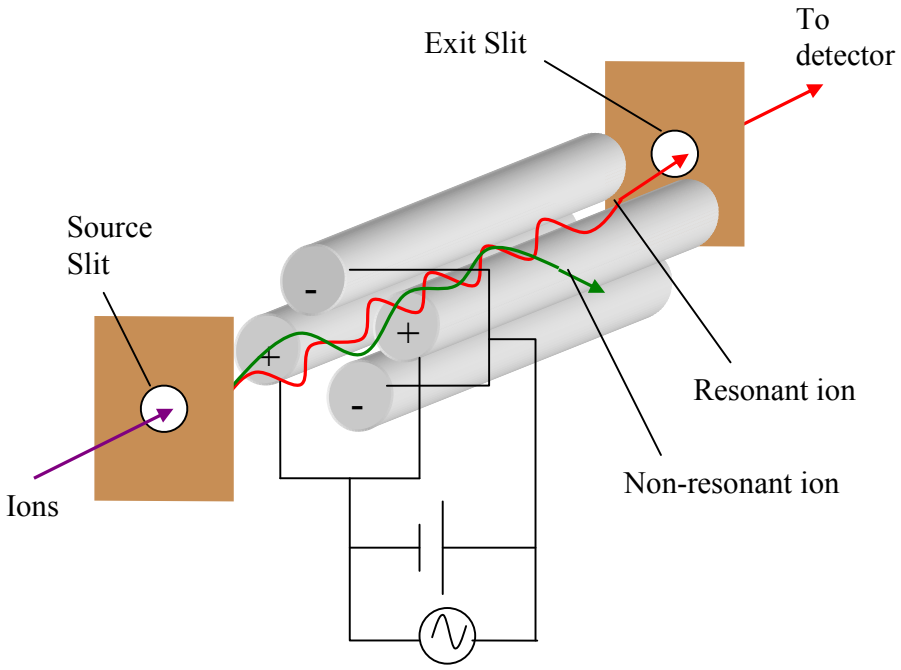


Figure 2.2: Schematic of quadrupole mass analyser.

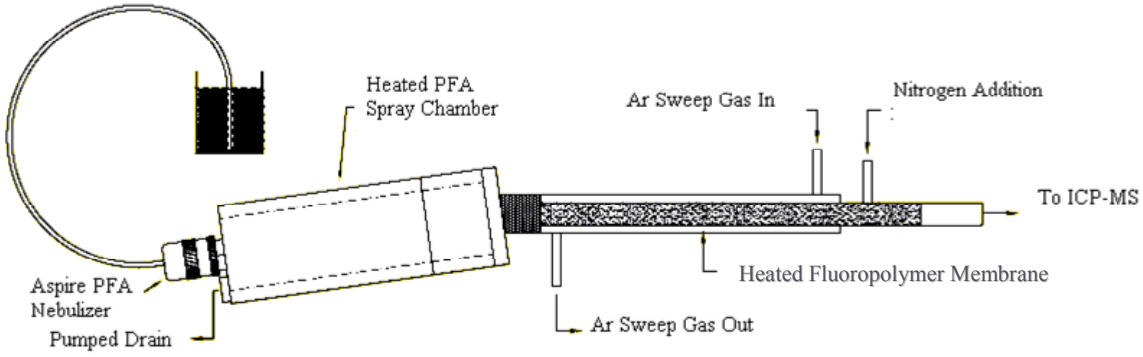


Figure 2.3: Schematic of the Aridus system.[26]

2.1.3 Laser Ablation

In the year 1985 A. L. Gray first used LA as a solid sampling technique.[27] This is developed to analyse solid materials via ICP-MS. It is a microanalytical technique used for the determination of trace elements in solid materials. It avoids the dissolution step normally required for preparing the sample. The principle of this method is to use the high powered laser to ablate the surface of a solid material and transport the dry aerosol into the ICP-MS. The main advantages of using LA are that it involves less interferences due to the absence of solvents and its superior detection limit.

When light energy from a laser with high power density focused onto a solid material inside a chamber, the photon induced energy is converted into thermal energy resulting in vaporization and removal of the material from the surface of the solid.[28] There are different types of lasers used. The first ones to be used were the ruby lasers producing IR laser light.[27] But due to the drawbacks of ruby lasers such as low sensitivity and poor precision, solid state Nd:YAG lasers were introduced later which are the most commonly used ones till now. They operate at 1064 nm and the generated energy is sufficient enough for ablating solid materials at various spatial resolutions. Nd:YAG lasers can be frequency-quadrupled so that UV laser light can be generated which is suitable for ablating most solid material and was used throughout this work.[4]

There are various advantages of using laser ablation. Samples can be directly analysed without the need of lengthy dissolution steps; the resulting dry aerosol generates less polyatomic interferences in comparison to liquid samples. Due to these advantages there are various applications of LA together with ICP-MS such as biological, environmental and geological fields. One of the latest biological applications is imaging of biological tissues for toxic elements.[29] It is also used recently in pharmaceutical studies, as a comparative study for determination of fake drugs.[7]

Nielsen *et al.*[30] first introduced the combination of gel electrophoresis technique with LA-ICP-MS for speciation of metal binding serum proteins. Human serum was enriched with Co and subjected to agarose gel electrophoresis in two dimensions. Nd: YAG laser (1064 nm) interfaced to ICP-MS was used for the analysis of these gels. The identification of the main Co binding serum proteins was performed by comparing the distribution map for Co with the protein distribution map obtained via Coomassie

Brilliant Blue staining. The capabilities of LA-ICP-MS for the detection of trace elements especially selenium in a gel after gel electrophoresis were studied by Chery *et al.*[31] Limits of detection, linearity and repeatability were evaluated for various elements (Li, V, Cr, Mn, Ni, Cu, Zn, As, Se, Mo, Pd, Ag, Cd, Pt, Tl and Pb). The detection limits ranged from 5 x 10^{-2} for ^{95}Mo to 90 fmol for ^{77}Se. Two ablation strategies were used for the identification of selenoproteins: single hole drilling relevant for spot ablation of 2D separation gels and line rastering relevant for 1D separation. The detection limit for Se was found to be 0.07 µg/g for spot ablation and 0.15 µg/g for line rastering.

In most of these applications, protein spots in gels were directly ablated and introduced to the ICP-MS for the detection of hetero-elements in proteins which is the straightforward method. But in order to reduce the high blank values obtained from direct analysis of gels, membranes after electroblotting can be used as an alternate method to analyse elements especially phosphorus. Even though an additional step is involved for blotting of proteins from gels, this method can be particularly used to achieve trace matrix separation. Since proteins are present in the upper layer of the membrane material after blotting they can be easily targeted by the laser. Additionally to this technique, LA-ICP-MS can be combined with immunoblotting method where multiple antigens can be concomitantly analysed with single labelled antibodies. The total analysis time of samples can be relatively reduced in comparison to the Electrochemiluminescence (ECL) method.

2.1.4 Calibration and Quantification Strategies in ICP-MS

ICP-MS is the most powerful technique for the analysis and quantification of trace elements in both solid and liquid samples. Proteins can be quantified either relatively or absolutely based on the quantification approach and the method adopted. The quantification of proteins based on relative quantification is usually performed by comparing the intensities in a test sample relative to the corresponding intensities in a standard used for calibration whereas absolute quantification gives the real absolute quantitative data within the unknown sample.

In analytical perspective, calibration forms the basis to perform quantification. Different types of calibration strategies such as internal, external, matrix-matched,

standard addition, isotope dilution and calibration by internal normalization are used with ICP-MS based methods.[28] The evaluation of simultaneous use of some of the methods as potential calibration methods was discussed in detail in literature.[32] Generally ICP-MS based calibrations are mainly based on internal standards, i.e. by adding known amount of elemental standard which is not usually present in the sample. For this purpose an element is selected similar to the analyte element in mass, ionization potential and chemical behaviour.[33] These standards are useful for analysis where the quantity of the sample analysed and the instrument response varies from run to run and is difficult to control. The concentration of the unknown can be determined by plotting analyte to internal standard signal ratio as a function of the analyte concentrations in the standard. For quantification of phosphorus using ICP-MS based methods sulphur has been often used as the internal standard, e.g. in the work of Becker *et al.* it was applied for the quantitative determination of phosphorus and other elements in proteins of yeast mitochondria using LA-ICP-MS.[34]

Commonly internal standardization is applied in combination with external calibration strategy for accurate quantification of analytes. In an external method, experiments are performed first with the full range of calibration standards and blanks and then the unknown samples are quantified with the calibration curve. This method is usually used when there is very little difference between the matrix in the standards and the unknown samples. When there are matrix effects or drifts in sensitivities of the instrument, standard addition or matrix-matched methods could be used an alternative. In the matrix matched method the matrix of the standard can be adjusted to match that of the sample and used for quantification. Recently matrix-matched laboratory standards were used by Becker *et al.* for quantitative determination of copper, zinc and other elements distributed in thin slices of human brain samples measured by LA-ICP-MS.[35] In standard addition method samples are spiked with known concentrations of analytes and the resulting regression line, which is obtained after plotting intensities against the concentration of the added spikes, gives the analyte concentration as the intercept of the x-axis. In isotope dilution analysis the sample is spiked with a solution of known quantity of a rare but highly enriched isotope and the concentration of the element of interest in the sample can be calculated using the isotopic ratio of the resulting solution and the

known isotopic ratio of the spike and the natural abundances. This method was used recently by Schaumlöffel *et al.*[36] for absolute quantification of sulphur-containing peptides after tryptic digestion and analysis by nanoHPLC-ICP-MS. Calibration can also be performed by normalizing the resulting intensities with the help of internal standards. In this study using LA-ICP-MS, the use of proteins and inorganic compounds as matrix-matched standards externally in blots as one of the calibration strategy was explored. In addition, by measuring internal standards in blots, the potential of calibration by internal normalization together with external calibration with labelled proteins was also tried for quantification of proteins.

2.1.5 Protein Quantification Methods

Three spectrophotometric methods were used in this work to determine the concentration of proteins in a solution. These include measurement of the proteins' intrinsic UV absorbance and 2 methods which generate a protein-dependent colour change; the Lowry assay (2.1.5.3) and the Bradford assay (2.1.5.2). The protein concentration using Lowry and Bradford assay was obtained using an UV/VIS spectrophotometer.

2.1.5.1 UV Absorbance at 280 nm

This method is applicable to purified proteins with exhibiting absorbance at 280 nm. Amino acids with aromatic rings, tyrosine and tryptophan are primarily responsible for the absorbance peak at 280 nm. Since these amino acid contents of various proteins vary within a narrow limit, the absorption peak at 280 nm has been used as a fast and sensitive method for protein concentration estimations. This method does not need any standard calibration curve, it is simple to perform and the sample is recoverable. The Beer-Lambert law states that

Absorbance $A = E \times C \times L$

where E is the extinction coefficient, C the concentration in mol/l and L the optical path length in cm. If the extinction coefficient is known, the absorbance gives the concentration of the protein for a 1 cm path length.

2.1.5.2 Bradford Assay

The Bradford dye assay is based on the equilibrium between three forms of Coomassie Blue G dye, i.e. absorbance shift in this dye when bound to basic (primarily arginine, lysine and histidine) and aromatic residues. Under strong acid conditions the dye exists in a stable doubly-protonated red form.[37,38] Upon binding to protein; however, it is most stable in an unprotonated, blue form. The three different absorbance maxima of the dye-protein complex correspond to three forms of the dye present, the red form which absorbs light at 470 nm, the single protonated neutral green form at 650 nm and the anionic (deprotonated) blue dye-protein complex at 595 nm.

The detection limits of this method range from 1-20 μg for micro assay and 20-200 μg for macro assay. It is faster, involves fewer mixing steps and gives a more stable colorimetric response compared to Lowry assay. However, there are limitations such as it is non-linear over wide ranges and its response to different proteins can vary widely.

2.1.5.3 Lowry Assay

The Lowry assay is based on reduction of Cu^{2+} to Cu^{1+} amides. Under alkaline conditions, Cu^{2+} deposits itself to the peptide bonds of protein to form the protein Cu^{2+} complex, which subsequently reduces Folin-Ciocalteu phosphomolybdic/phosphotungstic acid to heteropolymolybdenum blue by the copper catalyzed oxidation of aromatic acids.[37] The product can be detected colorimetrically by absorbance between 550 and 750 nm. The Lowry method is sensitive to pH changes and therefore the pH of the assay solution should be maintained at 10-10.5. The limitation of using this method is that it can't be used in presence of strong acids or ammonium sulphate which can interfere with the assay. The detection limit ranges from 2-100 μg.

2.1.6 Electrophoresis

2.1.6.1 Principle of Electrophoresis

Electrophoresis is a technique used to separate and sometimes purify macromolecules especially proteins and nucleic acids that differ in size, charge or conformation. As such it is one of the most widely used techniques in biochemistry and molecular biology. When charged molecules are placed in an electric field, they migrate

toward either the positive or negative electrode according to their charge. Proteins and nucleic acids are electrophoresed within a matrix or gel. The generally used support medium is cellulose or thin gels made up of either polyacrylamide or agarose, each of which has attributes suitable to particular tasks.[39] Cellulose is used as support medium for low molecular weight biochemical's such as amino acid and carbohydrates whereas agarose and polyacrylamide gels are widely used for larger molecules like proteins.

2.1.6.2 Polyacrylamide Gels

Polyacrylamide is a cross-linked polymer of acrylamide. Polyacrylamide gel is prepared by the free radical polymerization of acrylamide and the cross linking agent N N′ methylene bis acrylamide (bis). The structures of the compounds acrylamide and bis are shown in Figure 2.4a and b. The polymerization is started when ammonium per sulphate and tetramethylethylendiamine (TEMED) are added to the mixture. The length of the polymer chains is dictated by the concentration of acrylamide used, which is typically between 3.5 and 20%. Polyacrylamide gels are significantly more annoying to prepare than agarose gels. Acrylamide is a potent neurotoxin and should be handled with care. Polyacrylamide gels have a rather small range of separation, but very high resolving power. In case of DNA, polyacrylamide is used for separating fragments of less than about 500 bp. However, under appropriate conditions, fragments of DNA differing in length by a single base pair are easily resolved. In contrast to agarose, polyacrylamide gels are used extensively for separating and characterizing mixtures of proteins.[40] In addition to the properties of the molecules of interest, the gel pore size and buffer systems are the important parameters which need to be optimised in electrophoresis.

The pores in the gel are created by crosslinking of polyacrylamide with the bis to create a network of pores and their size is determined by the total amount of acrylamide present (%T) and the amount of the cross linker (%C). A schematic of a polyacrylamide network is shown in Figure 2.5. The pore size decreases when the total amount of acrylamide increases. The relative pore size of the gel is indicated by the %T which is the weight percentage of the total monomer including the crosslinker. %C is the crosslinker:acrylamide monomer ratio of the monomer solution. For example 8.5%T

means that there is a total of 8.5 g of acrylamide and bis per 100 ml of gel and 8.5%T:6%C means that a total of 8.5% acrylamide and bis and bis is 6% of the total.

The buffer system affects the migration of proteins and is used in the gel and the running buffer. There are continuous and discontinuous buffer systems. In continuous buffer system the same buffer is used at constant pH and concentration throughout for the preparation of the gel. It is made of one continuous %T. But most of the today's systems use discontinuous buffer systems where 2 types of buffer ions are used. Two types of gels, stacking and resolving gels are prepared with 2 types of buffer systems so that samples are compressed into a thin starting band and individual proteins are finely resolved and separated. In Native PAGE conditions, pH of the buffer systems is important as the migration of protein depends on both the size and charge of the protein. It should be maintained in such a way that the protein of interest is stable.

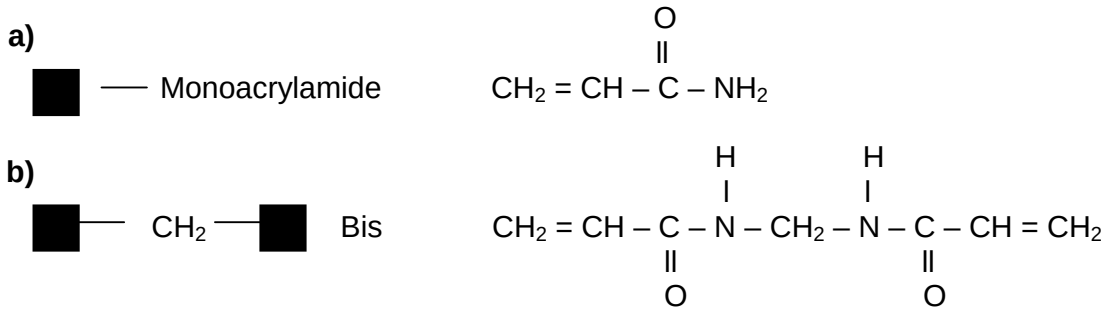


Figure 2.4: (a) Monoacrylamide and (b) Bis.

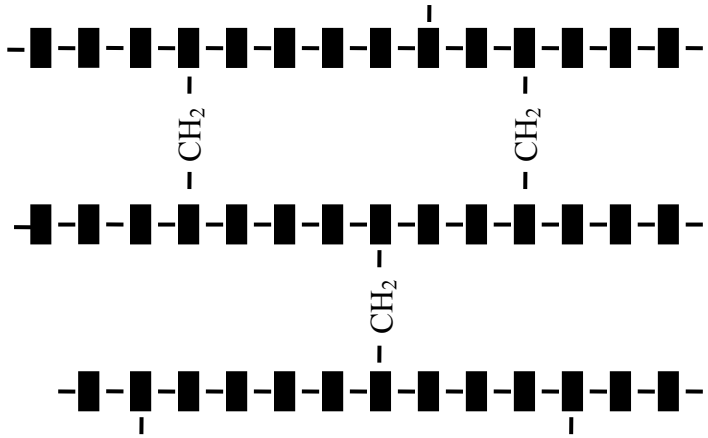


Figure 2.5: Polyacrylamide network.

2.1.6.3 Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis

The general electrophoresis techniques cannot be used to measure the molecular weight of the biological molecules because the mobility of a substance in the gel is influenced by both charge and size. In order to overcome this, the biological samples are treated so that they have a uniform charge and the electrophoretic mobility which then depends primarily on size. The molecular weight (MW) of proteins may be estimated if they are subjected to electrophoresis in the presence of a detergent sodium dodecyl sulphate and a reducing agent Dithiothreitol (DTT). SDS disrupts the secondary, tertiary and quaternary structure of the protein to produce a linear polypeptide chain coated with negatively charged SDS molecules. 1.5 g of SDS binds per gram of protein. DTT assists the protein denaturation by reducing all disulfide bonds.[41,42] The general principle of the SDS-PAGE is shown in Figure 2.6. After linearization proteins are transferred to a gel and when electric current is applied they migrate towards the anode because of the negative charge imparted on them by SDS. Usually 8 to 10 sample wells are present in each gel that is casted. A section of it is shown in the Figure 2.6.

MW of a protein can be determined by separating the protein sample on the same gel with a set of MW standards especially pre stained markers. After staining, gels are analysed to obtain the relative migration distance (R_f) values. R_f is the distance of the protein migrated through the gel divided by the migration distance of the dye front. The distance should be measured from the sample wells to the band of interest. The MW of the unknown protein is calculated using the standard curve obtained from plotting of log MW versus R_f values.

SDS-PAGE with discontinuous buffer system developed by Laemmli[54] is the most commonly used electrophoresis method. Preparations involve casting of two different layers of acrylamide between glass plates. The lower layer (separating or resolving gel) is responsible for actually separating polypeptides by size. The upper layer (stacking gel) includes the sample wells, and has the composition that causes the samples to become compressed (stacked) in order to have sharper bands and correspondingly better resolution among bands. The bands obtained from this technique are very specific even within a low range.

SDS-PAGE separates proteins according to the primary structure or size but not amino acid sequence. Hence different proteins with same number of amino acids or molecular weight cannot be separated with this method that results in inferior resolution.

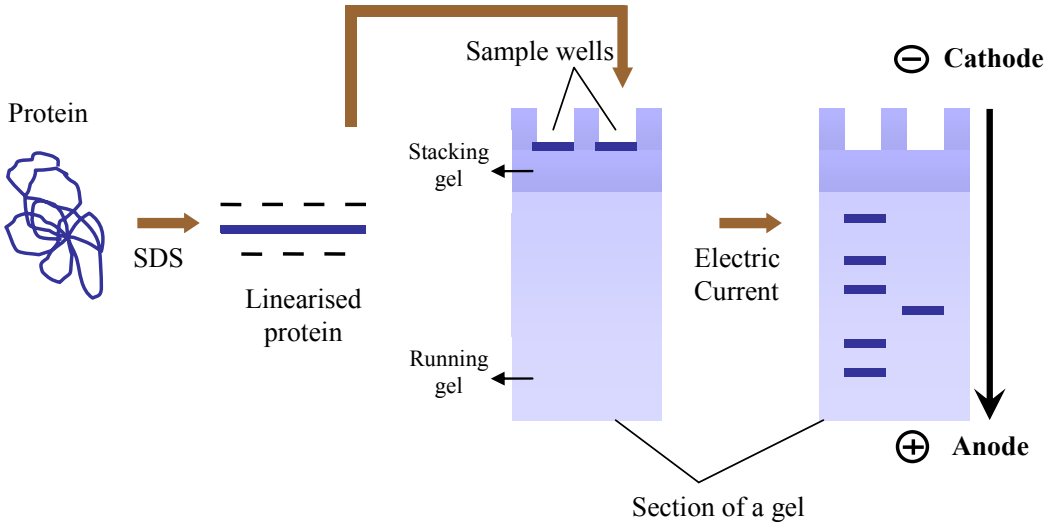


Figure 2.6: Principle of SDS-PAGE.

Electrophoresis can be performed in 2 ways, either by using horizontal or vertical systems. Horizontal systems can be used where it is affordable to use precast gels. Precast gels are available with different %T concentrations and in different formats. In this work, horizontal SDS-PAGE electrophoresis using precast gels was used and they were performed on the Multiphor II Flatbed Electrophoresis System (Amersham, Biosciences, Freiburg, Germany) connected to the power supply (2197, Amersham, Freiburg, Germany). Vertical SDS-PAGE separation system, Miniprotean III from Bio-Rad was used in this work where proteins are separated in smaller gels and in addition to the pre cast gels, manual casting of gels can also be performed with this device.

2.1.7 Blotting

The transfer of biological samples from a gel to a membrane and the detection on the membrane surface is termed as blotting and also referred as electroblotting. The transfer of protein in this way and its subsequent detection is referred to as Western

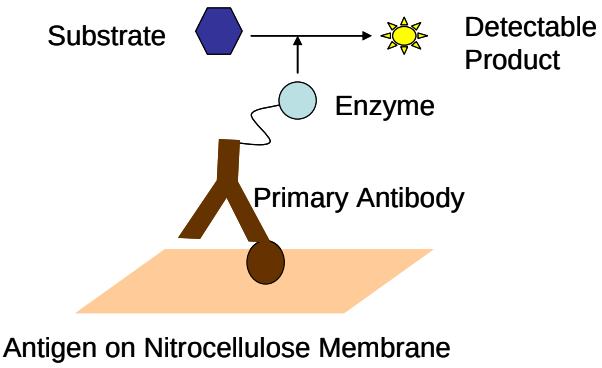
blotting. It is evolved from the term Southern blot which is a technique used for DNA detection and Northern blotting which is used for the detection of RNA on membranes. It is also referred as immunoblotting because an antibody can be used to detect the antigens on the membrane. This technique was first introduced by Towbin *et al.*[43] in 1979 who used this method for the electrophoretic transfer of ribosomal proteins from polyacrylamide gels to nitrocellulose (NC) membranes. By either using autoradiography or the peroxidase reaction product, the specific proteins could be detected. 100 pg of protein can be detected using the latter method. Two types of membranes are generally used for the transfer. One is nitrocellulose and the other is polyvinylidene fluoride (PVDF). Nitrocellulose membranes are mostly used as it provides high sensitivity with low background and binding is irreversible. Membranes are available in 2 pore sizes, 0.2 μm and 0.45 μm, and their use is dependent on the application. 0.2 μm is used for transfer of low molecular weight (<20 kDa) and 0.45 μm for transfer of high molecular weight proteins (>20 kDa).

Proteins can be transferred onto the membranes via semi-dry (SD) blotting or wet blotting techniques. As the name suggests, semi dry is done under dry conditions using filter discs and the wet blotting is done in complete wet conditions. Both methods have their own advantages and disadvantages, but the semi dry method is better suited as it requires less volume of buffer than wet (also known as tank) blotting. As lower volumes of buffer used there is lower background while detecting the proteins via their elemental composition by LA-ICP-MS which can further reduce the detection limits of the method. Hence SD blotting was mainly applied in this study and a comparison was performed with contact blotting method. SD blotting method offers fast as well as homogenous transfer of proteins and it can be used with discontinuous buffer system, so that smaller proteins as well as protein mixtures of very different sizes can be blotted more uniformly. Another advantage of semidry blotting is that it is highly efficient in quantitatively transferring large proteins of >200 kDa. The semidry blotting experiments in this study were performed using the graphite anode and cathode plates connected to the power supply (2197, Amersham, Freiburg, Germany).

Western blotting is the combination of above explained protein blotting methods with immunodetection methods. This technique is applied in various applications for

example in detection of protein expressions in cells and tissues. It is primarily used in medical diagnostic applications like HIV testing. Immunoblotting can be performed once the specific antibodies are available for the detection of antigens on membranes. Generally there are 2 ways of detection, direct and indirect detection. After separation of proteins using SDS-PAGE or 2D separation and blotting onto membranes, immunoblotting by use of antibodies can be applied to probe specific proteins. In the direct method, the applied primary antibody is already enzymatically labelled (Figure 2.7a). So after the substrate addition, the antigen can be directly detected. In the indirect method, two antibodies are used, primary and secondary antibody where the latter is labelled with an enzyme (Figure 2.7b). The substrate is then added which gives a detectable product. The first method is the quickest as it employs only one antibody and the cross reactivity of the secondary antibody can be eliminated. But with the second method, the sensitivity can be improved due to the fact that more than one secondary antibody can bind to the different epitopes of the primary antibody.

a)



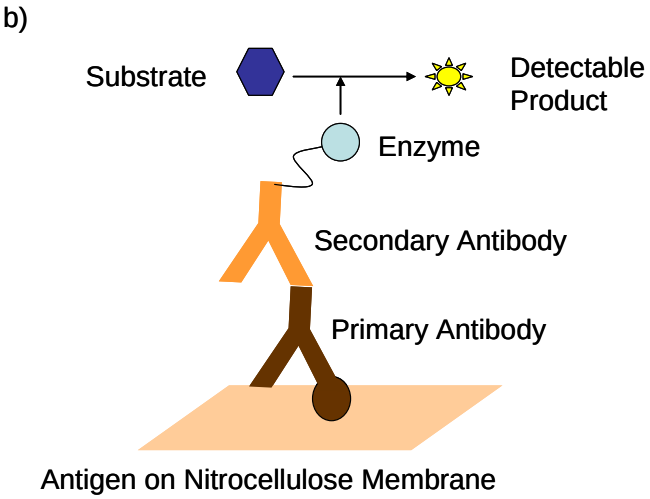


Figure 2.7: (a) Direct and (b) indirect detection of proteins using antibodies.

2.1.7.1 Electrochemiluminescence Detection

In Western blotting techniques different detection methods are applied such as Colorimetric, chemiluminescent, fluorescent or radioactivity based detection methods can be used. The sensitivity of the technique can be improved by using enhanced chemiluminescent (CL) based methods. One of the CL methods, electrochemiluminescence is used commonly in a variety of biological detection assays. ECL is a kind of luminescence caused by an electrochemical reaction. For the antigen detection, immunoblotting is performed with the primary or the secondary antibody which is linked already to the labelled enzyme horseradish peroxidase (HRP). When the blot is incubated with a luminol based substrate it results in the emission of light which can be captured and visualized by exposing the blot to a film or to an appropriate CCD camera. The substrate luminol is oxidised in presence of the enzyme HRP and hydrogen peroxide which results in the product 3-aminophthalate that emits light at 425 nm. The detection limit can be improved with the help of various other enzymatic detection methods such as using biotin-streptavidin peroxidase system. The benefits of using this method is that it is sensitive, fast, easy and inexpensive and it has a high dynamic range and low detection limits. Nevertheless there are disadvantages like only one specific antigen can be probed at the same time and one may not detect all low affinity antibodies. In this work the Western Lightning Chemiluminescence Reagent Plus was used to

visualize the bands of the labelled-antibody and images were acquired by the ChemiLux gel imager CSX-1400M (INTAS, Göttingen, Germany).

2.1.8 Gel Scanner

The imaging of the stained gels was performed using the fluorescent laser scanner FLA-5100 and a 532 nm laser (Fujifilm Life sciences, Duesseldorf, Germany). The system includes a red laser (635 nm) as standard and green (532 nm), blue (473 nm) and near infrared (670 nm) as optional lasers. A resolution of 10 to 200 microns (1 micron corresponds to a pixel size of 1 µm) can be set according to the requirement and it has a dynamic range of 5 orders of magnitude. The Coomassie stained gels are placed on a 40 x 46 cm stage (Fluor stage in Figure 2.8) and a specialized fluorescent board is placed over it. The laser light is reflected by a mirror into the optical head which moves under the stage. The optical head directs the laser light onto the sample. When the green second harmonic generation laser (532 nm) at photon multiplier tube (PMT) setting of 250 V is applied, the excitation and fluorescence are decreased at parts of the gel according to the density of the stain. The light emitted from the sample travels via a mirror through the filter to the PMT which is placed besides the optical head on the left side and detected. A positive image can be obtained from the negative image using the Image reader software. The schematic of the gel scanner is shown in Figure 2.8.

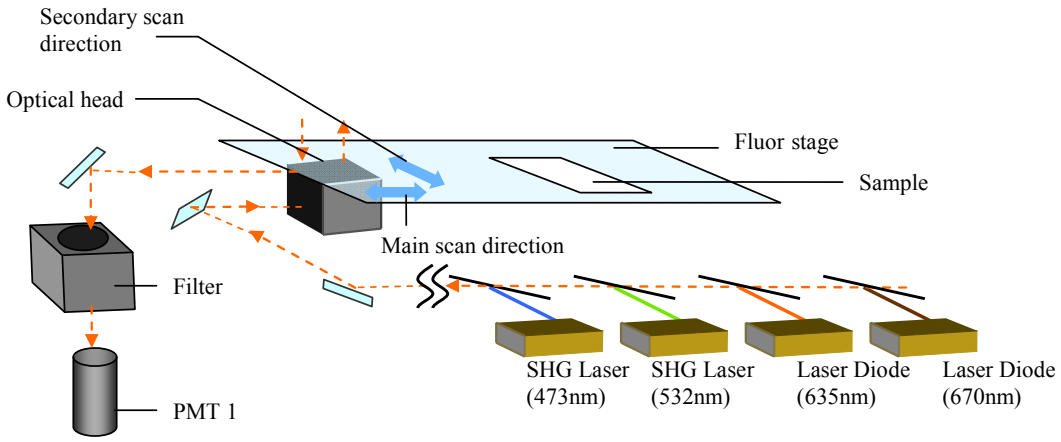


Figure 2.8: Schematics of the FLA-5100 gel scanner.

2.1.9 White light Interferometer

The craters of the laser scanned blot membranes were measured with a white-light interferometric (WLI) microscope NewView 5010 (Zygo, Middlefield, USA).WLI is a powerful tool for optical measurements and works with a light source with broad spectral bandwidth. Due to that the coherence length of the source is short and good contrast fringes will be obtained only when the two paths of the interferometer are closely matched in length. WLI is a technique for non-contact measurement of surface topography at high vertical and moderate lateral resolution. It provides fast, quantitative, surface texture measurement and analysis on many types of surfaces, in just seconds. The major drawback of scanning WLI measurement is that only a single surface height is being measured at a time and a large number of measurements and calculations are required to determine a large range of surface height values. Nevertheless modern computers with specialized software can solve these problems. The main applications are for the measurement of chromatic dispersion and distances.

2.1.10 Antibody Structure

Antibodies are proteins produced by the immune system for the defense of the organism against foreign species. They consist mainly of amino acids but also contain carbohydrate residues. An antibody is approximately 150 kDa protein consists of two heavy (50 kDa) and two light chains (25 kDa) couple to each other by disulfide bonds, forming a characteristic Y-shape (Figure 2.9). When subjected to SDS-PAGE, antibody is split into heavy and light chain fragments and thus it should give two bands, one at approximately 50 kDa and another at approximately 25 kDa.

A small region of 110-130 amino acids at the tips of the Y-shaped antibody varies with different antibodies. This part of the antibody determines the specificity of the antibody which is designed to specifically bind antigens by a lock-and-key mechanism. Each target requires a different recognition structure and therefore a greater variability is observed at the tips of the antibody. The rest of the antibody shows little variation. Antibodies are also called as immunoglobulins and classified into five classes IgG, IgA, IgM, IgD and IgE depends on the composition of their constant region. A wide variety of

commercially available pre labelled specific antibodies can be purchased for quantitative assays.

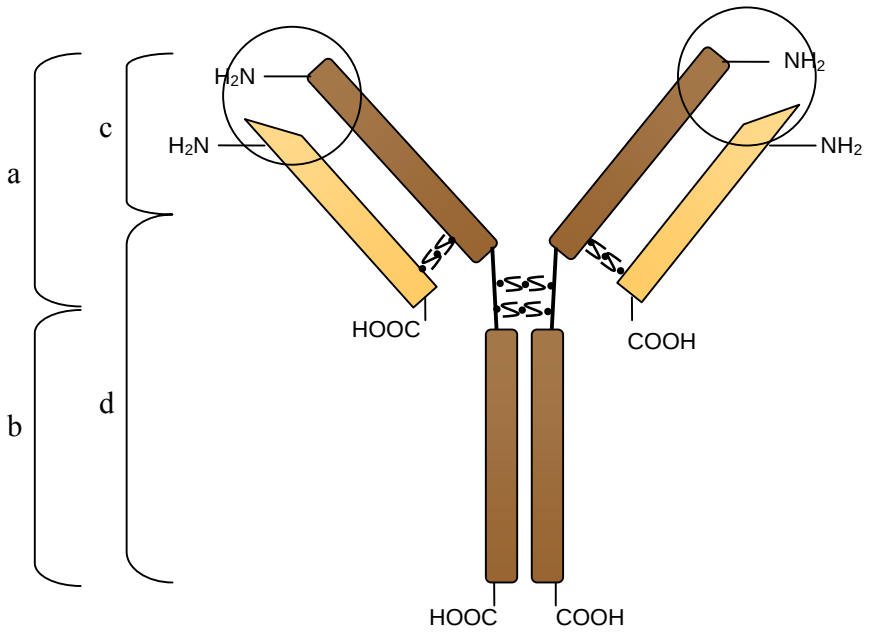


Figure 2.9: Schematic of an antibody structure. Two heavy chains (dark) are linked by disulfide bridges in the central part, Hinge region. Light chains are bound to the heavy chains by other disulfide bridges. a) Fragment antigen binding (Fab) region, b) Fragment crystallisable (Fc) region, c) variable and d) constant regions. The tips of the variable regions (denoted by circle) interact with the antigen strongly.

2.1.11 Labelling using Bioconjugation Chemistry

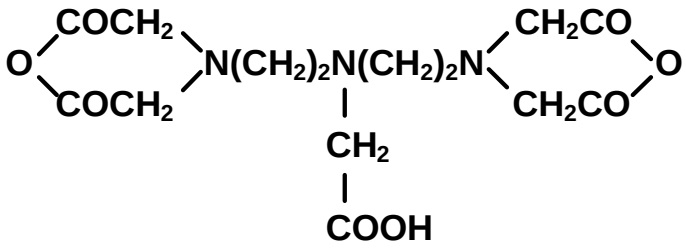
The covalent attachment of chelating compounds to antibodies and other proteins is termed as the labelling of proteins. It enables the detection of proteins and among different methods, labelling with chelating compounds has been widely studied and used in the medical, biological and analytical fields. The term chelate is generally reserved for complexes in which the metal ion is bound to two or more atoms of the chelating agent, although the bonds may be any combination of coordination or ionic bonds. There are numbers of chelates available of which 2 were chosen for detecting proteins via LA-ICP-MS using well established biochemical methods. The first one used here is diethylenediaminepentaacetic acid dianhydride and second one is 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-tetraaceticacid. They are bi-

functional, which means that they have a reactive functional group for targeting the proteins and a metal chelating group.

2.1.11.1 Diethylenediaminepentaacetic Acid

DTPA is the chelating agent that is used extensively in medical sciences for the removal of radioactive materials from the human body. It can be used to label the protein with elements. It was previously used for radiolabelling of antibodies.[55] The structure of this molecule and the DTPA-protein complex are shown in Figure 2.10a and b. It consists of a diethylenetriamine backbone modified with five carboxymethyl groups. Its conjugate base has a high affinity for metal cations. After binding to the lanthanide it exists in a penta anionic form, i.e. all five carboxylic acid groups are deprotonated. This bi-functional chelating agent contains 2 amine reactive anhydride groups. It can react with the N terminal amine groups of the proteins to form amide linkages. The anhydride rings open to create multivalent metal chelating arms able to bind tightly to metals in a coordination complex. A study with respect to LA-ICP-MS detection was performed in this work for detection of compounds which do not have detectable hetero-elements. This labelling strategy can be used for optimising the detection conditions for LA-ICP-MS method and also for the potential multielemental determination.

a)



b)

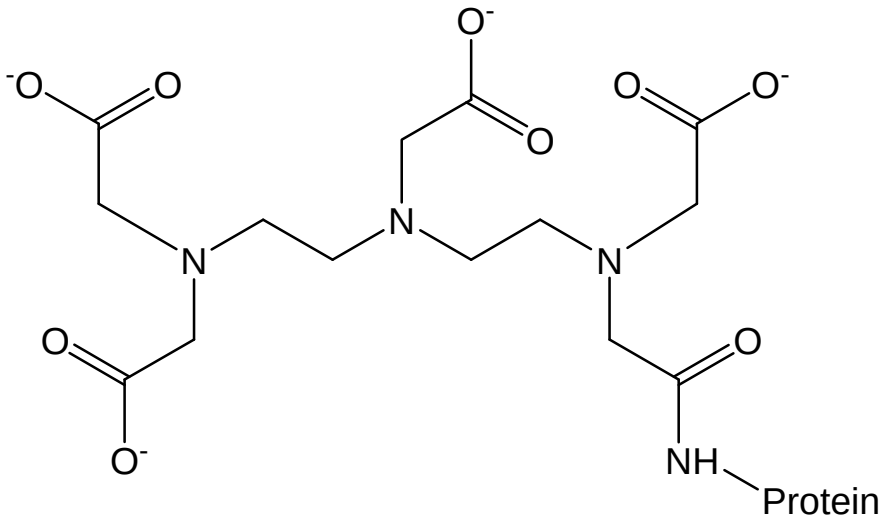


Figure 2.10: (a) Diethylenetriaminepentaacetic acid dianhydride and (b) DTPA-protein complex.

2.1.11.2 2-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10 - tetraaceticacid

The second method used here is based on chelating compounds in particular p-SCN-Bn-DOTA. These chelating agents are bi-functional, i.e. they contain a metal chelating group and a second functional group which is chemically reactive in nature, which binds to a specific biological target molecule, here to proteins and antibodies. In medicine they are very often used in combination with radiopharmaceuticals and contrast improving agents for instance in cancer therapies or imaging applications.[44]

The reaction scheme of the mechanism of labelling proteins followed in this work using p-SCN-Bn-DOTA is shown in Figure 2.11. The chelating compound is bi-functional; the first one involved in binding a metal, for instance europium, and the other function is used to bind to a protein via a reactive linker molecule, SCN in this case. This type of linker molecule binds covalently preferable to amino groups of the protein. The reactive group of DOTA is an isothiocyanate group attached to a benzene ring. As shown in Figure 2.11 in the first step the amino group of the protein reacts with the SCN group to form a phenyl thiocarbamoyl group. This reaction strongly depends on the pH value because below a pH of 7.5 only the end-terminal amino group is charged and will bind, whereas with increasing pH value above 9 the lysine residue will react and at even higher pH values also the arginine residue is charged. In the second step a three valent metal

cation is added to the protein-DOTA complex and 3 of the 4 carboxyl groups of the macrocyclic DOTA will form a coordinative binding with the threefold charged metal. The remaining fourth carboxyl group is then carrying still a negative charge.

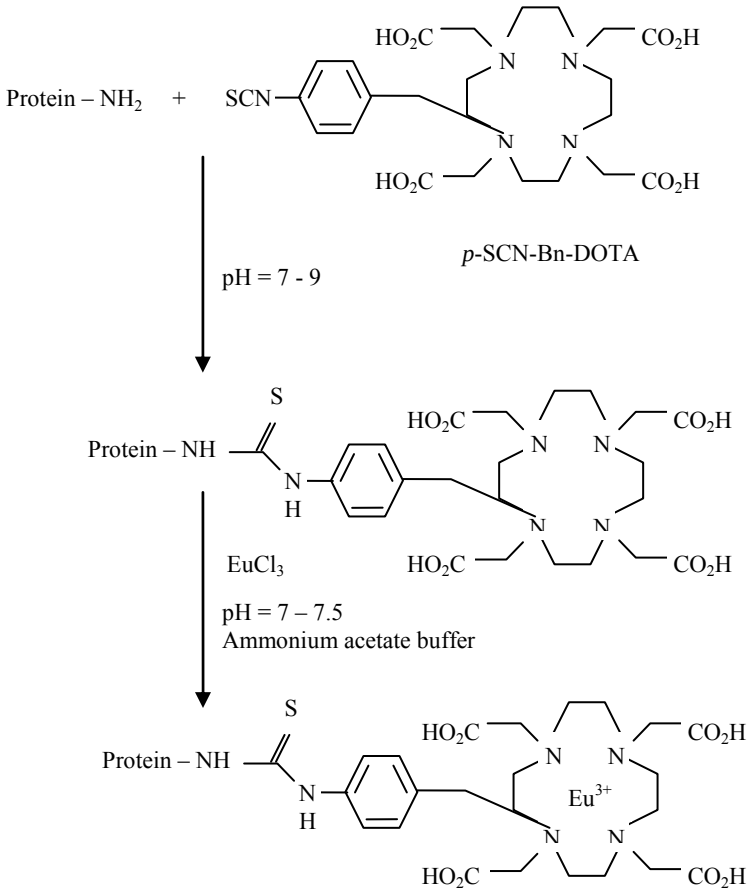


Figure 2.11: Schematics of labelling procedure (adopted from[45]).

2.1.11.3 Iodination

Iodination of proteins has been well known for a long time and has already often been applied, but mainly in combination with radioactive ^{125}I labels, for instance in the pioneering work of Markwell[46]. The chemistry is very well described in textbook[47] and in original publication[48]. The scheme of a protein is shown in literature of Jakubowski *et al.* [49] which contains two binding sites for iodine. The reaction is based on electrophilic substitution of iodine to histidine and tyrosine. Starting with NaI and a protein in solution, the reactive species I^+ is generated by use of an oxidizing compound. Reaction is then possible at two different reaction sites of both amino acid residues: at the ortho-

positions of tyrosine and at the 2,5 positions of the imidazole-ring in the histidine residue. This method is applied in combination with LA-ICP-MS for detection of the stable iodine isotope ^{127}I, thus minimizing the risk of radioactive compounds and extending the application to a conventional laboratory environment. It was used here for labelling antibodies which was then applied for detection of antigens by LA-ICP-MS methods.

2.2 Experimental

2.2.1 Inductively Coupled Plasma Mass Spectrometry

The elemental analysis was carried out using two ICP-MS systems,

(1) Finnigan Element-2 (Thermo Fisher Scientific, Bremen, Germany)

It is a high resolution double focusing magnetic sector field ICP-MS. Laser ablation was coupled to this system for analysing blot membranes and liquid sample analysis was performed with Aridus desolvation system using microconcentric nebulizer (Micromist, Glass Expansion Ltd., Melbourne, Australia) and cyclonic spray chamber (Cinnabar, Glass Expansion Ltd., Melbourne, Australia).

Figure 2.12: Element-2 ICP-MS.[50]

(2) PlasmaQuad II/III (Thermo Fisher Scientific (formerly Fisons/VG-Elemental), Bremen, Germany)

It is a quadrupole ICP-MS. It was used only for liquid sample analysis of certain elements. Liquid sample analysis was performed using Meinhard concentric nebulizer and Scott spray chamber.

Figure 2.13: Plasma Quad III ICP-MS.[51]

2.2.2 Protein Quantification Methods

2.2.2.1 Bradford-Assay

The protocol for this protein quantification method is described in more detail in the manual of the company Sigma Aldrich.[52]

The UV absorbance was carried out using the Cary 500 UV/VIS spectrophotometer (Varian Deutschland GmbH, Darmstadt, Germany). All solutions were prepared using ultra pure water (Millipore, Bedford, MA, USA).

1) Solution

Working solution: Bio-Rad Coomassie solution in ethanol and phosphoric acid

Protein Standard: 1.0 mg/ml Bovine Serum Albumin (BSA) in water

2) Protocol

In order to obtain the standard curve the BSA solution was prepared in the following concentrations – 2.5, 5.0, 7.5, 15.0 and 20 mg/ml. For zero value only water was used. Experiments were performed in polystyrene micro cuvettes.

Table 2-1: Amount of sample used for Bradford Assay.

Solutions	Samples	BSA standards	Zero value
Samples	800 µl	-	-
BSA standards	-	800 µl	-
water			800 µl
Coomassie solution	200 µl	200 µl	200 µl

After a gentle mix solutions were incubated at room temperature for 3 min. Then the extinction was measured with the photometer at wavelength 595 nm.

3) Evaluation

The calibration curve was directly obtained from the photometer software or by using Excel application. If the measured extinctions of the sample lie beyond this standard calibration curve then samples were diluted with different dilution factors and again measured.

2.2.2.2 Protein Absorbance Assay (280 nm)

This assay was used for low amounts of samples. 2 µl of sample was enough for the measurement. For that purpose Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Bremen, Germany) was used. It has in built calibration available for many proteins. This instrument gives the UV spectrum, measures the protein absorbance at 280 nm and calculates the concentration (mg/ml).

2.2.2.3 Lowry-Assay

The protocol is described in more detail in the biochemical methods book of Cooper.[53]

1) Solution

Solution A: 100 g/l Na_2CO_3

Solution B: 10 g/l $CuSO_4 . 5H_2O$

Solution C: 20 g/l K-Na-Tartrate . $4H_2O$

Working solution: 1 part solution B, 1 part solution C and 20 parts of solution A mixed together (done just before use)

FCP reagent: 10% (v/v) Folin-Ciocalteu's phenol reagent

Protein standard: 1.0 mg/ml BSA in water

2) Protocol

In order to obtain the standard curve the BSA solution was prepared in the following concentrations – 0.25, 0.50, 0.75 and 1.00 mg/ml. For zero value water was used. Polystyrene micro cuvettes were used for the measurement.

Table 2-2: Amount of sample and sequence of steps involved in Lowry assay.

Solutions	Samples	BSA standards	Zero value
Samples	200 µl	-	-
BSA standards	-	200 µl	-
water			200 µl
Working solution	200 µl	200 µl	200 µl
It was then incubated at room temperature for 15 min			
FCP reagent	600 µl	600 µl	600 µl
Then incubated at room temperature for 45 min			
Extinction was measured at wavelength 550 nm			

3) Evaluation

The calibration curve was directly obtained from the photometer software or by using Excel application. If the measured extinctions of the sample lie beyond this standard calibration curve then samples were diluted with different dilution factors and measured again. For samples and standards with concentrations less than 100 µg/ml, extinction was measured at 650 nm.

2.2.3 Gel Electrophoresis

Two types of electrophoresis, vertical and horizontal were followed in this work and the protocols of the experiments were designed according to Laemmli.[54]

2.2.3.1 Vertical Electrophoresis

2.2.3.1.1 Preparations of Buffer and Solutions

Separating buffer: 181.5 g/l Tris-HCl

Stacking buffer: 60.5 g/l Tris-HCl

10% SDS solution: 100 g/l sodium dodecyl sulphate

10% APS-solution: 100 µg/ml ammoniumpersulphate (freshly prepared)

Acrylamide/Bis (30%T, 2.67%C) 29.2 g Acrylamide
0.8 g N'N'-bis-methylene-acrylamide

Diluted in 100 ml water, filtered and stored at 4 °C in the dark (maximum 30 days)

Sample buffer (SDS reducing buffer)
(twofold): 30.3 g/l Tris-base
40.0 g/l sodium dodecyl sulphate
240.0 g/l Glycerine
6.2 g/l Dithiothreitol
pH 8.8 (3M HCl)
little grains of bromophenol blue

Electrode buffer (tenfold): 30.3 g/l Tris-base
144.0 g/l Glycin
10.0 g/l sodium dodecyl sulphate
Stored at 4 °C

50 ml was diluted with 450 ml water for each electrophoresis run.

TEMED

2.2.3.1.2 Preparation of Gels

a) Separation gel:

Dependent on the required pore size of the gels homogenous gel was prepared using the following chemicals (Table 2-3) and poured into the gel chamber immediately after 50 µl 10% APS solution and 10 µl TEMED were added (for a 10 ml monomer solution). It was poured in such a way that roughly 1 cm area was left free so that stacking gel can be added later prior to placing of the comb for sample loading. Immediately after pouring the solution, water was added in the free area left to avoid

contacting with air. The contact of air with the solution may cause abnormal polymerization reactions. The polymerization time for the reaction was 1 h.

b) 6% stacking gel:

After the removal of water, stacking gel was prepared using the following chemicals and poured into the gel chamber immediately after 50 µl 10% APS solution and 10 µl TEMED were added (for a 10 ml monomer solution).

5.4 ml water

2.0 ml 30% Acrylamide / Bis – stock solution

2.5 ml stacking buffer (0.5 M Tris HCl, pH 6.8)

100 µl 10% (w/v) SDS

Then a comb was inserted into the stacking gel solution carefully without making any air bubbles. The polymerization reaction time was 45 min. If the gel was shrunken during this time then some more stacking gel solution were added.

Table 2-3: Preparation of separating gel.

Percent gel (%)	H_2O (ml)	30% degassed acrylamide / bis (ml)	Separating buffer (ml)	10% w/v SDS (ml)
10	4.1	3.7	3.1	2.4
11	3.3	3.7	4.3	5.0
13	2.5	2.5	2.5	2.5
15	0.1	0.1	0.1	0.1

2.2.3.1.3 Sample Preparation

The sample and the marker were mixed with SDS-sample buffer (1:1 by volume) and kept in a water bath for 3 min at 95 °C. This step was to denature the proteins. If it was necessary to isolate native proteins the heat denaturation and addition of DTT in the sample buffer were avoided.

A dual colour molecular weight marker (Bio-Rad, Munich, Germany) was used for calibration of the molecular weight scale in SDS-PAGE. It contains ten highly pure recombinant protein bands with 10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kDa. It was mixed 1:1 (by volume) with water and about 10 µl was used for electrophoresis.

2.2.3.1.4 Running Conditions for Electrophoresis

The gel cassette sandwich after casting the gel was removed from the casting chamber, rinsed with water and placed carefully into the electrode assembly. It was then filled with the electrode buffers. 20 - 30 µl sample or marker was taken in a pipette and injected into the wells carefully. The electrophoresis was started with constant current of 20 mA and continued until the protein enters the separating gel. Then a current of 40 mA was applied with a voltage of around 150 - 200 V. The electrophoresis was stopped when the bromophenol blue dye front in the sample buffer reaches the end of the gel.

2.2.3.2 Horizontal Electrophoresis

2.2.3.2.1 Preparation of Gel, Buffer and Solutions

Rehydration buffer: 36.34 g/l Tris
1.0 g/l SDS
0.1 g/l sodium azide
pH 8.0 (adjusted with acetic acid)

Anode buffer: 36.34 g/l Tris
1.0 g/l SDS
0.1 g/l sodium azide
pH 8.4 (adjusted with acetic acid)

Cathode buffer: 9.69 g/l Tris
143.34 g/l Tricine
1.0 g/l SDS
0.1 g/l Sodium azide

Sample buffer (stock solution): 3.0 g/100 ml Tris
pH 7.5 (adjusted with 96% acetic acid)

SDS sample buffer: 5 ml stock solution
0.5 g SDS
7.7 mg DTT
Diluted in 50 ml water, freshly prepared

Precast CleanGels (T = 10%) were purchased from Electrophorese Technik (ETC, Kirchentellinsfurt, Germany). Before the running of the electrophoresis the dry readily

available clean gel must be rehydrated. The gel was submerged in 25 ml rehydration buffer with the plastic part facing upwards for about 60 min. The gel was placed in such a way that there are no air bubbles. After 60 min the gel was dried with filter paper before placing on the horizontal electrophoresis running apparatus.

2.2.3.2.2 Sample Preparation

A total volume of 20 µl was prepared with 10 µl of the SDS sample buffer and 10 µl of the sample protein. It was then heated up to 95 °C for 3 min for denaturation experiments.

2.2.3.2.3 Running Conditions for Electrophoresis

Initially the electrophoresis plate of the unit was water cooled for 15 min. For the positioning of the gel 500 µl of benzene was placed on the cooled plate. After that the gel was placed on the plate with the sample pocket positioned on the cathode side. The anode and cathode stripes were soaked with 20 ml respective buffers. The cathode stripe was placed on the cathode side where the sample pocket was positioned and the anode stripe was placed on the anode side of the gel so that 5 mm of the gel is covered. Any air bubbles present were removed by pressing the stripes with the gel gently. 8 µl of the dual colour marker was loaded in the first and the last pocket in each of the experiments. 10 µl of sample was loaded onto each sample pocket. The electrode cables were connected and the whole unit was covered. Initially 20 mA current was used for around 15 min. The actual current to be used was calculated according to the size of the gel and maintained for 2 h.

2.2.4 Staining of Gels

2.2.4.1 Coomassie Blue Staining

Two types of Coomassie blue staining were used. The first one was prepared according to the protocol of Westermaier.[39]

1) Solutions

Fixing solution: 12% (v/v) Trichloro acetic acid

Stock solution: 980 ml 2% Phosphoric acid

	100 g Ammonium phosphate
	1 g Coomassie Brilliantblue G250 in 20 ml water
	Solutions not filtered
Colouring solution:	160 ml Colouring stock solution
	40 ml Methanol
Washing solution:	12.1 g/l Tris-base
	pH 6.5 (2% Phosphoric acid)
Rinsing solution:	25% (v/v) Methanol

2) Protocol

The gel was kept in a box and about 50-100 ml each of the above mentioned solutions was added.

Table 2-4: Procedure for Coomassie Blue staining of gels.

Step	Solutions	Duration
Fixing	Fixing solution	60 min
Colouring	Colouring solution	Over night
Washing	Washing solution	1-3 min
Rinsing	Rinsing solution	Max. 1 min

Another type of Coomassie staining method was followed in this work using Roti-Blue solution. After electrophoresis, gel was transported to the staining box where the complete staining of the gel took place with incubation of the gel in 100 ml staining solution for 2 to 15 h. The staining solutions contained 20 ml Roti-Blue (Coomassie Blue), 20 ml methanol and 60 ml water. The staining is based on the electrostatic interaction between the anion of Coomassie solution and the protonated amino groups of proteins. The gel was destained in a destaining solution for 5 min. The destaining solution contained 25 ml methanol with 75 ml water. The gel with the blue bands was scanned with a scanner and quantified or it was placed in a stabilizing solution in order to preserve it for a long time. The stabilizer solution is a 20% ammonium sulphate solution. In order to dry the gel it was placed in a solution for 30 min with 10 ml glycerine (99.5%), 20 ml ethanol and 70 ml water. It was placed in a cellophane sheet for future reference.

2.2.5 Semidry Blotting

2.2.5.1 Preparation of Buffers and Solutions

Anode I: 0.3 mol/l Tris (18.15 g) and 20% methanol (100 ml), filled up to 500 ml with water.

Anode II: 25 mmol/l Tris (3.03 g) and 20% methanol (200 ml), filled up to 1000 ml with water.

Cathode: 40 mmol/l 6 aminohexanacid (2.6 g) and 20% methanol (100 ml) filled up to 500 ml with water.

2.2.5.2 Running Conditions for Blotting

After resolving the proteins by SDS-PAGE, separated proteins were transferred onto nitrocellulose or PVDF membranes. The membrane binds and immobilizes the proteins in the same pattern as in the gel. The following procedure was optimised and used for all Western blotting experiments.

After electrophoresis the gel was placed in blotting buffer (anode II). Nitrocellulose membrane and filter papers were cut with respect to the gel size. Filter papers were soaked in the corresponding buffer solutions. The gel and membrane were placed in an Anode II containing box. The graphite anode was wetted with water. The membrane and gel, sandwich termed as transfer unit (Trans) and filter papers were placed on the graphite as shown in Figure 2.14 with the gel surface facing downwards. Air bubbles were removed by gently rolling a glass rod over the top. It was then covered finally with a cathode graphite electrode. The blotting current used was calculated accordingly with 0.8 mA/cm^2 typically 235 mA and it was run for 1 h at RT. The transfer of proteins was complete after 1 h and then followed either by staining or by further reaction with primary antibodies for the Western blotting experiment.

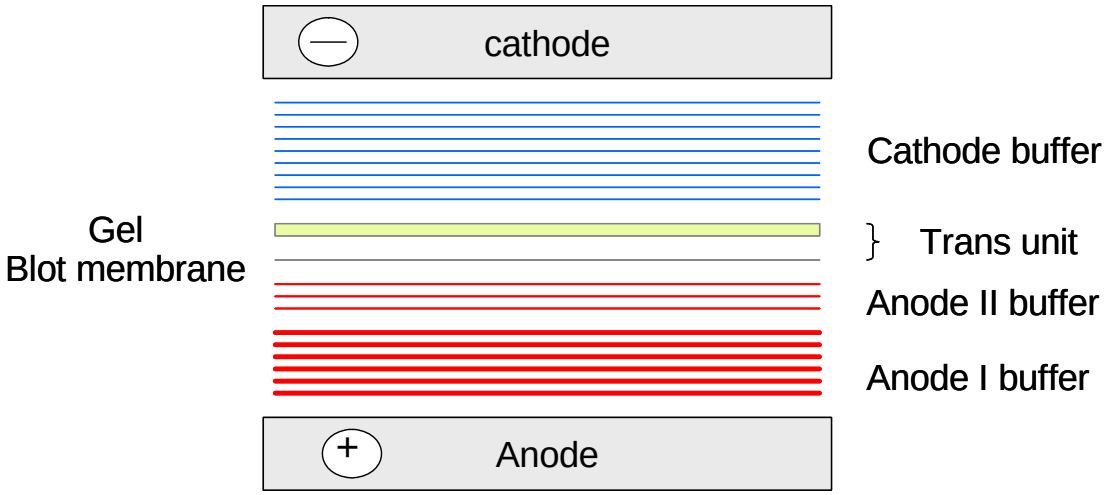


Figure 2.14: Semi dry blotting assembly.

2.2.6 Contact Blotting

After separation the gel and the membrane were soaked in anode II buffer (explained in section 2.2.5.1) for 3 - 5 min. The gel was then placed on a glass plate. Next the membrane was placed on the gel surface. Then the filter papers were placed over the blotting-foil. Above the filter papers another glass plate was placed. Finally a weight was placed on top of the upper glass plate. It was performed from 4 hours (h) to overnight.

2.2.7 Immunoblotting

The semi dry blotting experiment was followed by the immunoblotting experiment for the detection of proteins using labelled antibody experiments. After blotting, the nitrocellulose sheets were incubated over night in PBS (9.5 mM sodium phosphate buffer, pH 6.9, 137 mM NaCl, 2.7 mM KCl), 0.1% Tween 20, 5% milk powder followed by 2 short washing steps with water and then 3 washing steps with PBS/0.1% Tween 20 for 10 min each. The blot membrane was then exposed for 2 h to the labelled antibody (anti-CYP1A1 or anti- CYP2E1) diluted in PBS at a concentration of 1 µg/ml. After that it was washed again with PBS/0.1% Tween 20 3 times.

2.2.8 Indian Ink Staining

After the transfer of proteins from the gel to the membrane, the membranes were stained in order to visualize the protein spots. Although originally needed for

visualization, we found out that metal contaminations being present in the stain can be applied for LA-ICP-MS based detection.

Buffer: 0.05 M Tris HCl, pH 8.0
9.76 g NaCl / l
0.5 ml Tween 20 / l

Staining solution: 250 ml above buffer, 2.5 ml acetic acid 96%, 250 µl Pelican Fount ink, pH 4.0

The membrane after blotting was kept in a plastic box with 0.2 M NaOH for 5 min without shaking. After that it was washed with water. It was then washed 4 times with buffer for 10 min with in between washing with water. Then it was kept in 70 ml staining solution overnight or for a minimum time of 5 h. It was then washed with water for a short time. It was then air dried and ready for LA-ICP-MS measurements. PBS buffer was avoided for experiments where also phosphorus needs to be measured.

2.2.9 Labelling of Proteins

2.2.9.1 Labelling with DOTA and Lanthanides

2.2.9.1.1 Buffers and Stock Solutions

0.5 M Tris-HCl, pH 9.0 – 9.5

0.1 M Tris-HCl, pH 7.5

0.5 M acetate buffer, pH 7 – 7.5

Carbonate-bicarbonate buffer, pH 9

A) 0.1 M sodium carbonate solution

B) 0.1 M sodium hydrogen carbonate solution

C) 10 ml A and 115 ml B were mixed together with 375 ml water.

DOTA stock solution: DOTA (Macrocyclics, Dallas, USA) was diluted in 1 ml carbonate-bicarbonate buffer. The DOTA stock solution was further diluted according to the requirement. 200 µl of stock solution was diluted with carbonate-bicarbonate buffer solution in order to obtain a 40% solution which was used for further steps.

Europium stock solution: 3.664 mg $EuCl_3 \cdot 6\ H_2O$ (Holmium: 2.699 mg $HOCl_3.6H_2O$ and Terbium: 2.732 mg $TbCl_3.6H_2O$) was diluted in 1 ml acetate buffer. 1 µl of the stock solution thus contained 10 nmol europium (holmium and terbium).
BSA stock solution: 1 mg BSA was diluted in 1 ml carbonate-bicarbonate buffer. The concentration was thus 15.1 nmol/ml.

2.2.9.1.2 Procedure

2.2.9.1.2.1 Reaction of Proteins and antibodies with DOTA (Step 1)

In the first step the reaction of the linker with the protein was optimised in the following way. 397.3 µl (BSA) stock solution (= 6 nmol), 411 µl DOTA solution (= 240 nmol; 40 times $c_{protein}$) and carbonate-bicarbonate buffer (pH 9) were added in an Eppendorf vessel as to give a final volume of 1 ml. Alternatively 10, 20, 80 and 100 times of DOTA in molar excess to protein amount were used. In case of antibody about 0.4 mg antibody was used for labelling corresponding to about 2.7 nmol antibody. A shaker was used for the whole reaction time. For stopping the reaction 1 ml of 0.5 M Tris-buffer was added.

The excess DOTA was removed from the protein solution by application of a size exclusion column (PD-10 Desalting column, Amersham, Freiburg, Germany). This column was conditioned and cleaned by 10 times washing with 3.5 ml 0.5 M acetate buffer. The volume of the reaction solution was increased to 2.5 ml by adding acetate buffer and loaded onto the column. The eluent was discarded and the DOTA protein complex was eluted in 3.5 ml acetate buffer. In the next step ultra-filtration was applied using ultra-filtration tubes (Ultracel YM-10 membrane; cut-off 10 kDa, Millipore, Schwalbach, Germany) at 3,000 to 4,000 rpm and 15 °C. For further washing two times 400 µl acetate buffer was used. The ultra-filtration was stopped once a final volume of about 400 to 500 µl was reached. Due to possible protein losses the total amount of protein was determined by use of the Bradford Assay, which is not interfered by the labelling compounds.

2.2.9.1.2.2 Reaction of Lanthanide with DOTA (Step 2)

The molar amount of the protein from the above reaction step was calculated from the resulting concentration and different ratios of excess lanthanide were added for different experiments. The reaction between lanthanides and DOTA protein complex was performed with acetate buffer (pH 7 - 7.5) for 30 min at 37 °C unless otherwise mentioned and loaded onto a purified PD 10 column. The reaction time chosen was 30 min at 37 °C unless otherwise mentioned. The eluent was discarded and the labelled protein was eluted when 3.5 ml of 0.1 M Tris buffer (pH 7.5) was applied onto the column. In the next step the sample was concentrated using the ultra-filtration tubes (15 °C, 3,000 to 4,000 rpm, 1 h). It was washed more than 5 times with 0.1 M Tris buffer. The final volume obtained was around 500 µl. The whole procedure of the above 2 reaction steps is shown in the Figure 2.15 for antibody labelling.

2.2.9.2 Labelling using DTPA

For a comparative study labelling of protein was performed using DTPA. The procedure was followed as explained in literature.[55] BSA with a final amount of 3.03 nmol was buffered at pH 7.0 with 0.05M bicarbonate buffer and added to the solid anhydride (6.06 nmol and 12.12 nmol for 2 separate experiments) and the mixture was agitated for 2-5 min. The free DTPA from the mixture was removed by using the gel filtration column. Europium solution was added to the resulting product from the column so that the molar amount was in 4 fold excess compared to the BSA amount. The buffer used was 0.5 M acetate buffer at pH 6.0. The reaction time was 1 min.

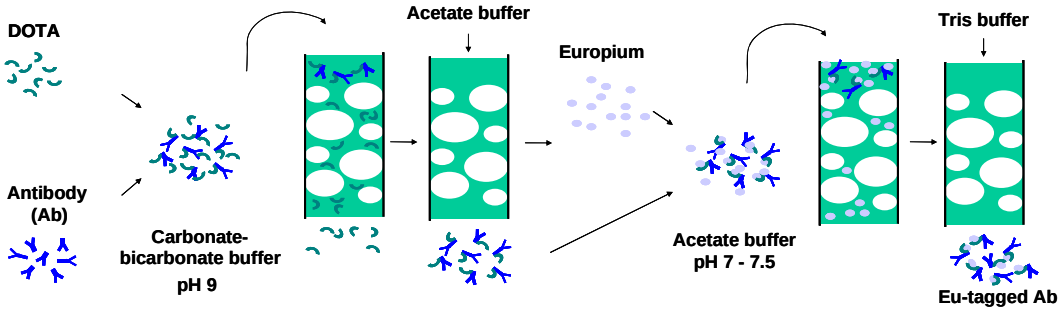


Figure 2.15: Schematics of the whole procedure followed for the labelling of protein and antibodies using DOTA compound.

2.2.10 Quantification of Elements by Means of Quadrupole ICP-MS

2.2.10.1 Sample Preparation

2.2.10.1.1 Preparation of Buffers and Solutions

Europium stock solution: A solution with europium concentration of 0.01 mg/ml in 0.1 M Tris buffer was prepared.

1% TritonX stock solution: 100 ml TritonX in 10 ml water

Internal standards: The concentration of indium and cerium stock solution from the manufacture is 1 µg/µl. From this 100 µl was taken, diluted and made up to 10 ml with 3% nitric acid. 10 µl of this solution thus contained 100 ng indium (cerium).

2.2.10.1.2 Calibration and Quantification

The range for calibration was chosen to be 0 to 500 ng/ml europium. It was prepared from the stock solution with 0.1 M Tris buffer and the final volume was adjusted to be 5 ml. Apart from that each europium standard solution had 20 ppb indium (cerium), 0.05% Triton X and a pH of 5.0 was maintained with 0.1 M nitric acid.

2.2.10.1.3 Protein Samples

100 to 200 µl samples were diluted with 0.1 M Tris buffer and made up to 5 ml. Each sample solutions had 20 ppb indium (cerium) and 0.05% Triton X prepared from the stock and a pH of 5.0 was maintained with 0.1 M nitric acid.

2.2.10.1.4 Measurements

The sample was injected into the ICP MS via the nebulizer. The flow rate of the peristaltic pump was fixed at 0.4 ml/min. There were frequent washes between the standard and sample measurement with a blank solution and also after each complete experiment.

2.2.11 Quantification of Elements by Means of ICP-SF-MS

2.2.11.1 Sample Preparation

2.2.11.1.1 Preparation of Buffers and Solutions

Phosphorus stock solution: A solution with PO_4 concentration of 1 mg/ml in 0.1 M Tris buffer was prepared.

1% TritonX stock solution: 100 ml TritonX in 10 ml water

Internal standards: The concentration of rhodium solution from the manufacture was 1 µg/µl. From this 100 µl was taken, diluted and made up to 10 ml with 3% nitric acid. 10 µl of this solution thus contained 100 ng rhodium.

2.2.11.1.2 Calibration and Quantification

The range for calibration was chosen to be 0 to 200 ng/ml phosphorus. It was prepared from the stock solution with 0.1 M Tris buffer and the final volume was adjusted to be 10 ml. Apart from that phosphorus standard solutions had 10 ppb rhodium, 0.05% Triton X and a pH of 5.0 was maintained with 0.1 M nitric acid.

2.2.11.1.3 Phospho-protein Samples

The phospho-protein samples used were α-casein, β-casein, ovalbumin (Sigma Aldrich, Taufkirchen, Germany) and pepsin (Dunn Labortechnik, Asbach, Germany). 100 to 200 µl samples were diluted with 0.1 M Tris buffer and made up to 5 ml. Each sample solution had phosphorus, 20 ppb rhodium and 0.05% Triton X prepared from stock and a pH of 5.0 was maintained with 0.1 M nitric acid.

2.2.11.1.4 Measurements

The sample was injected into the ICP-SF-MS via the Aridus system. The flow rate of the peristaltic pump was fixed at 0.1 ml/min. There were frequent washes between the standard and sample measurement with a blank solution and also after each complete experiment.

2.2.12 Laser Ablation ICP-MS

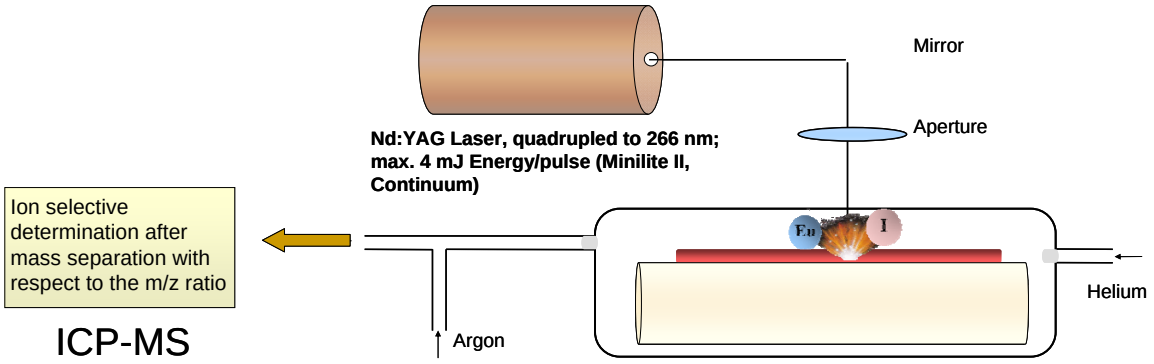


Figure 2.16: Schematic setup of the laser ablation system with the cell used in this work.

For laser ablation a flash lamp pumped Nd:YAG laser operated at the fourth harmonic wavelength was used (266 nm), achieving sufficient laser energy of about 3 mJ and a pulse width of 3 to 5 ns (Minilite II, Continuum, Santa Clara, USA). The laser was operated in a Q-switched mode and with its maximum repetition rate of 15 Hz. The general setup of a LA system used in this study is shown in Figure 2.16.

The homebuilt laser ablation chamber and its optimisation were already described.[56] The cell was equipped with a polytetrafluoroethylene (PTFE) cylinder which has a diameter of 38.16 mm and a length of 140 mm (Figure 2.17). Membranes were mounted on this cylinder. This cylindrical format allowed free rotation around the axis with a help of a cylindrical knob and gave access to ablation of all positions on the membrane surfaces in 2 directions (linear translation and rotation). At the same time low cell volume could be maintained to 11.3 cm^3. The cylinder was placed inside a closed chamber and the cell volume was reduced further with a PTFE insert. There was a rectangular quartz window at the top of the chamber with a length of 140 mm, a width of 20 mm and a thickness of 5 mm (Figure 2.18). The length of the channel was 120 mm so that membranes with a size up to 120 mm X 120 mm could be analysed in one run. The blot membranes were placed on the PTFE cylinder with the surface of the membrane in the focus of the laser beam. The cell was mounted on an X-Y-Z table with µm resolution. The laser was focussed onto the membrane in the chamber using a mirror and a lens system. The photo of the cell and the lens system used is shown in Figure 2.18.

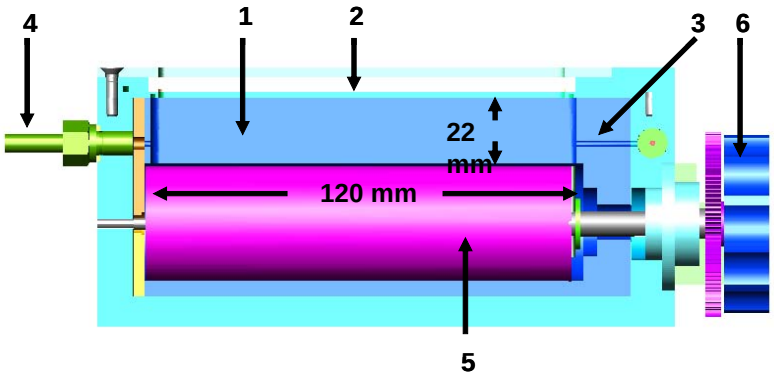


Figure 2.17: Ablation cell, (1) PTFE insert, (2) quartz window, (3) gas inlet, (4) gas outlet, (5) membrane holder, (6) rotary knob.[56]

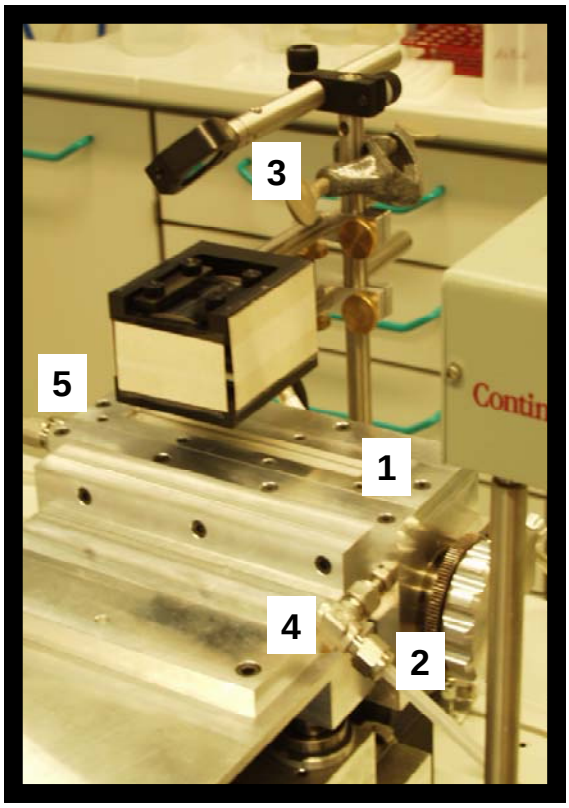


Figure 2.18: Photo of the cell, (1) quartz window, (2) rotary knob, (4) gas inlet and (5) gas outlet and (3) lens system.

The cell was coupled to the ICP torch via polyethylene (PE) tubing with a length of 50 cm and an internal diameter of 4 mm. Helium (He) was used as a carrier gas with a flow rate of 1.3 l/min. It was used as it has a positive effect on reducing the temporal signal dispersion.[57] It also has a positive effect on the laser plume expansion dynamic, the transport of the aerosol to the ICP and guarantees a more efficient vaporization in the

ICP due to a higher thermal diffusivity.[58] For daily tuning of the instrumental conditions, the Aridus (Cetac) was used as a sample introduction system producing a nearly dry aerosol, thus plasma conditions should be not equal but similar to the dry aerosol produced by the laser. The sample outlet of the nebulizer was mixed with the He flow behind the chamber by use of a T-connector. The argon flow rate of the Aridus was set to 1 l/min.

To establish a good spatial resolution during line-scanning, a small crater diameter is advantageous, while for highest signal intensity a larger crater volume would be better to increase the amount of ablated material. To solve this problem beam shaping was used to produce a laser beam of oval shape, with higher width than length. For beam shaping, two planar-convex cylindrical lenses were used, mounted one upon the other in crossed direction. The length of the resulting crater was about 100 µm in scan direction and the width was adjustable between 100 and 600 µm which was influenced by the laser energy used.

The whole chamber was moved relative to the fixed laser beam with a forward speed of 1 mm/s. The blot membranes were subsequently rastered line by line with a distance of 1 mm in-between, e.g. a blot after laser ablation is shown in Figure 2.19. It is an Indian ink stained membrane which had the protein marker spots after SDS-PAGE and semi dry blotting. 8 scans with 1 mm distance were needed in order to ablate the whole electrophoretic lane. The blot was not damaged after one scan and the time taken to complete this experiment was 20 min. It can be seen that the black spots coming from the stain are completely ablated by creating a crater with a depth of approximately 65 µm.

For ICP-MS a sector field instrument type Element-2 was used. Due to the spectral interferences being present at m/z 31 phosphorus was detected at a medium resolution (MR) of 4,000 if not mentioned otherwise. Rhodium, lithium, europium, terbium, iodine and holmium were measured at low resolution. Carbon ($^{13}C^{+}$) was measured at medium resolution. The instrumental parameters which were used for different experiments are shown under experimental sections of each chapter.

Figure 2.19: Photo of a laser ablated Indian ink stained membrane.

2.2.12.1 Data Acquisition

For all the experiments unless and otherwise stated the following parameters were chosen for the method. Both pulse counting and analogue modes were chosen as the detection type and Escan mode was selected as the scan type as it requires virtually no magnetic settling time. In this scanning mode magnetic field is constant and the accelerating voltage can be varied. For the phosphorus intensity measurement at MR 10 channels per peak were chosen with an integration window of 60% and mass window of 100%. Hence, 6 channels per peak from the centre of the maximum peak height were selected for a single data point. For the Low resolution (LR) experiments an integration window of 80% was chosen with 8 channels per peak which were taken into account for a single data point. The segment duration (data acquisition duration per mass) was varied according to the number of elements measured and the mass resolution required. For example for 10 channels with sample time of 10 ms and 100% mass window, the total segment duration will be 100 ms. The number of data points was chosen according to the length of the blot. For example for a blot of 58 mm length with a scan speed of 1 mm/s, with the above set of conditions for the MR measurement, the total calculated data points were 665.

For imaging, all separated intensity data sets for each laser trace were copied together by means of a Matlab software routine (The MathWorks, USA) developed by Ingo Feldmann and Peter Lampen from ISAS, which additionally enables a fast preview imaging of the elemental distribution of the measured blot membrane during measurement. The measured intensity time profile data of the ICP-MS was exported to the program Origin (Additive GmbH, Friedrichsdorf, Germany), where intensity time

profiles as well as colour-coded surface plots were obtained by transforming time into a millimetre scale using the translation velocity selected.

For quantitative evaluation of the results, the intensities for each y-data point (time interval) of all laser traces belonging to one protein spot were averaged - again by means of a Matlab routine - resulting in the intensity-time relation of one electrophoretic lane. In a second step, all peaks of interest were integrated over time or distance using commercially available chromatography software (EuroChrom 2000, Knauer, Berlin).

Chapter 3

Method Development for Application of LA-ICP-MS in Phosphorylation Studies

As was already mentioned, a new LA cell was developed and used for imaging of elemental distribution in electrophoretic protein spots blotted onto membranes.[56] Initially with this cell a calibration approach by use of standard phosphorus containing proteins for quantification of phospho-proteins has been described. But most of the other analytical figures have not been investigated in detail yet.

Thus it was the aim of this study 1) to optimise the working conditions to achieve good detection limits, 2) to develop a quantification scheme for phosphoproteomic studies, 3) to investigate the analytical figures of merit such as reproducibility, sensitivity of the method and 4) to apply the method for a typical application in phosphoproteomics. For the latter topic the human urothelial carcinoma cell line 5637 was selected and time resolved measurements were performed.

3.1 Introduction

Phosphorylation is the most important post-translational modification of proteins and therefore quantitative measurement of the phosphorylation state of proteins is a key issue in many life science applications. The significance is underlined by the fact that a new term and research field, namely phosphoproteomics, has been established.[59,60,61] Dynamics and activity of the proteome are not merely defined by expression levels of proteins, because phosphorylation has a fundamental impact on their activity and life-span. When a phosphate group is added it becomes a phosphorylated protein changing its confirmation and reactivity. Phosphorylation occurs mainly on the serine, tyrosine and threonine, and to a minor extend on the histidine, arginine or lysine residues of the protein. Phosphorylation of specific amino acid residues in enzymes functions as a molecular switch either turning on or turning off the catalytic activity.[62] The enzyme kinase is involved in the addition of the phosphate group to the protein and the enzyme phosphatase is involved in the removal of the phosphate group from the protein. Cellular

signal transduction processes are largely a cascade of protein phosphorylation starting at the receptor level down to transcription factors and proteins control the function of transcription factors.[63] At last, phosphorylation may result in accelerated or retarded degradation of proteins by the proteasome complex. In this case, the phosphorylation status determines the steady state level of a protein.[64] This enumeration of protein phosphorylation effects clearly demonstrates the significance of the phosphorylation status of the proteome for assessing the biochemical activity status of cells or tissues. Thus, quantitative methods in phosphoproteomics are needed in order to identify characteristics of different physiological and pathological states.

3.1.1 Detection Methods for Phospho-proteins

Determination of the phosphorylation status of the proteome or of specific sub-proteomes requires 1) separation of proteins and 2) quantitation of the phosphorylation degree of discrete proteins. Overall detection of phosphorylated forms of proteins is based on reaction of blotted proteins with fluorescence dye labelled antibodies directed against phospho-serine, phospho-threonine and phospho-tyrosine residues. While anti-phospho-tyrosine-antibodies work well, antibodies directed against phospho-serine and phospho-threonine often show weak affinities for their antigens.[63] Besides this, there are some more facts that aggravate the quantitative analysis of the phosphorylation status of proteins only by means of available antibodies. This concerns phosphorylation of amino acid residues other than serine, threonine and tyrosine such as histidine, lysine, hydroxylysine and hydroxyproline.

Some of these problems can be overcome by the use of molecular mass spectrometry in combination with electrophoretic separation of protein samples and techniques for enrichment of phospho-proteins.[65] Molecular mass spectrometry is most often applied for phosphoproteomics after an enzymatic digest of the protein spot in the gel, followed by analysis of the resulting polypeptides including scanning for phosphorylated forms.[66] This can be laborious and time consuming. Additionally, the mass spectrometric response of a phospho-peptide may be suppressed by the presence of other peptides in a complex mixture. This was shown by Wind and co-workers, who analysed a mixture of a tryptic digest of two recombinant proteins after LC separation by

electrospray ionisation mass spectrometry and by ICP-MS.[67] They could show, that the signal measured by ICP-MS was very well representing the expected content of the observed peptides and by that they could compare the ICP-MS sensitivities with those measured by ESI-MS using identical LC conditions. From this comparison they found that the ESI ionisation efficiency of tryptic peptides increases in proportion to the LC retention time and decreases by the presence of an additional basic residue, which makes it difficult to use ESI-MS for quantification. This demonstrates that for quantification of phosphorylated polypeptides and proteins ICP-MS looks very promising and it has already been applied for this purpose, as it is discussed in the following.

A sector field ICP-MS coupled to reversed phase high performance liquid chromatography was already used for the first time at ISAS as an element specific detector for quantitative detection of phosphorylated polypeptides.[22] By use of sulphur, being present in the amino acids methionine and cysteine of the peptides, as an internal standard this method was extended by Wind *et al.* where sulphur was measured together with phosphorus for improved quantification. After calibration by use of standards an easy and straightforward determination of the phosphorylation state was possible by measuring the P/S ratio.[68]

An alternative approach was presented by Bandura *et al.* and with their LC approach they could detect sub nano-molar amounts of phosphorylated polypeptides in a digest. In the investigation of total protein extracts from cultured malignant cells and from human malignant tissue they found an increased global degree of phosphorylation compared to controls.[69]

Pröfrock *et al.* investigated various nebulizers for application of nano and capillary liquid chromatography and measured the phosphorylation state of tryptic digests of β-casein using a reaction cell instrument in an energy discriminating mode and sub-pmol detection of the digests was possible.[70]

These examples show that LC-ICP-MS plays already an important role for detection of phosphorylated polypeptides in enzymatic digests most often of protein standards only. For many applications standards are not available and therefore techniques are needed which can measure, qualitatively and quantitatively, the phosphorylation status already in the gel or directly on a blot membrane.

Sample introduction from a gel or a blot membrane into an ICP-MS is in principle possible by use of laser ablation. This sample introduction technique was used first for metalloproteins separated by immuno-electrophoresis by McLeod and co-workers.[30] LA-ICP-MS for qualitative detection of phosphorus in β-casein was first presented by Marshall and co-workers in protein separation by SDS-PAGE and blotted onto a membrane[71] using a reaction cell instrument pressurized with hydrogen and helium. The limits of detection (LOD) for phosphorus were dominated by high blanks.

Becker *et al.* applied LA to detect phosphorylated proteins qualitatively and quantitatively by drilling a hole into the gel just in the centre of the electrophoretic spot, which was made visible by silver staining.[72] They applied this method for analysis of the human tau protein, which is a key protein in the formation of neurofibrillary tangles in Alzheimer disease. By this method 17 phosphorylation sites of the tau protein were quantified.

More recently LA was also applied for 1D and 2D electrophoretic gel separation to detect cadmium-binding proteins,[73] metalloproteins[74] and selenoproteins,[31] as to mention a couple of applications. More details and a more complete overview are given in a review, which was published recently.[75] Becker *et al.* applied laser ablation for direct detection of metals, sulphur and phosphorus in human brain proteins using again the medium resolution of the sector field device.[76] Semi-quantitative calibration was performed by standards nebulised in a pneumatic nebulizer and added to the laser ablated aerosol. A limit of detection of 0.18 µg/g for phosphorus was determined in the gel blank.[34] The group of McLeod applied whole gel elution of phosphorylated proteins as an alternative to LA for quantification by application of calibration strategy using liquid standards.[77] An additional LC coupling by use of activated alumina was also applied for reduction of phosphate contaminations from the gel and from the buffers.[77]

Krüger *et al.*[78] applied laser ablation ICP-MS for detection of phosphorylated proteins after separation by SDS-PAGE and blotting onto membranes. This technology was used in the meantime to determine the phosphorylation state of the cytoplasmic proteome of selected bacterial and eukaryotic cells.[78] The finding in this case was that the eukaryotic cells exhibit a significantly higher phosphorylation degree compared to the bacterial proteome. Sulphur was used here as an internal standard.

Phosphorylated proteins were not only detected in gels but after blotting onto membranes. This looks promising due to several reasons. Most important a trace matrix separation can be achieved to reduce phosphate blanks from phosphate buffers used for sample preparation in sodium dodecyl sulphate polyacrylamide gel electrophoresis. Additionally the proteins were enriched in a thin surface layer, which looked promising if laser ablation was used for sample introduction. Nevertheless losses during blotting were often mentioned as a limiting factor.[79] In a previous work LA-ICP-MS was already applied at ISAS for detection of phosphorylated proteins separated by SDS-PAGE and blotted onto membranes.[21] A linear relation between the measured intensity and the total amount of β-casein protein loaded onto the gel was measured and could be used as a calibration for quantification of identical proteins. From the calibration curve a limit of detection of about 5 pmol was estimated from the signal to noise ratio.[21] A new laser ablation cell was applied in this work to achieve promising levels of detection from blot membranes using high resolution mode of the HR-ICP-MS. The $^{31}P^{+}$ signal obtained could give quantitative information both with respect to relative and absolute amounts of phosphorus present in phospho-proteins.

3.1.2 Application

3.1.2.1 Dynamic and Steady State Phosphorylation at Cellular Level

Dynamics and function of the cellular proteome are not merely defined by protein expression levels which can be adapted to the cell's needs by induction or suppression of gene activation. In addition to transcriptional regulation, post-translational modifications of proteins have an important impact on protein function. Such modifications can be reversible or irreversible and can influence the activity, the turn-over or the sub-cellular localization of a protein. Protein phosphorylation appears to play the major role among protein modifications with respect to regulation of protein function, and nearly 30% of all proteins expressed in eukaryotic cells are phosphorylated to some extent.[80] Very sensitive cellular processes such as cell cycle control are largely governed by kinase-mediated protein phosphorylation events. Thus phosphorylation is highly a dynamic and complex process and since it is important for cell function, there is a demand on methods which allow analysing the alterations in the phosphorylation state of the proteome. With respect

to analytical strategies and methods to be developed, one is confronted with the following facts: 1) the number of known proteins which are modified by phosphorylation is high and increases continuously. 2) Phosphorylation within proteins occurs at different amino acid residues such as serine, threonine, tyrosine and histidine. 3) One and the same protein molecule can be phosphorylated at multiple sites.

3.1.2.2 Epidermal Growth Factor and Phosphorylation

Effects on cells by extra cellular stimuli can be mediated by membrane bound receptors. As a consequence of ligand binding to the receptor, a cascade of intracellular biochemical responses is elicited. This is also the case for effects evoked by epidermal growth factor (EGF) which was so far well investigated and was used here as a reference. When this EGF binds to its receptor (EGFR) signals are transmitted via phosphorylation and dephosphorylation events which are then involved in regulating tumour growth and metastasis. EGFR is overexpressed for instance in the majority of solid tumours including breast cancer. Such overexpression causes intense signal generation and activation of downstream signalling pathways, resulting in cells that have more aggressive growth and invasiveness characteristics. When the receptor gets activated the tyrosine kinase part of the receptor in turn gets activated which phosphorylates proteins in the signal transduction pathway leading to activation of genes that regulate cell proliferation, angiogenesis, motility and metastasis. The most comprehensive characterization concerning the EGF stimuli was presented by Olsen and co-workers.[81] This group detected quantitatively by use of organic mass spectrometry 6,600 phosphorylation sites on 2,244 proteins and also determined their temporal dynamics (after 0, 5 and 10 min) after stimulating HeLa (human cervix epithelial adenocarcinoma) cells.

Intracellular signalling is significantly based on post-transcriptional modifications of proteins already present in the cell at the time point of stimulation either in the active or inactive form resulting in fast enzyme-driven responses. In the course of reactions, involved proteins can be altered at multiple sites by multiple types of modifications including phosphorylation, acetylation, methylation, ubiquitination and sumoylation.[82]

3.1.2.3 Oxidative Stress by H_2O_2 and Phosphorylation

Different pathways are switched on by hydrogen peroxide and by EGF. Interestingly, H_2O_2 is able to induce tyrosine-phosphorylation of the EGF-receptor. Because serine and threonine remain non-phosphorylated upon H_2O_2–action, specifically only the phosphoinositol pathway via phospholipase gamma is initiated, while the MAPK pathway remains unaffected.[83] Thus, although H_2O_2 and EGF show distinct effects and act on different processes there are also common pathways influenced by both, but a direct comparison is not available because never ever the same cell system was investigated under identical conditions.

3.2 Experimental

3.2.1 Protein Purification

Most proteins from the stock, which were used as standards, contain inorganic phosphorus and therefore had to be purified if used for calibration. Protein purificiation was performed by using the Centriprep ultrafiltration column with a cut-off of 3,000 Da. The proteins in 0.125% ammonia solution were initially centrifuged at 3,000 g for 20 min. The sample was washed with 0.125% ammonia solution various times. When the retentate volume in the Centriprep columns reached around 0.5 ml the centrifugation was stopped and the sample was decanted quantitatively into a separate flask. After purification the concentration of the protein was measured with the Bradford assay. Stock solutions of β-casein, α-casein, ovalbumin and pepsin were prepared with 13.2, 11.8, 11.6 and 16.2 mg/ml. For storage, the filtrate and the retentate solutions were freeze dried at –20 °C for 24 h until they became a solid residue.

The phosphorus content of the purified proteins was quantified by means of ICP-MS in diluted protein solutions as described in section 2.2.11, and in all cases the value measured was lower than the theoretical value. The estimated values are given in the Table 3-1.

Table 3-1: Measured amount of phosphorus quantified by ICP-MS in-solution measurements.

Protein	Amount Detected pmol/ml	Measured value pmol phosphorus/pmol of protein	Theoretical value pmol phosphorus/pmol of protein
α-casein	930	7.4	8
β-casein	761	3.8	5
Pepsin	1415	1.4	1
ovalbumin	762	1.5	2

3.2.2 Gel Electrophoresis

Vertical electrophoresis was performed as explained in section 2.2.3.1 using a discontinuous buffer system with 10% separating and 6% stacking gels. Different concentrations of separating gels were tried for the cell culture experiments. The one which was used in section 3.3.4.1 had 11% of separating gel so that proteins with broader molecular weight range can be separated. Different concentrations of protein (Table 3-1) from the phospho-protein stock (section 3.2.1) were prepared so that a final volume of 20 µl (10 µl sample + 10 µl sample buffer) had the required phosphorus amounts and this volume was applied for most of the vertical electrophoresis experiments (section 2.2.3.1.3) if not mentioned otherwise. Only for the experiments discussed in section 3.3.4, 8 µl from each cell lysate sample was used for separation. The horizontal electrophoresis with 10% precast gels was used for separating proteins in section 3.3.4.2 as gels of large size can be used.

Nitrocellulose membrane was used for the transfer of proteins from gels for all experiments except the comparison experiment in section 3.3.1.4 where polyvinylidene membrane was used. The semidry and contact blotting procedures were performed as explained in section 2.2.5 and 2.2.6, respectively.

3.2.3 Cell Culture

The human urothelial carcinoma cell line 5637 (CLS Cell lines Service, Heidelberg, Germany) was provided by PD Dr. P. Roos from IfADo. This cell line was grown in medium sized culture flasks on RPMI 1640 (with glutamine)

supplemented with 10% FCS and 1% penicillin/streptomycine in an incubator (37 °C, 5% CO_2). For the experiments, culture flasks containing 2 million cells in 25 ml culture medium were used. After 24 h of culture, if there was enough growth, cells were serum deprived for another 24 h. In independent experiments, cells were subsequently treated either with hydrogen peroxide (100 µM), EGF (100 ng/ml = 8.1 nM) for 7.5 min or hydrogen peroxide (100 µM) for 2, 4 and 7.5 min. Effector concentrations and incubation times were selected according to Li *et al.,*[84] Wilson *et al.*[85] and Olsen *et al*.[81] Incubations were stopped by discarding the culture medium, an intermediate quick washing step with ice-cold 20 mM Tris-buffer (pH 7.5) and by final disruption of cells in SDS-containing electrophoresis sample buffer diluted 1:2 with 20 mM Tris-buffer (pH 7.5). The resulting suspension was heated for 5 min at 95 °C. Aliquots were used for subsequent analysis or kept frozen at –20 °C until use. The protein concentrations were determined by Lowry assay as explained in section 2.2.2.3. It is important to note that no additional sample pre-treatment was performed with the cell lysate in order not to alter the phosphorylation state or the protein profile. But this has a severe consequence: all phosphorus containing bio-molecules such as ATP, DNA or RNA are still present in the samples and can contribute to blank values. The experiments were performed at IfADo in collaboration with the group of PD Dr. Roos.

3.2.4 Laser Ablation ICP-MS Optimisation

For optimisation, Aridus (Cetac) was used as a sample introduction system producing a nearly dry aerosol, thus plasma conditions should be not equal but similar to the dry aerosol produced by the laser. To optimise the analytical conditions of the LA-ICP-MS, several parameters such as gas flow rates, applied power of the ICP-MS, torch position and multiplier voltages were varied. The instrumental parameters for the laser ablation and the ICP-MS used for better optimised performance are shown in Table 3-2. Between the experiments laser energy was controlled using a laser energy meter. From time to time ICP-MS instrument was calibrated over the entire mass spectrum using multielemental standards. An overview about the laser setup, real time analysis, scan settings, detection mode and data acquisition was given in section 2.2.12. For the

experiments, the absolute intensities obtained for different blot membranes can only be compared when they were measured with similar optimisation conditions. The positioning of the laser ablation cell, the starting up of the cell movement and the triggering of laser were manually performed except experiment shown in section 3.3.4.1, where a microprocessor control unit was used for positioning the laser cell and the whole procedure of ablating a full membrane was automated including the triggering of laser. .

Table 3-2: Instrumental parameters.

Laser ablation system	
Laser energy	3.5 mJ
Make-up gas flow rate (Ar)	1 l/min
Carrier gas flow rate (He)	1.05 l/min
Repetition rate, shots per second	15 Hz
Translation velocity	1 mm/s
Distance between line scans	1 mm
Crater diameter	~500 µm
ICP-MS system	**ELEMENT 2**
Torch position	
X,Y,Z	2.85 mm, 0.85 mm, -5 mm
Incident power	1,200 W
Cooling gas flow rate	16 l/min
Auxiliary gas flow rate	1.3 l/min
Ni skimmer cone diameter	1 mm
Ni sampler cone diameter	0.7 mm
Isotopes measured	$^{31}P^{+}$, $^{13}C^{+}$
Total measurement time per peak including settling time	101 ms
Mass resolution	4,000 (MR) 400 (LR)

3.3 Results and Discussion

3.3.1 Optimisation of LA

3.3.1.1 Scanning Conditions

In principle, the ablation cell, which was already developed[56] and which is used for the imaging of $^{31}P^{+}$ distribution after blotting onto large size membranes, should have the following features. It should provide a reasonable local resolution so that adjacent electrophoretic spots can be separated for which a sufficient lateral resolution is required. Additionally, it should provide an as high as possible but constant sensitivity independent of the location of the electrophoretic spot in the cell. All these conditions had been successfully optimised. An optimisation of generator forward power, carrier and make-up gas flow rates for the cell had been performed. It is advantageous to use high He gas flow rates as they have a positive influence on the basis peak width and enable fast sample wash-out from the cell. Continuous ablation with high laser repetition frequencies was preferred since it showed less intensity losses from gas inlet to gas outlet than single shot ablation[56] and in addition a better representation of the electrophoretic spots could be acquired. The cell can be used with a fastest laser scan velocity of 2 mm/s. But in order to have more data points from the electrophoretic spots investigated and to maintain a good local resolution, the line-scan velocity of 1 mm/s was used. This line-scan speed is much faster in comparison to values from literature[71] (60 µm/s) and it can be used to reduce the total analysis time for the detection of separated electrophoretic spots. The optimised parameters were given in the section 3.2.4 which resulted in highest peak area and thus in a signal with moderate peak width.

In order to reduce the measurement time, back and forth scanning of the laser beam looks advantageous. Thus, at the beginning bi-directional scanning was checked using $^{13}C^{+}$ as a marker for laser ablation conditions and signal dispersion. This element is present in the membrane materials and can be utilised to see if this element can be used as an internal standard.

Two measurements were performed at one end of an NC membrane where there are no protein spots present after subjected to blotting. The first one was by scanning in

direction from the gas inlet to the outlet (forward), the next in the reverse direction (Figure 3.1). The $^{13}C^{+}$ intensity measured in forward direction shows a constant signal for a length of the scan line of about 6 cm. But when the membrane was scanned in a reverse direction, a certain drift in the $^{13}C^{+}$ intensities could be seen. This phenomenon can possibly be attributed to particles trapped in the dead volume in a turbulent zone. Although time can be saved for the whole measurement, scanning in reverse direction was not performed to avoid this drift effect of the signal intensity.

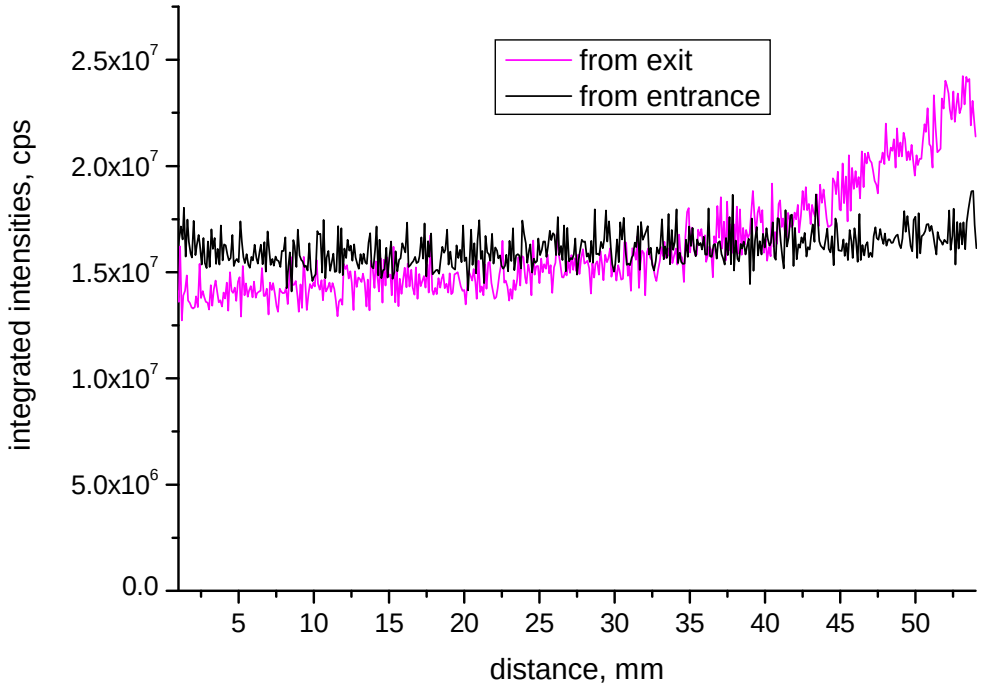


Figure 3.1: $^{13}C^{+}$ trace on an NC membrane in the forward and the reverse direction.[86]

Concerning this measurement, $^{13}C^{+}$ can possibly be used as an internal standard particularly for experiments where measurements are performed in both directions. As a proof for using $^{13}C^{+}$ as the internal standard, another second element should be available for comparison. $^{13}C^{+}$ was not applied here as to spend most time of our relatively slow scanning sector instrument to measure the analytical element. But it will be applied in experiments for quantification of Cytochromes P450 isozymes shown in chapter 5. In these experiments a line scan of $^{13}C^{+}$ was measured at medium resolution at the end of each of the NC membranes before start of the experiment. The obtained intensities were averaged and can be used to normalize with the resulted sample intensities from different blots. Sulphur is an important marker element present in most proteins, but can not be

used as an internal standard for calibration and determination of the phosphorylation state here, because a very high S blank signal was caused from the SDS used for separation in PAGE. This problem can only be overcome if a native PAGE is applied.

3.3.1.2 $^{31}P^+$ Intensity Measured with Low and Medium Mass Resolution

Detection of $^{31}P^+$ is hampered in plasma-based spectrometry, since it features a high first ionization potential (10.484 eV) and therefore low ionization efficiency in an argon-based plasma and various polyatomic interferences in particular $^{15}N^{16}O^+$ are limiting as well. However, the commercial availability of high-resolution instrumentation and collision/reaction cell ICP-MS has essentially solved this interference problem.[69,87,88]

In principle a MR of 4,000 as it is provided with the Element 2 used here is sufficient to overcome the most prominent interferences. However, due to the nitrocellulose matrix also matrix related spectral interferences such as from $^{15}N^{16}O^+$, $^{13}C^{18}O^+$, $^{14}N^{16}O^1H^+$ and $^{14}N^{17}O^+$ can be expected and therefore the mass window of phosphorus at m/z 31 was detected by using a magnetic scan and the medium resolution mode. A mass range covering the most important interferences of phosphorus, $^{15}N^{16}O^+$, $^{13}C^{18}O^+$ and $^{14}N^{16}O^1H^+$, was measured using medium mass resolution. The result is shown in Figure 3.2 for an NC membrane on which β-casein with a total amount of about 150 pmol of phosphorus was blotted after 1D SDS-PAGE separation. This spectrum was obtained from the data points measured during ablation of the blank area of the membrane. Three peaks are seen, the first one belongs to phosphorus at 30.973 Da, the second one can be attributed to $^{15}N^{16}O^+$ at mass 30.995 Da and the third is $^{14}N^{16}O^1H^+$ at mass 31.0053 Da. From Figure 3.2 it can be seen that the interfering signals of $^{15}N^{16}O^+$ and $^{14}N^{16}O^1H^+$ are below 1% of the intensity of phosphorus blank. This is surprising, because by ablation of the NC membrane all critical elements like C, N and O were introduced into the plasma. Nevertheless, it seems that the amount of oxygen from the membrane is not high enough to form oxides at the levels of phosphorus signals.

To compare the detection limits in low (R = 400) and medium (R = 4000) resolutions, two spots of above mentioned standard proteins were measured. Per spot 8 line scans were summed up and the results are shown in Table 3-3. The peak height measured at low resolution was 8 times higher than those of the medium resolution.

Nevertheless, at medium resolution a limit of detection of 1.5 pmol could be achieved which is lower than those of 4 pmol calculated for the low resolution mode. This is the reason why in this study the medium resolution mode was used further on. Nevertheless, this measurement also shows that phosphorylation of proteins can be studied with quadrupole instruments even without reaction cell if LA is used for sample introduction.

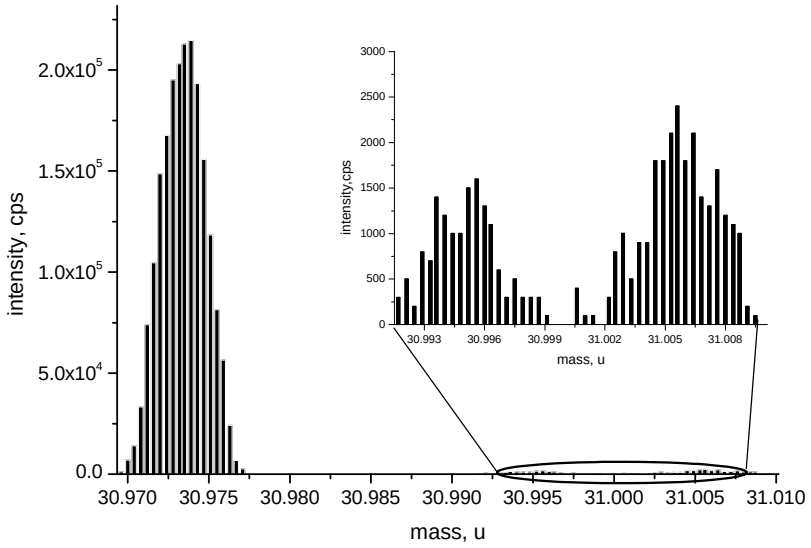


Figure 3.2: Mass window of $^{31}P^+$, $^{15}N^{16}O^+$, $^{13}C^{18}O^+$ and $^{14}N^{16}O^1H^+$ during measurement of the blank of an NC membrane using LA-ICP-SFMS (medium resolution mode).[86]

Table 3-3: Intensity and estimated limits of detection in medium resolution and low resolution: application of 152 pmol of phosphorus in β-casein.

Parameters	Low resolution	Medium resolution
Peak height (cps)	$38 \cdot 10^6$	$4.81 \cdot 10^6$
Standard deviation	$0.33 \cdot 10^6$	$0.015 \cdot 10^6$
Background level (cps)	$2.6 \cdot 10^6$	$2.2 \cdot 10^5$
LOD (pmol)	4	1.5

3.3.1.3 Reproducibility

Reproducibility means the quality of giving reproducible and consistent results from adopting a specific experimental procedure. It was not known at the beginning of the study how reproducible PAGE separations like sample preparation, dilution steps and sample application into the wells are. It is important to know how signal generation by a

pulsed laser ablation and drift effects of the ICP-MS might contribute additionally to worsening of the standard deviation of signals. Therefore, the reproducibility of signal responses from LA processes of the blots analysed with ICP-MS is necessary to prove the accuracy of this method. For this purpose 7 samples of 20 pmol β-casein each (corresponding to a total phosphorus content of 76 pmol) were separated by 1D SDS-PAGE and blotted onto an NC membrane using conditions described in section 3.2.2. The $^{31}P^{+}$ intensity of the protein spot area was measured during a line scan with a length of 57 mm. The whole membrane area (57 mm x 53 mm) was covered by 53 line scans with a distance of 1 mm in-between. In addition to the return back time of the chamber and dead time of around 1 min (required for synchronization with data acquisition of ICP-MS) a total instrumental analysis time of 65 min was necessary. From this measurement (Figure 3.3) the peak area of the phosphorus signals with those of single line scans was compared. The single line scans for the measurement were chosen from the centre of the protein spot. The mean value of all the integrated peak profile areas of the accumulated signal of 7 single line scans was found to be $2.5 \cdot 10^6$ compared to $4.5 \cdot 10^5$ counts of the single line scan. The relative standard deviation over all 7 spots comparing the mean values resulting from multiple spots with single scan amounted to 6.1% which was surprisingly low and demonstrated that laser ablation and SDS separations are quite reproducible. When only single line scans were evaluated the relative standard deviation was increasing to 10%. This is the reason, why integration is always performed with multiple scans across a protein spot in the following experiments. Apart from the laser scanning conditions on the same blot, reproducibility of the gel electrophoresis and blotting conditions are also necessary to prove the accuracy of the method. So two different electrophoretic separations and blotting were performed with 152 pmol β-casein, as explained in section 3.2.2 using vertical electrophoresis. $^{31}P^{+}$ intensity of the protein spot areas was measured for the two blots using LA-ICP-MS on the same day and shown in Figure 3.4. The intensities of the whole area of the protein spots obtained from the multiple scans were summated and found to have $7.56 \cdot 10^6$ cps and $8.77 \cdot 10^6$ cps for blots a and b shown in Figure 3.4, respectively. The relative standard deviation over 2 spots comparing the mean values resulting from multiple spots amounts to approximately 11% which is low and demonstrates that electrophoresis and blotting is quite

reproducible. But more blots should be compared and it should also be measured together in the same laser ablation experiment with less time interval in order to avoid any other effects. Alternatively internal standards such as $^{13}C^{+}$ can be used for normalization of obtained intensities from different blots irrespective of the electrophoresis and blotting procedures.

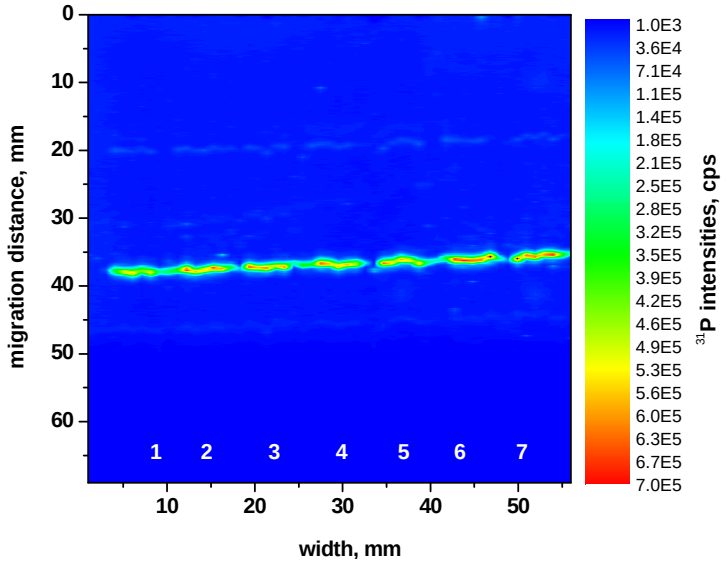


Figure 3.3: $^{31}P^{+}$ intensity distributions of 7 samples of 20 pmol β-casein each (76 pmol phosphorus amount) measured by LA-ICP-MS on an NC membrane after SDS-PAGE and blotting.[86]

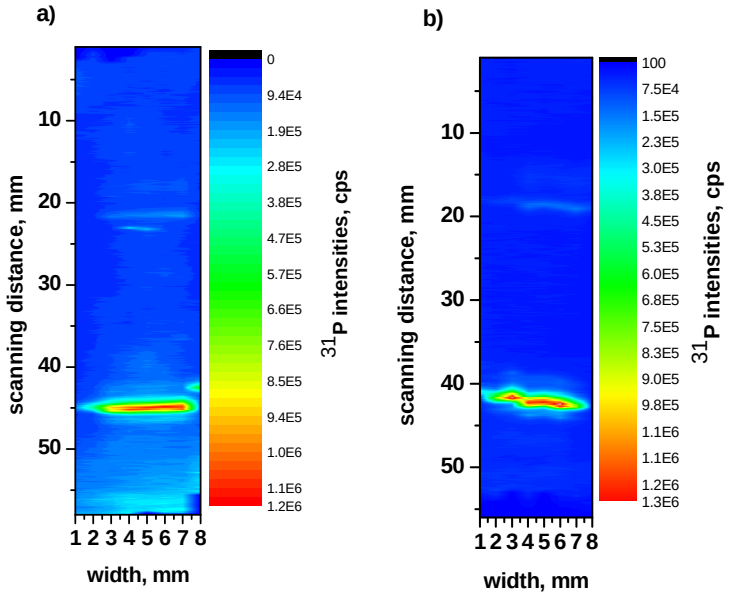


Figure 3.4: $^{31}P^{+}$ intensity distributions of 40 pmol β-casein (152 pmol phosphorus amount) on 2 different membranes measured by LA-ICP-MS after SDS-PAGE and blotting.

3.3.1.4 Comparison of PVDF and NC Membrane Characteristics for LA on Phosphorylated Proteins

For blotting, different membrane materials are commercially available but most often either PVDF or NC are applied, because they provide a stable binding of proteins on the surface, so that losses of proteins penetrating the membrane material are reduced as much as possible. The binding capacity of both materials is similar but depends on the manufacturer. Typical values are 70 - 150 µg/cm for NC and 125 - 300 µg/cm for PVDF. Additionally, from the analytical point of view, the material should have a couple of features with respect to laser ablation, laser energy and detection by ICP-MS. For instance, it should show good ablation properties and should have low blanks for the elements of interest. Furthermore it should not cause additional spectral interferences. Therefore, both materials were investigated after separation and blotting of 40 pmol of β-casein (Figure 3.5). From the accumulated $^{31}P^{+}$ intensity it can be found that for the same laser energy the $^{31}P^{+}$ intensity of the protein spot area is more than a factor of 13 higher for the NC in comparison to the PDVF material. This strong difference can only partially be explained by the different ablation behaviour of the membrane material. After the first laser scans the depths of the craters in NC and PVDF membranes were measured by a white light interferometer, they were found to be to be 65 µm and 31 µm, respectively (Figure 3.6a and b). For PVDF, a second scan on the same laser trace reached a depth of 54 µm, while for NC the membrane was drilled through. From the crater profile for a laser trace of 10 mm length a volume of 0.19 mm^3 for NC and only 0.074 mm^3 for PVDF was calculated, thus 2.5 fold more material was ablated in case of an NC membrane. That shows that both materials behave quite differently as the chosen laser energy is more suitable for NC membranes. One reason for this different behaviour might be that NC decomposes easily after heating to temperatures above 185 °C whereas PVDF decomposes at around 425 °C.

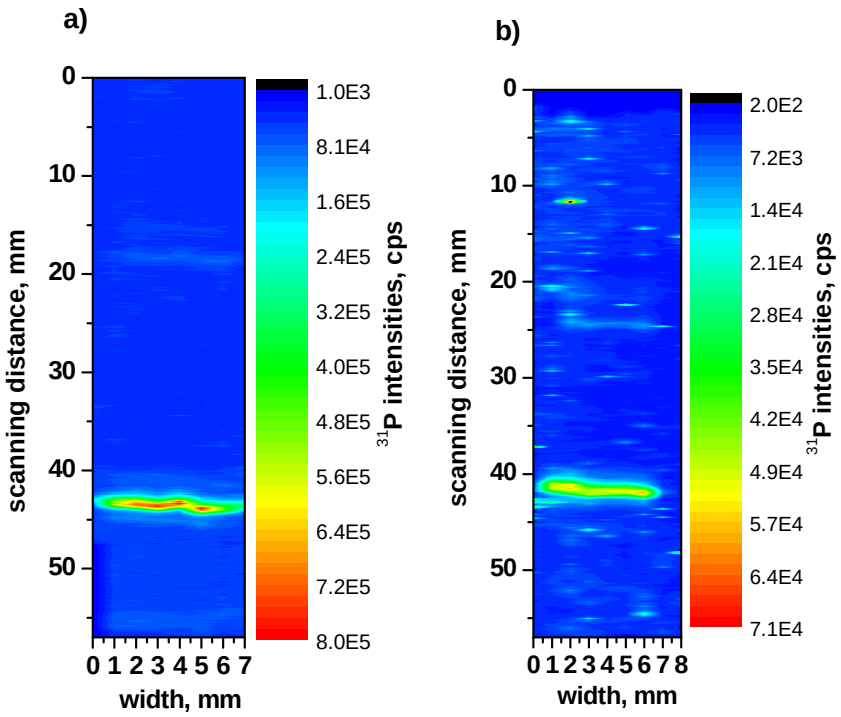
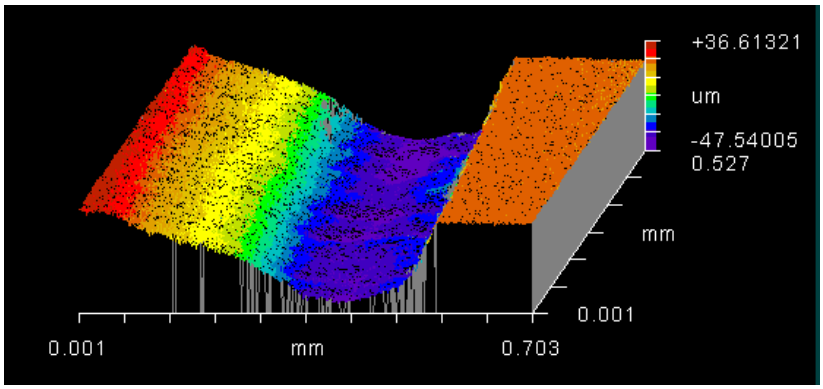


Figure 3.5: $^{31}P^{+}$ intensity distributions of 40 pmol of β-casein after blotting to an (a) NC and (b) PVDF membrane and subjected to LA-ICP-MS.[86]

In a similar experiment the depth distributions of the protein spot were investigated by ablating every scan line a few times until the membrane material in the crater was completely ablated. This was done for both membrane materials, but because of the different ablation behaviours, the depth resolution was strongly different for both materials, because the same laser energy was used for both experiments. The resulting time profile is shown only for the PVDF material in Figure 3.7, because no protein related signal was detected for the NC material during the second scan. Considering the intensities of all the scans to be 100%, only 56% of the intensities could be obtained in the first scan (depth of 31 µm), whereas with the second scan – reaching a depth of 54 µm – 30% was detected. With the third scan this value dropped to 9% only, but the depth was not measured in this case. In total five scans were needed to penetrate this material. From the results of this investigation it could be concluded that on both membrane materials the proteins were enriched within a depth of the upper 70 µm.

a)



b)

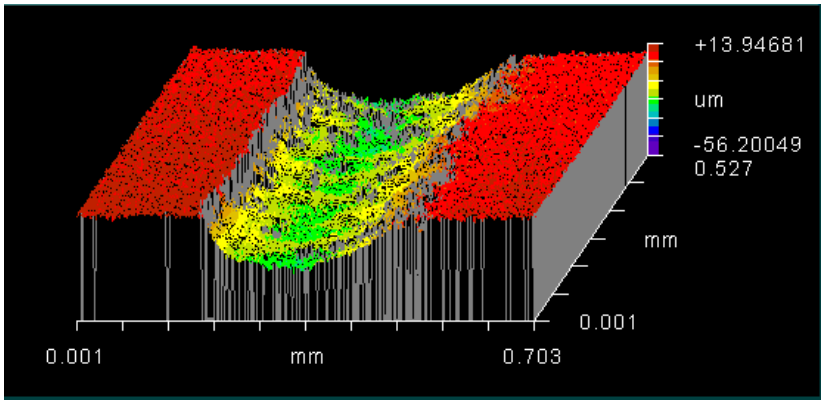


Figure 3.6: (a) Crater profile of a laser trace on an NC membrane measured by means of a white light interferometer, (b) Crater profile of a laser trace on PVDF membrane measured by means of a white light interferometer.[86]

The value of 56% in the first scan was less than the value reported by Müller *et al.*,[89] who had also used PVDF membranes and found recovery rates of gold-conjugated antibodies in the first scan line of 78.6%. However, the depth distribution of proteins can also depend on the applied protein and the amount.

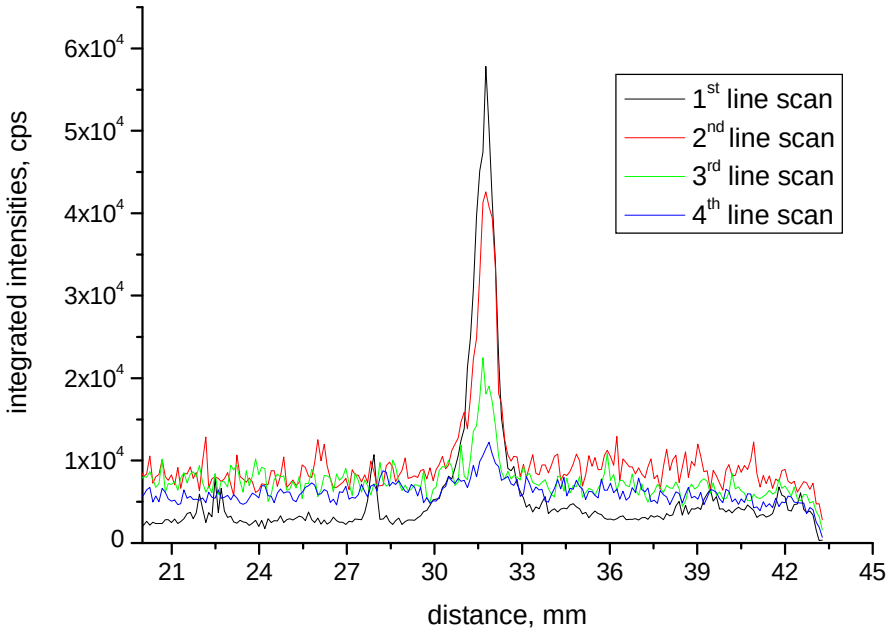


Figure 3.7: Multiple line scans of a protein spot of 40 pmol β-casein on PVDF membrane.[86]

From a pragmatic point of view the selection of the material is quite straightforward. Although both membranes enrich the protein in a thin surface layer, NC membranes are more suitable with the present lens system used so that for not too high amounts of protein loaded onto the membrane single scanning of the protein spot per sample is even sufficient and guarantees highest sensitivity, too. Additionally it was shown in Figure 3.2, that no additional spectral interferences are generated for NC membranes. This is the reason why this material was exclusively used further on.

3.3.1.5 Comparison of Different Blotting Processes for LA detection

There are different types of blotting processes available for the transfer of proteins from gels to membranes but contact and electro blotting methods were investigated and compared here to achieve better detection levels for phospho-proteins using LA-ICP-MS. Contact blotting is the simplest and it involves the diffusion principle. It is already known that contact blotting is less efficient than the electroblotting methods. However it was not clear how proteins are distributed in the upper layer of the membranes after using different blotting methods. As it was discussed in the previous section, this is important to optimise the laser ablation processes of membranes. Therefore an experiment was

performed, to check the distribution of the proteins in the membranes after different blotting methods and to find conditions of highest signal intensities.

Two sets of protein samples, pepsin and β-casein, containing each about 50 pmol phosphorus were subjected to 1D separation. One set of samples was subjected to contact blotting and the other to semidry blotting. In order to avoid the interferences from the ICP-MS for $^{31}P^{+}$ measurement at MR, membranes were not stained. After that LA was performed on the blots which were mounted on the same cylinder. The resulting phosphorus intensity plots are shown in Figure 3.8a and b for semidry and contact blotting, respectively. Trace 1 corresponds to β−casein and trace 2 to pepsin. From the intensity profile it is obvious that the semidry blotting procedure shows the highest signal intensity. From the integration data of these samples, it was found that the obtained intensity for β−casein using semidry blotting is ~$1.01 \cdot 10^6$ cps which is 3 times higher than those of contact blotting ($3.35 \cdot 10^5$ cps). It was also similar for pepsin where it was 2.9 times higher for semidry blotting ($1.16 \cdot 10^6$ cps) than those of contact blotting ($3.9 \cdot 10^5$ cps). Therefore semidry blotting was selected here for all further investigations.

3.3.1.6 Sensitivity and Limits of Detection

Sensitivity is the ratio of the net signal obtained to the concentration of a specific element. The detection limit of any method is indirectly proportional to its sensitivity. Thus for getting better detection limits, sensitivity has to be optimised. Sensitivity of a method is controlled by factors such as the instrument design and the various optimisation conditions used for the analysis such as the settings on the ion optics, alignment of torch positions etc. For any quantitative analysis, sensitivity is one of the significant parameters to be considered. The benefit of using HR-ICP-MS is that it is highly sensitive but the consistency of the resulting sensitivity should be checked with respect to the analysed samples, here different phospho-proteins. So far, only pepsin and β-casein were applied and therefore in the next experiments other phospho-proteins have to be investigated with respect to sensitivity. Since the molecular weight and structure of the phospho-proteins vary, this study was needed to develop quantification schemes for as many phospho-proteins as possible. The following experiment was designed keeping in mind all the optimisation procedures discussed in the above topics. As previously

discussed a nitrocellulose membrane was chosen and for the blotting the semi dry blotting method was adopted.

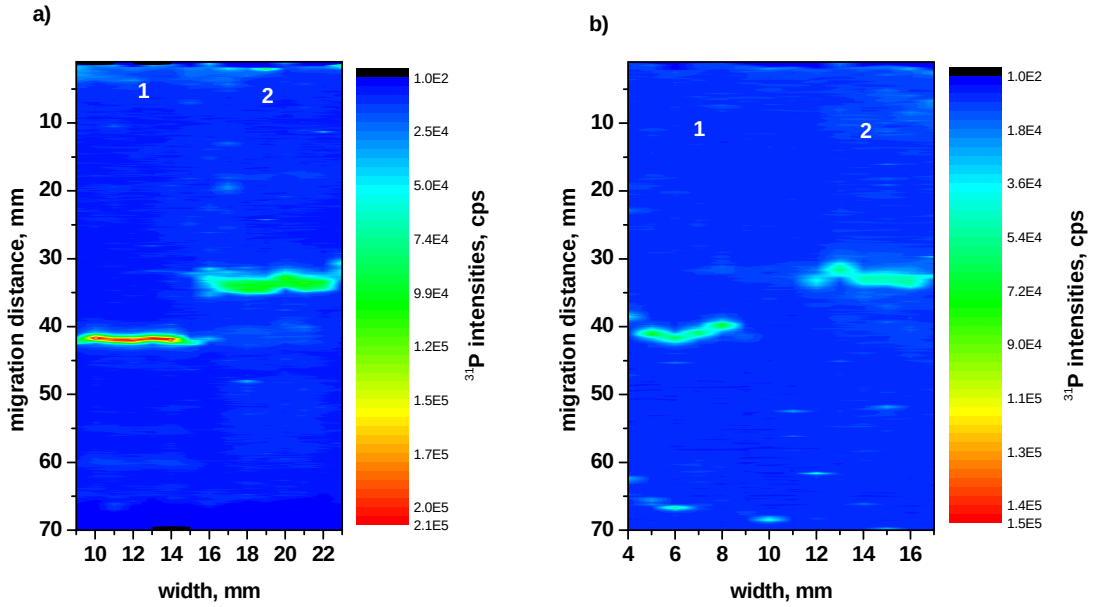


Figure 3.8: (a) $^{31}P^+$ image of semidry blotting experiments with different proteins (1) β-casein and (2) pepsin, but with same amount of phosphorus, 50 pmol and (b) the same samples with contact blotting experiment.

The phospho-proteins pepsin, α-casein and ovalbumin with a final phosphorus content of 45, 451 and 88 pmol were SDS-PAGE separated and blotted. The 2 dimensional plot of the $^{31}P^+$ intensities at MR were obtained after LA-ICP-MS measurement. 8 scans were performed for each protein spot in 1 mm distance. From Figure 3.9 different intensity spots can be seen dependent on the phosphorus content. For example α-casein which has 8 theoretical phosphorus residues shows a more intense spot and the signal intensity obtained is higher compared to the other 2 proteins. For ovalbumin, the spot is broader which is also true for pepsin. Apart from these spots some impurities were still detected which can be neglected. Additionally some spikes (denoted by arrows in Figure 3.9) could be seen coming from the minute particles present inside the cell during the laser ablation process.

Each of these spots was cumulatively integrated. The integrated intensity value was calculated from the cumulative line scan obtained from the Matlab routine. This is shown in Figure 3.10. The integrated values are mentioned in the figure with the

respective phosphorus amount loaded. The integrated intensity for α-casein was obviously higher when compared with those of the other 2 proteins which have similar intensity distributions. The sensitivity of the method with respect to each protein was estimated using the peak area and found to be consistent (ovalbumin-2.16 · 10^4 cps/pmol, pepsin-2.67 · 10^4 cps/pmol and α-casein-2.44 · 10^4 cps/pmol) with a relative standard deviation of 11.6%. Each protein exhibits different distribution on the membrane, thus for calibration and quantification the whole peak area was taken into consideration for integration. The LOD of the method calculated using the 3σ definition for α-casein was 1.4 pmol whereas for pepsin and ovalbumin it was 4.4 and 6.8 pmol, respectively.

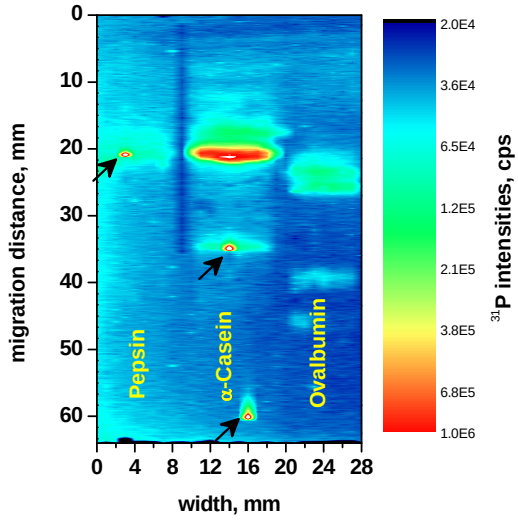


Figure 3.9: $^{31}P^+$ images of the blot obtained after LA-ICP-MS. Prior proteins were separated using SDS-PAGE and semi dry blotted. Amount of protein loaded: 1 µg with a final phosphorus amount of 45, 451 and 88 pmol of pepsin, α-casein and ovalbumin. Arrow represents the spikes obtained during the laser ablation process.

This method was also suitable for proteins which are present in the same lane on the membrane rather than in 3 lanes, which was shown above. The 2 different phospho-proteins, ovalbumin and α-casein, were mixed together (with final phosphorus amounts of 75 and 92 pmol, respectively) and separated by SDS-PAGE separation and blotted. The $^{31}P^+$ image of the blot after LA-ICP-MS is shown in Figure 3.11. The integration of the spot area was performed and the sensitivities achieved for the method were consistent with a relative standard deviation of 4.4% for the phosphorus amounts of different phospho-proteins measured (α-casein: 6529 cps/pmol, ovalbumin: 7373 cps/pmol). Peak

area was considered for the calculation of sensitivity. The LOD of the method calculated using the 3σ definition for α-casein was 0.43 pmol whereas for ovalbumin it was 1.86 pmol which was better than the experiment where electrophoretic separation of three proteins were done in different lanes.

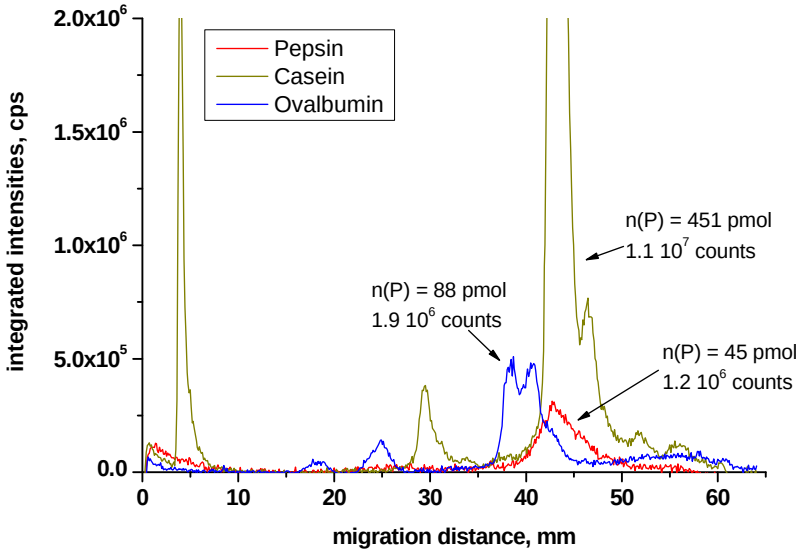


Figure 3.10: Integrated phosphorus intensities as a function of migration distance for the 3 proteins shown in Figure 3.9.

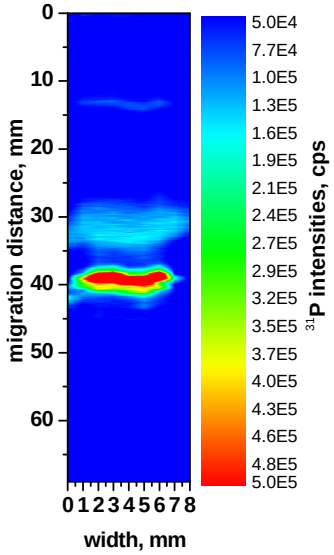


Figure 3.11: $^{31}P^{+}$ images of the blot obtained after LA-ICP-MS. Samples: Mixture of protein ovalbumin and α-casein loaded with a final phosphorus amount of 75 and 92 pmol, respectively.

3.3.2 Calibration and Quantification Strategy

For calibration different concepts are described in literature. For instance, Becker *et al.* have used an ultrasonically generated aerosol simultaneously introduced with the ablated aerosol for quantification.[72] For application of this approach it is a prerequisite that the laser aerosol size distribution is very well represented by the pneumatically generated aerosol as well as it does not change during ablation. Additionally transport, vaporization and ionisation must be identical for both types of aerosols, which is hard to achieve. From this point of view all those concepts are preferred in this work where the signal of the standard and of the target protein is coming from a laser ablation process of the same matrix.

For gel applications a couple of papers have already shown the suitability of a calibration by matrix matched standards. For instance, Becker *et al.* have recently also used an alternative calibration approach for phosphorus determinations in proteins.[34] In this purpose the $^{31}P^{+}$ intensity of the standard protein, in this case ovalbumin was dotted with total amounts ranging from 0.1 ng up to 500 ng on a gel after separation of the proteins. From the slope of the linear calibration graph and the gel blank they calculated a LOD for phosphorus of 0.18 µg/g.

Marshall *et al.* have used the phosphorus containing protein β-casein for calibration and a limit of detection of 16 pmol has been estimated.[71] The direct ablation of the gel was limited by a too high blank value of phosphorus in the gel. Bandura *et al.*[69] have used the protein phosvitin in 1D separation for a calibration calculating limits of detection at 50 – 100 pmol level.

Alternatively, Chéry *et al.* have used hydrated gels with multielement standard solutions for a calibration and calculation of limits of detection under multielement conditions.[31] This technique using metal standards hydrated in a gel cannot be used here, because metals are lost during the blot procedure due to the fact that they are moving to the cathode, whereas SDS treated proteins move to the anode. A calibration and quantification approach was chosen where the signal of the standard and of the target protein was coming from a laser ablation process of the same matrix.

Therefore two different calibration procedures, 1) dotting and 2) blotting were investigated here for comparison. The first procedure was based on the use of standards

directly dotted by a pipette onto the surface of the membrane and in the second procedure a protein with well known phosphorus content was PAGE separated, blotted and then used to calibrate the samples which were present on the same blot membrane.

For the dotting experiment, 5 µl of three solutions in 5 series, ranging from 19 to 152 pmol phosphorus in β-casein, 17.5 to 140 pmol phosphorus in pepsin and 25 to 200 pmol phosphorus in an inorganic phosphorus solution, were dotted directly onto an NC membrane and were ablated after drying.

Images of the phosphorus intensity distribution measured are shown in Figure 3.12 for the dotting experiment. It can be seen from the dots that each protein is inhomogeneously distributed in the protein spots. Nevertheless, after integration of the spot area the calibration graphs are linear and the sensitivities for β-casein, pepsin as well as inorganic phosphorus fit well with not more than 20% deviation (Figure 3.13). Coefficients of determination (R^2) of 0.9915, 0.9823 and 0.9898 could be obtained for PO_4^{3-}, β-casein and pepsin, respectively. As a test analysis a SDS-PAGE separated α-casein (Figure 3.14) with a total phosphorus amount of 93 pmol was used as unknown sample and quantified after blotting and laser ablation using the calibration curves.

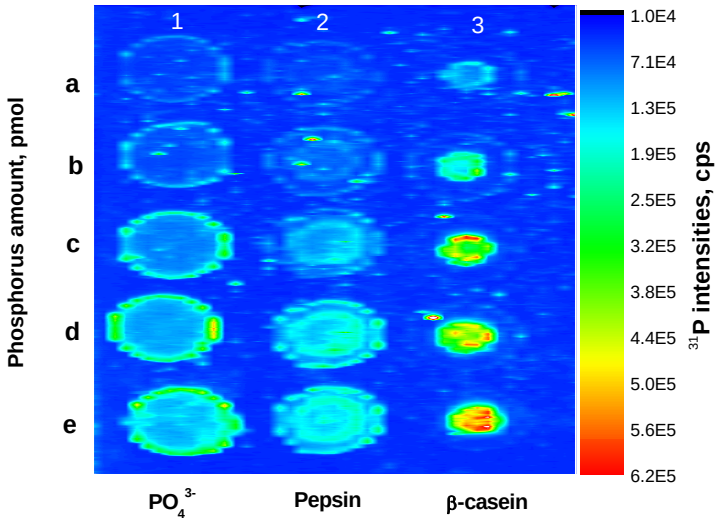


Figure 3.12: $^{31}P^+$ images of dotting experiment with different phosphorus amount of (1) PO_4^{3-}, [a-25, b-50, c-100, d-150, e-200 pmol] (2) pepsin [a-17.5, b-35, c-70, d-105 and e-140 pmol] and (3) β-casein [a-19, b-38, c-76, d-114, e-152 pmol].[86]

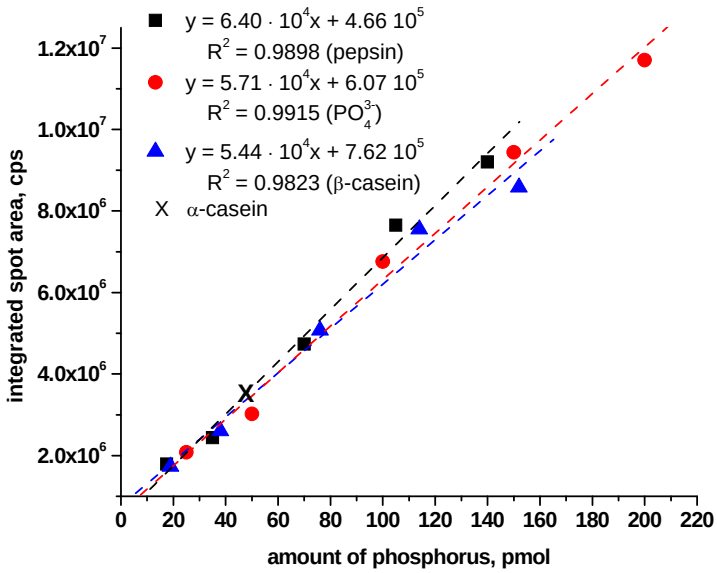


Figure 3.13: Calibration graph obtained after integrating spot areas from Figure 3.12.

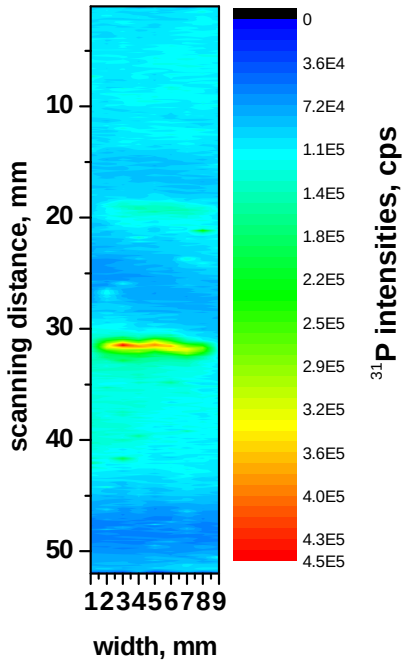


Figure 3.14: $^{31}P^{+}$ images of an α-casein spot with phosphorus amount of 93 pmol on an NC membrane.

Using the calibration of PO_4^{3-}, pepsin and β-casein 47, 44 and 47 pmol phosphorus amounts of α-casein were calculated, respectively. All results show that by

dotting, the amount of phosphorus in the protein is underestimated and only a fraction can be found (around 55%).

As an alternative approach, the standards were separated and blotted together with the test sample to compensate for the incomplete protein transfer. For this purpose β-casein with increasing total amounts of phosphorus ranging from 38 to 304 pmol were blotted together with α-casein with a total phosphorus amount of 139 pmol. The phosphorus intensity distribution is shown in Figure 3.15 for different protein spots on an NC membrane after 1D SDS-PAGE separations. Using the calibration (R^2 = 0.985) a total amount of 126 pmol of phosphorus was determined for the α-casein sample. Even though the sensitivity (Figure 3.16) in this calibration was lower compared to the dotting experiment, the quantified α-casein amount was in better agreement with the amount chosen for this experiment. A linear dynamic range was observed using this method for proteins containing phosphorus amounts of up to 300 pmol. The number of phosphate residues was calculated for α-casein using the two calibration strategies. The β-casein PAGE separated calibration strategy shows 7.4 phosphate residues in α-casein which is in good agreement with the theoretical value of 8, whereas by external dot calibration this value would be only 4.4.

From the much better accuracy which was achieved using SDS-PAGE and blotting of standards compared to the experiments where dotting was applied for calibration it can be concluded that not only the standards are ablated and transported in the same way as the test sample, but also that possible blotting losses are compensated if proteins are chosen as standards. This is the reason why this quantification strategy was preferred in this work. So far it is not clear yet, if this calibration strategy is valid only for proteins with a similar molecular weight, because the proteins selected here covered only a small range of the molecular weight scale of 23 to 35 kDa and thus it needed to be investigated for each set of target proteins.

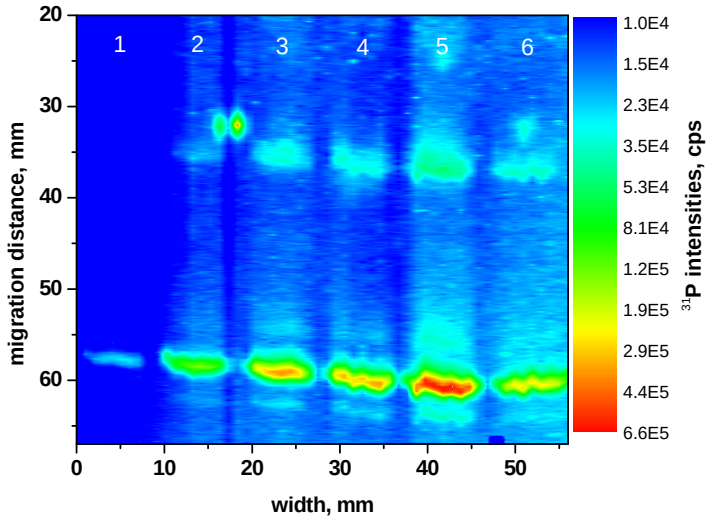


Figure 3.15: $^{31}P^{+}$ images of α- and β-casein spots on an NC membrane (1) 38, (2) 76, (3) 152, (4) 228, (5) 304 pmol phosphorus in β-casein and (6) 139 pmol phosphorus in α-casein.[86]

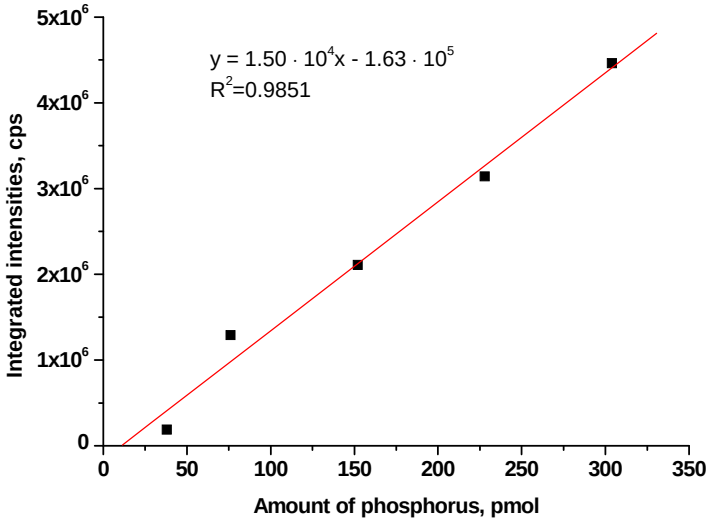
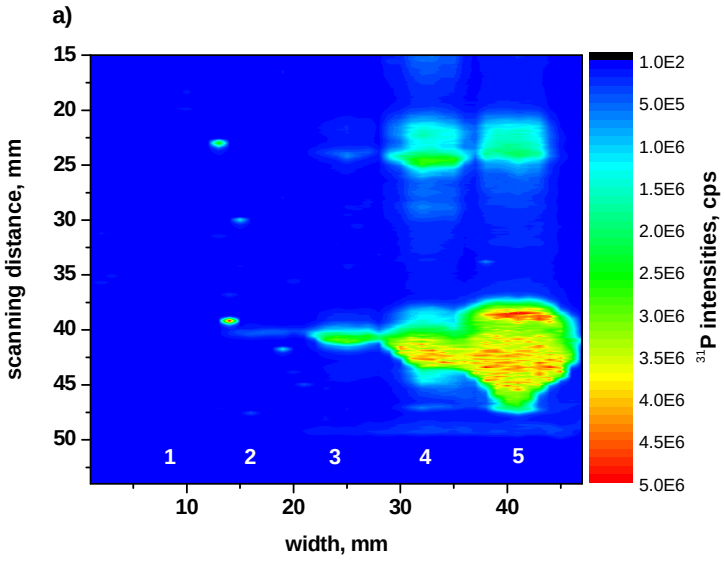


Figure 3.16: Calibration graph obtained after integrating β-casein spot areas from Figure 3.15.

3.3.3 Linear dynamic range

It was already discussed in the previous experiment that a linear dynamic range was observed for proteins containing up to 300 pmol phosphorus amounts. Therefore, the next experiment was performed to see at which point the membrane becomes overloaded. For this purpose β-casein with amounts of 1, 10, 100, 1,000 and 10,000 pmol (3.8 to

38000 pmol phosphorus amounts) was separated and blotted onto 2 NC membranes, one behind the other. The $^{31}P^{+}$ intensity distributions are shown in Figure 3.17a and b. The integrated intensities for phosphorus of the protein spots are shown in a graph in Figure 3.17c. From this figure it can be seen that the graph deviates from linearity already for total protein amounts of more than 100 pmol (380 pmol phosphorus amount). In addition to that quite significant intensities could also be observed in the second membrane. The main spot area of spot numbers 4 and 5 are about 0.3 and 0.8 cm^2 and they contain 1,000 and 10,000 pmol of the protein, respectively (Figure 3.17a). Thus, a protein loading of around 80 µg/cm and 300 µg/cm can be estimated where the latter is beyond the upper limits of the NC membranes capacity. For too high amounts of proteins they are penetrating deeper and at least pass through the membrane into the second membrane and possibly also to the anode filter causing a severe loss. This means that one has to determine always the linear dynamic range for a specific membrane material, a given amount of protein and blotting conditions, if quantitative results are needed.



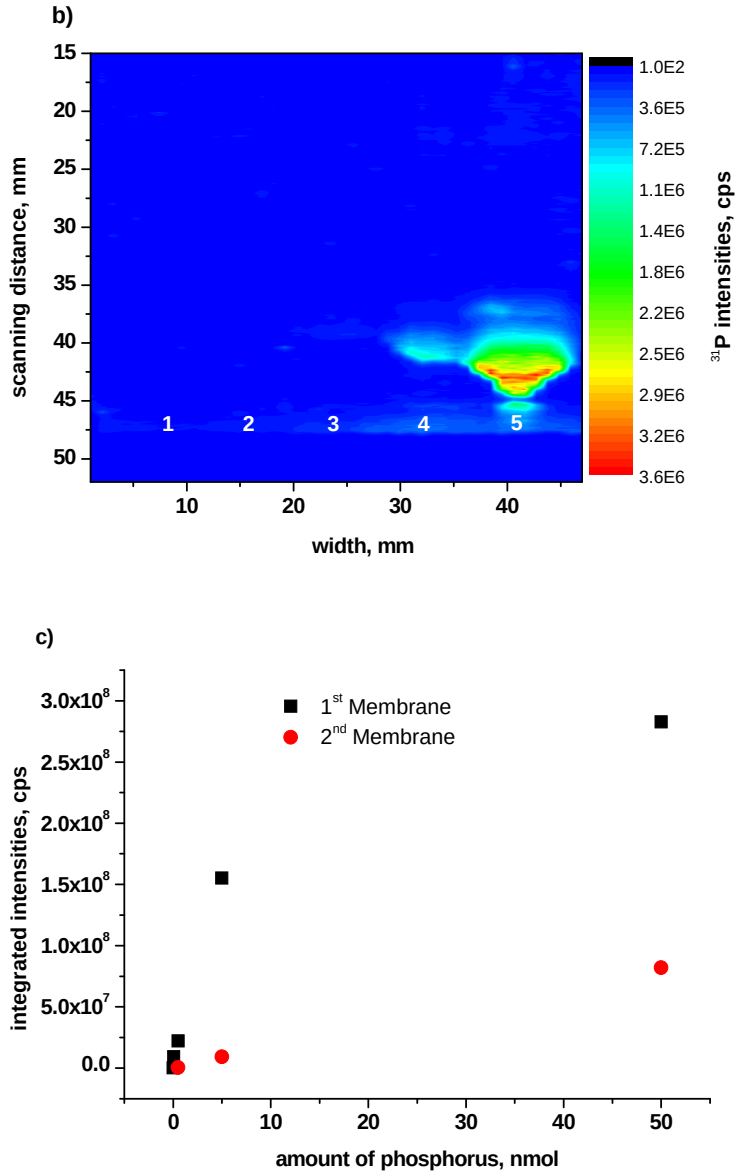


Figure 3.17: $^{31}P^{+}$ intensity distributions of increasing amounts of β-casein in the (a) first membrane and (b) second membrane. (c) Integrated intensities of the spot in the first and the second membrane.[86]

3.3.4 Application

3.3.4.1 Effects of Hydrogen Peroxide on the Total Protein Phosphorylation State in Urothelial Cells

In an initial experiment the time-dependent effect on the phosphoproteome of urothelial cells upon exposure to hydrogen peroxide was investigated. The aim of the

experiment was to define a suitable time window in which clear effects of H_2O_2 on protein phosphorylation can be measured by LA-ICP-MS. As has been mentioned previously, protein phosphorylation events usually occur within a few minutes upon stimulation of cells with oxydizing compounds. Therefore, the human urothelial 5637 cells were treated with 100 µM H_2O_2 for 2, 4 and 7.5 min. The cells were lysed immediately after exposure in SDS-containing electrophoresis buffer. The cell lysates were subjected to SDS-PAGE, electro-blotting and subsequent laser ablation ICP-MS for phosphorus detection. The scanned Coomassie stained gel and the resulting $^{31}P^+$ intensity distribution of the whole cell proteins after treatment with H_2O_2 are shown in Figure 3.18a and b. The white spots seen at the end of the low molecular weight range belong to phosphorus contaminations in the running front. Comparing both figures it is hard to see any direct correlation between phosphorus signals (Figure 3.18b) and protein bands (Figure 3.18a), but this demonstrates already that the most abundant proteins are not carrying most of the phosphorus. Another feature of the 1D separations can also be seen in Figure 3.18a. Although similar total protein amounts were used and loaded into each well of the gel, the individual electrophoretic lanes show slight differences in protein content and mobility, and the latter complicates the calibration of the molecular weight scale and also has an implication for Figure 3.18b. Although there were no changes in the protein expression pattern visible in the Coomassie stained gel (Figure 3.18a), significant differences could be seen for the corresponding $^{31}P^+$ distribution (Figure 3.18b). In comparison to the control, all $^{31}P^+$ intensities in lane 1 to 3 were increasing which can be attributed to an increase of the whole phosphoproteome.

Concerning the question how much the phosphoproteome of the stressed cell line is increasing, the $^{31}P^+$ intensities of each electrophoretic lane were integrated and normalized by the integrated protein content of each lysate using the stained gel (Figure 3.18a) and to the most intense protein band. Due to a high $^{31}P^+$ background and a too low amount chosen the recalibration standard could not be used here for quantification. Therefore, Figure 3.19 shows a plot of the integrated measured intensities (normalized to 1 with reference to lane 4) in each lane, i.e. in the untreated cells (control) and the cells treated for 2, 4 and 7.5 min with H_2O_2. From this figure it

becomes obvious that during 7.5 min the phosphorus intensity increases by a factor of about 1.3 and is highest already after 4 min. In Figure 3.19 a fast response of the cells to H_2O_2-dependent oxidative stress is observed and this demonstrates that the sensitivity of the method described here is already sufficient to measure differences time resolved and the selected time window of H_2O_2-induced effects appears to be suitable for further investigations.

a) b)

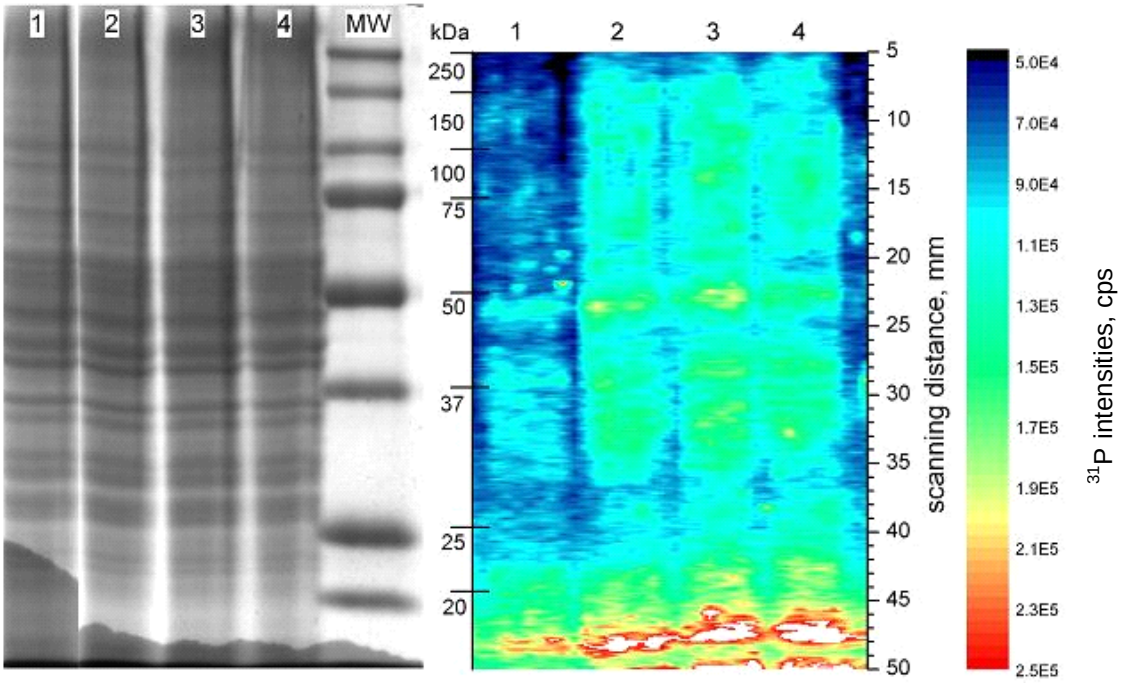


Figure 3.18: (a) Proteins from cell lysates of human 5637 urothelial cells were separated by SDS-PAGE and stained with Coomassie Blue and scanned with a gel scanner (1) control, treated with H_2O_2 for (2) 4, (3) 7.5 and (4) 2 min (MW) dual colour marker. (b) $^{31}P^+$ distribution of whole cell proteins separated by SDS-PAGE and subsequently blotted onto NC membranes. $^{31}P^+$ was detected by ICP-MS after laser ablation.

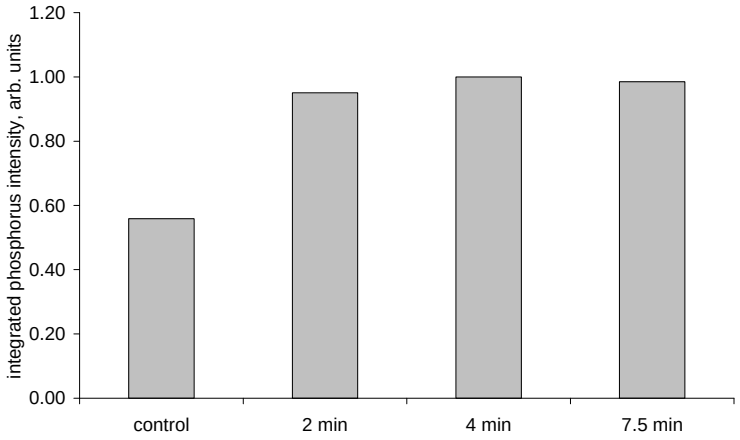


Figure 3.19: Integrated total phosphorus intensity (normalised to 1) of all proteins after H_2O_2 treatment of 5637 urothelial cells for 2, 4 and 7.5 min.

So far, the first data shown in Figure 3.18b also provides molecular weight resolved information. For this purpose, not the whole electrophoretic lane but some selected molecular weight ranges were integrated.

Figure 3.20 shows how the extent of phosphorylation differs between protein fractions in different molecular weight ranges. The low molecular weight fraction below 25 kDa is not shown in this figure, because this fraction was coming too close to the running front and therefore contained many different charged low molecular weight bio-molecules and not only proteins.

Again as mentioned before, we do not know if all phospho-proteins in the range from 25 to 250 kDa have the same blotting efficiency, therefore an absolute comparison is not possible yet. Thus relative changes in the 5 different fractions will only be discussed here. The largest increase of the normalised phosphorus intensity was seen in the high molecular weight range in the fractions 55 to 90 and 90 to 250 kDa. In the fraction from 43 to 55 kDa a very well pronounced band was visible around 50 kDa in all 4 samples, which showed a fast increase in the first 4 min and remained more or less constant for the rest of the time. Overall, a treatment time of 7.5 min was selected for the next experiment just as to work under more or less static conditions.

In the next experiment this method was applied to evaluate changes in the phosphoproteome by treatment of the urothelial cell by two different stimuli.

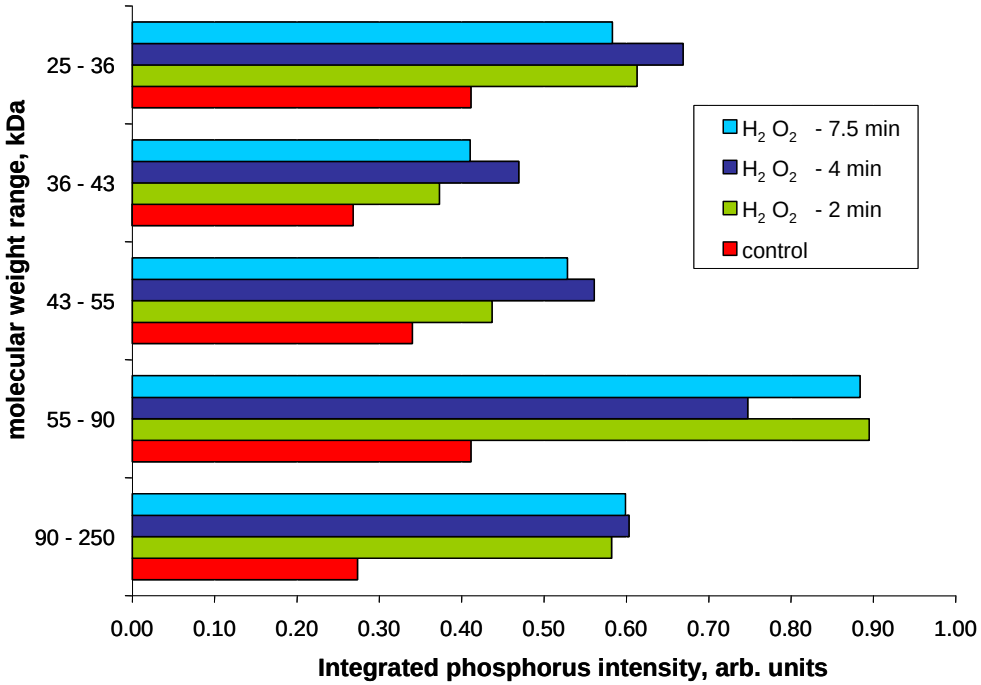


Figure 3.20: Integrated total phosphorus intensity (normalised to 1) of different protein fractions obtained by SDS-PAGE of whole cellular proteins from untreated and H_2O_2 treated 5637 urothelial cells at 2, 4 and 7.5 min.

3.3.4.2 Modulation of the Protein Phosphorylation Status of Cells by Treatment with Epidermal Growth Factor and H_2O_2

As already mentioned, cells can respond specifically to different external stimuli. Following exposure, xenobiotics may cross the plasma membrane and exert their effects inside the cells. Compounds mediating their effects via membrane bound receptors switch on respective signalling pathways which mostly include multiple protein phosphorylations. The peptide hormone EGF was used for cell stimulation as a reference system. EGF binds to receptors of the ErbB-family, including EGFR (=ErbB1), which are coupled to different signalling pathways.[83,90] The aim of the next experiment was to show alterations in the phospho-protein pattern of EGF-treated 5637 urothelial cells and to compare them with changes observed upon H_2O_2-treatment. Because distinct pathways are affected by EGF and H_2O_2, a differential phospho-protein profile can be expected upon exposure to the two compounds.

For the experiment cells were grown again in 3 different cell culture flasks. One was used as control and the other 2 were treated with either EGF (8.1 nM) or

H_2O_2 (100 µM) for 7.5 min. After treatment the cells of the cultures were lysed and the proteins were separated by SDS-PAGE. Protein and phospho-protein patterns were visualized by Coomassie staining of the gels and by $^{31}P^+$ detection after protein transfer to an NC membrane by electro-blotting, respectively. The results are shown in Figure 3.21a and b. It should be mentioned that due to the previous experiment, two conditions were improved here. First, a higher amount was taken for the recalibration standard. Additionally, the PAGE separation was slightly modified to better resolve the low molecular weight range.

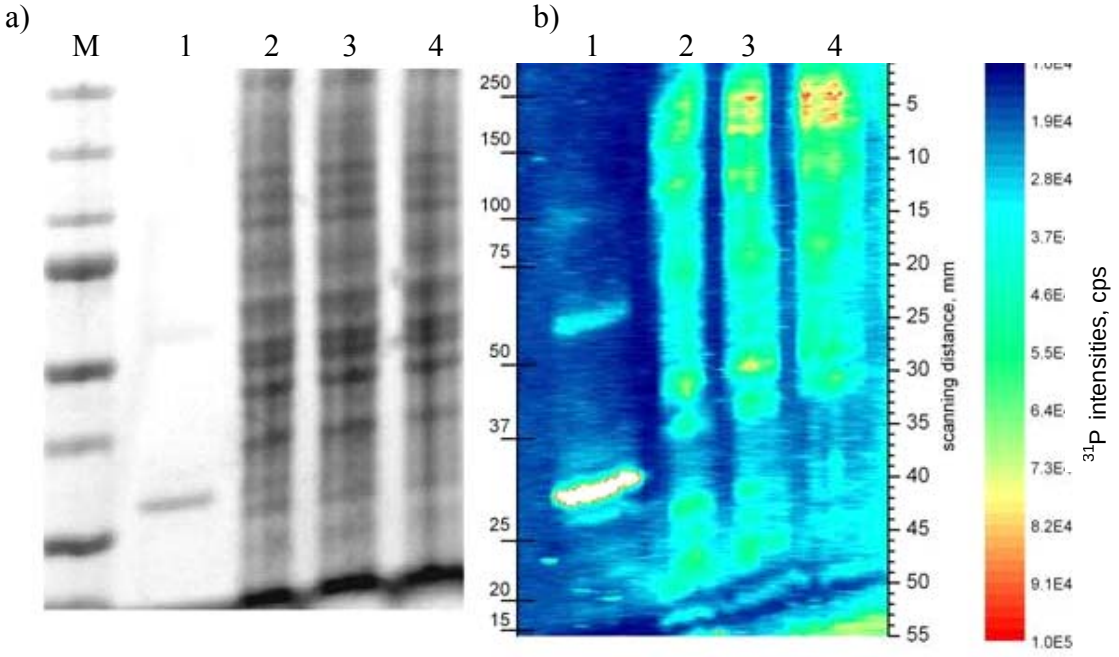


Figure 3.21: Analysis of the protein and phospho-protein pattern of 5637 urothelial cells, either untreated (2) or treated with EGF (3) and H_2O_2 (4). (a) Proteins separated by SDS-PAGE and stained with Coomassie Blue. M is the dual colour marker loaded on to the gel. (b) $^{31}P^+$ map of the same samples after laser ablation of the proteins blotted onto NC membranes. 40 pmol of β-casein corresponding to a phosphorus content of 152 pmol was used as standard (1).

As can be seen from the Coomassie stained gel (Figure 3.21a), again there are no noteworthy alterations in the general protein expression pattern by the short time treatments with EGF or H_2O_2 compared to the control cells (Figure 3.21a). Again this measurement was used for calibration of the weight scale as well as for normalization of the total protein content in the sample. At a first glance, it is obvious, that both compounds lead to a general increase in phosphorylated proteins (Figure 3.21b).

A more detailed analysis showed that EGF and H_2O_2 act differentially on the phosphoproteome. Based on proteins of different molecular weight ranges different types of response patterns elicited by EGF and hydrogen peroxide can be delineated as shown in Figure 3.22. The data shown was normalized to the total amount of proteins in the EGF sample used (lane 2 from Figure 3.21) to overcome the changes in cell culture experiments. For the low molecular weight range (15 to 18 kDa) a change was observed only for EGF but not for H_2O_2 compared to the control, whereas an increase of the phosphoproteome was seen for H_2O_2 in all different molecular weight ranges above 35 kDa, with the highest response in the highest range.

A decrease (dephosphorylation) of the phosphoproteome is observed for both stimuli mainly in the low molecular weight range from 18 to 25 kDa. Effects which are specific for either EGF or H_2O_2 were particularly clear in the two different molecular weight ranges of 40 to 50 and 95 to 300 kDa. In the high molecular weight range H_2O_2 caused a higher increase in the phosphoproteome compared to EGF. Although the total amounts of phosphorus did not show significant differences, which would lead to the misinterpretation that EGF is not causing any effect, the size fractioned representation showed a quite different phosphoproteome pattern for both samples. Additionally, if the same amount of stimuli was taken into account, the epidermal growth factor showed a much higher response by many orders of magnitude because only 8.1 nM were applied, which showed that this signalling cascade was causing a more severe effect on phosphorylation and de-phosphorylation processes.

Differential effects of H_2O_2 and EGF on the cellular phosphoproteome were seen with the LA-ICP-MS-method. Pathways which are influenced by H_2O_2 have already been discussed in section 3.1.2. However, although H_2O_2 and EGF showed distinct effects and acted on different processes there were also many common proteins which got phosphorylated in same levels by influence of both as can be seen from the results presented in Figure 3.22 in the molecular weight range from 25 to 40 kDa.

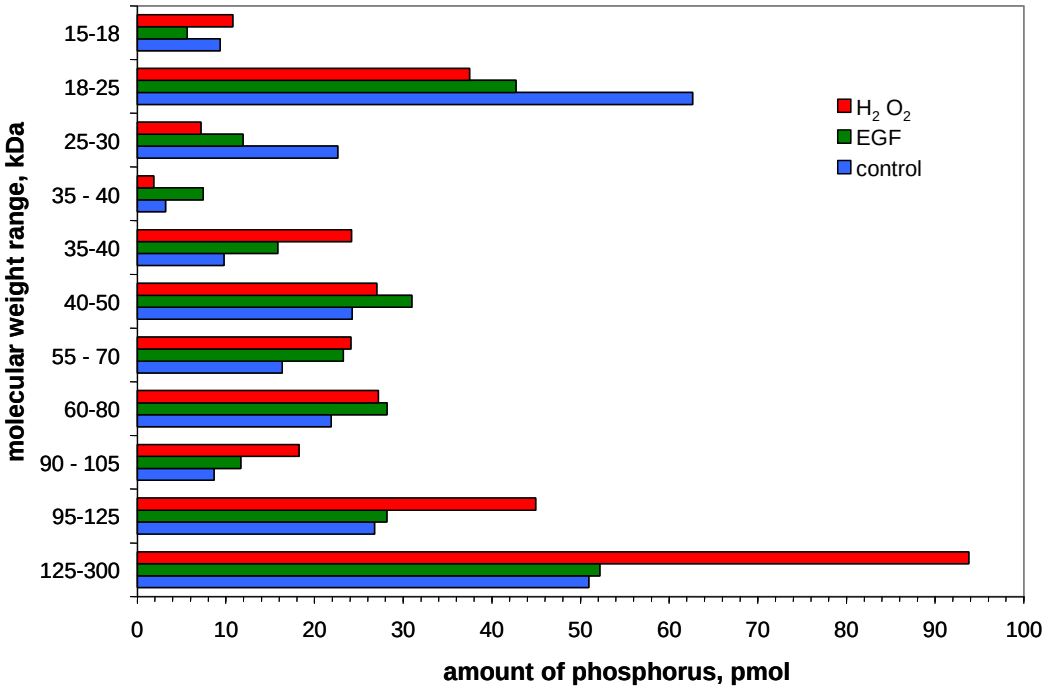


Figure 3.22: Phosphorus content of different protein fractions obtained by SDS-PAGE of whole cellular proteins from untreated and EGF or H_2O_2-treated 5637 urothelial cells. Separated proteins were blotted onto NC membranes. $^{31}P^+$ was detected by ICP-MS after laser ablation and quantified via one point calibration.

Overall, the results of the first preliminary experiments did not allow identifying specific proteins yet which are involved in signalling effects. On the other hand, all examples from literature mentioned in section 3.1.2 were concentrated on specific proteins, which most often were isolated and identified using a labelled antibody, without knowing if this target protein was the most relevant one. The data here allowed identifying in which molecular weight range effects are visible at which time scale and to which extent. This information could be used in a next step to select the most relevant proteins in a signalling pathway of phospho-proteins for further analysis.

Summarizing, these first investigations demonstrate already that the detection power achieved by using LA of blot membranes in combination with ICP-MS is a promising tool to study chemical stress induced in cancer cell lines time and molecular weight resolved. The specific alterations of the cellular phosphoproteome could be detected by LA-ICP-MS after a simple 1D-electrophoretic separation of the whole unfractionated cellular protein mixture. Keeping in mind that only 8 µl of cell

lysate was applied, the results shown here correspond to about 30,000 cells per sample only, assuming doubling of the cell number within the time range of exposure. Thus, the procedure is fast and requires low amounts of cell material, but still improvements are needed for biological studies. The sensitivity achieved is quite sufficient to observe changes in protein groups even after a few minutes treatment. A higher time resolution, in levels of a few seconds, is still a future goal, but this was limited in this investigation mainly by the speed and reproducibility of sample preparation. For instance, although we tried to load the same amount of protein to each well of the separating gel, differences of up to 30% were measured in the stained gel (calculated from the integrated intensities using gel scanner software) which could be attributed to differences in the cell cultures. Additionally reproducibility of the electrophoresis and the blotting experiments showed a relative standard deviation of 11%. Another problem was related to the separation and can be seen in Figure 3.21. Due to the high amount of protein loaded to the wells, the running front and all bands are curved, which can be compensated by software tools to a certain extent only and therefore complicates the evaluation of the data. A faster method to stop the kinase and phosphatase activity instantaneously during sample preparation would also be needed for this purpose.

In the work presented here, the integrated net phosphorylation status of a group of proteins can only be seen and not of single well defined protein as a result of phosphorylation and dephosphorylation reactions within this group. However, this is already an important improvement compared to the work of Bandura *et al.*, where only the total phosphorylation after digestion of a tyrosine kinase assay was measured.[69] Nevertheless, by this technique it was already possible by Bandura *et al.* to provide a distinguishable difference between malignant cell lines and primary cell cultures.[91]

In principle, laser ablation ICP-MS is sensitive enough to measure protein phosphorylation in single protein spots. This was demonstrated already in the work of Becker and coworkers, who investigated LA of single protein spots of the human tau protein directly in the gel after 2D separation.[72] This protein contained 17 phosphorylation sites, which was identified by Fourier transform ion cyclotron

resonance mass spectrometry. In a more recent work, Becker *et al.* detected more than 17 phosphorus containing proteins in a set of 176 protein spots from a human brain sample,[92] but comparison with this work is not possible, because the amount of sample taken into account was not defined.

Concerning improvements of LOD, here it was limited by phosphorus blanks coming from the membrane material and from the unfractionated cell lysate. So far, phosphorus free membrane material is not yet commercially available, and the development of additional sample treatment could avoid the impurities. Alternatively, improvements in terms of sensitivity seem also to be promising. For instance, Elliot *et al.* showed that an increase of the laser beam diameter from 100 µm to 780 µm resulted in an improvement of the limits of detection by one order of magnitude. In our case further increase in the laser beam, which was already 500 µm wide, was not possible due to the limited laser output energy. Another approach to improve limits of detection looks promising and was described by Elliot *et al.*,[93] who used whole gel elution. In this technique a whole protein spots was electroeluted from the gel into small volume containers and improvements were then possible if high efficiency pneumatic nebulisers were applied as this was discussed for instance by Pröfrock *et al.*[70]

For future work, also the assignment of alterations of the phosphorylation status to individual proteins is of major interest but this requires a higher resolution of the PAGE separations. Concerning resolution, usually 2D-gel electrophoresis is applied to achieve this goal, but this would give the risk that the amount of phosphorus now measured in a band is then distributed over several protein spots thus coming closer to the limit of detection. From this point of view, also improvements of detection power are required. Concerning this problem, both reductions of phosphorus blanks as well as improvements in terms of sensitivity are needed to measure single protein phosphorylation. Apart from improving the LOD, significant improvements seem to be possible mainly during the sample preparation step itself. For instance, the enrichment of phospho-proteins looks most promising. Various different techniques are available for this purpose, among which affinity based purification is the most promising approach.[81,94,95]

3.4 Concluding Remarks

LA-ICP-MS was investigated here for imaging of the intensity distribution of $^{31}P^{+}$ in electrophoretic spots of phospho-proteins after 1D PAGE separation and electroblotting onto commercial available blot membranes.

The parameters such as He gas flow rates and torch positions were optimised with the Aridus system before laser ablation and the sample preparation steps such as blotting and membrane materials were chosen to be suitable for better laser ablation conditions. In addition the power chosen for the method was also suited for better detection levels. The LA line-scan speed used was 1 mm/s which was much better in comparison to the previous investigations.[71] The detection of phosphorus containing proteins by LA-ICP-MS was nearly not disturbed by molecular interferences when measured at medium mass resolution.

With the laser setup used throughout this investigation NC membranes were found to be advantageous compared to PVDF membranes and semidry blotting method was beneficial. The laser ablated areas on the membranes were studied and compared using white light interferometer. With the above mentioned optimised conditions, similar sensitivities of different phospho-proteins were achieved for all the proteins investigated if normalized to P. The sensitivity achieved for phosphorus in pepsin was calculated and found to be about $2.67 \cdot 10^4$ cps/pmol.

A limit of detection of about 1.5 pmol for β-casein was calculated using the 3σ definition. Reproducibility of the whole method - including electrophoretic separation, blotting and laser ablation – showed relative standard deviation between 6% and 10% if the whole protein spot area was taken into account. Linearity could be achieved for a range of about 20 to 380 pmol phosphorus in proteins.

Two calibration procedures, dotting and blotting for quantification of unknown amounts of phosphorus containing proteins, were compared. If β-casein was PAGE separated and used as calibration protein the blotting calibration strategy showed 7.4 phosphate residues in the test protein α-casein which is in good agreement with the theoretical value of 8, whereas by external dot calibration this value was only 4.4.

After elaboration of the calibration method it was already applied for a first application to study the change in the phosphoproteome of a single cancer cell line after EGF hormone and oxidative stress stimuli by H_2O_2 quantitatively using a protein standard for calibration. Although both stimuli have already been investigated in the literature, this was never done before with one and the same cell line, but this is a prerequisite to identify similarities and differences in the signalling pathways. H_2O_2 (100 µM) induced an increase in total phosphorylation by a factor of 1.3 after treatment of cells for 7.5 min and in the fraction from 43 to 55 kDa a very well pronounced band was visible around 50 kDa in all the samples investigated. H_2O_2 caused more phosphorylation than EGF (8.1 nM) in the high molecular weight range above 90 kDa. It was shown that to a certain extent a calibration procedure using a protein standard during the same electrophoretic run compensates blot losses and incomplete ablations. In the results presented here, the measurement performed to study the hormone and oxidative stress stimuli allowed a time resolution at the scale of a few minutes and of course molecular size fractionation. At the moment the quantification methods investigated can be applied only for one dimensional SDS-PAGE, but for this study a much higher separation power for instance by application of 2 D PAGE is required in future.

But finally, as to give a more realistic assessment, the application chosen here was only a proof of principle experiment and it was only used to see relative changes of phospho-protein bands. Validation by conventional methods is needed to verify our findings.

Further improvements are still needed to further reduce the limit of detection of phosphorus by LA-ICP-MS, for instance by applying improved sample preparation techniques in cell culture experiments in order to reduce the phosphate blanks. Additionally phospho-protein purification and enrichment methods can be applied for prefractionating the total cellular proteins.

So far, this new method was applied only for cell cultures. More complex samples such as tissues or body fluids need to be investigated to show that the quantification concept elaborated in this work can really be applied for studies in phosphoproteomics.

Chapter 4

Method Development for Application of LA-ICP-MS in Screening and quantification of Labelled Proteins and Antibodies

In the previous chapter the hetero-element phosphorus present in the protein was utilized to detect and quantify proteins. But for proteins with no or too low number of hetero-elements, labelling methods can be applied for detection of bio-molecules by LA-ICP-MS in combination with separation methods. The main drawback of most of the recent labelling approaches discussed in literature is that the chemicals are not commercially available or only a very limited number of labelled antibodies can be bought from stock.[96] The controlled labelling of proteins is a prerequisite for its quantification. The aim is therefore to elaborate different labelling methods in order to simultaneously detect and quantify proteins from a mixture using ICP-MS. For this reason, it is necessary to investigate different labelling procedures using chelating complexes such as DTPA or DOTA and Indian ink staining. The procedures for labelling of proteins via DOTA for e.g. molar ratios, reaction time, temperature and buffer were optimised in this work for application of LA-ICP-MS.

On the one hand this study is of special interest for Western blotting where a protein is detected via a labelled antibody (Ab). On the other hand, the quantitative determination of different proteins in an organism, tissue or cell culture can be obtained i.e., differentially labelled proteins allow simultaneous detection using ICP-MS, thereby up and down regulation of different proteins can be studied at a given point of time.

4.1 Introduction

4.1.1 Indian Ink Staining

For the quantification of proteins on membranes in the subnanogram and nanogram range sensitive dyes like aurodye, ferridye and Indian ink are commonly used among which Indian ink staining is a very sensitive and cheap staining method.[97]

Indian ink is made of carbon black which is believed to bind with proteins through its functional quinone groups.[98] These activated carbon nanoparticles play a major role in purification of water, food, pharmaceuticals and fine chemicals because of their high surface area and porosity. Apart from binding with proteins it also contains other elements in high concentrations. Although these elements are not so abundant they are present in stable amounts. A screening of these elements by ICP-MS can help to find the abundant and stable element in the carbon black. Carbon black is essentially elemental carbon in the form of fine particles having a semi-graphitic structure in the form of randomly oriented condensed rings loosely held and with open edges having unsatisfied carbon bonds providing sites for chemical activity. Along with these sites there are also hydroxyl and carboxyl ions and sulphur in functional groups and small quantities of condensed hydrocarbons are adsorbed on the surface of the carbon black.[99] Its use is explored by Lönnberg *et al.*[100] who estimated the antibody concentration in the attomole range. They used the combination of carbon black as the label with flatbed scanner as the quantitative test system. Originally Indian ink was used in our work for visualisation of protein spots but then it was found that, it contains lithium as a catalyst in high amounts which can be used for LA-ICP-MS detection as well.

4.1.2 Labelling of Proteins and Antibodies

Labelling of proteins is used in proteomics and in metallomics for quantitative detection of proteins. It is the joining of bio-molecules to proteins or peptides by chemical, metabolic or enzymatic means. It is also termed as heteroatom-tagged proteomics. In recent times this was discussed in various articles, e.g. of Prange *et al.*,[9] Sanz-Medel,[101] Baranov *et al.*[102] etc.

Until recent times MALDI-TOF and ESI-MS methods are the only methods used extensively in quantitative proteomics research and their role in this field is unquestionable. Methods such as SILAC[103] and isotopic[104] coded tags are used for quantitative proteomics in combination with organic MS. The stable isotopes ^{13}C, ^{15}N, ^{2}H or ^{18}O are used as internal standards for the above methods either before or after enzymatic digestion. After separation by liquid chromatography methods, quantification of proteins can be obtained from MS via the mass differences between the heavy and

light samples. As an alternative method for quantification, element coding tags[105] are used together with organic MS. Since there are many monoisotopic lanthanides multiplexing of samples is highly possible which results in simultaneous detection of proteins. They are highly stable and show all a similar chemistry, so that a method once developed for one lanthanide element might be applicable for the others as well.

As a complementary tool, ICP-MS has already been used for quantitative detection of proteins via the bi-functional chelating agents. The advantage of using ICP-MS is that compound independent calibration methods can be performed for quantification of proteins because of its matrix independency. The sensitivity of the method can be improved when lanthanides are used because there are no interferences which is not the case for the naturally tagged proteins. For analytical detection of proteins the most common use of chelating compounds was the combination with radioactive tracers. Among the large variety of chelating compounds, McDevitt *et al.*[45] had compared the yield of binding ^{225}Ac in chelates based on DTPA, 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrapropionic acid (DOTPA), 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetrapropionic acid (TETPA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetramethylene-phosphonic acid (DOTMP), α-(5-isothiocyanato-2-methoxyphenyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (MeO-DOTA-NCS) and p-SCN-Bn-DOTA. In a second step they assessed also the yield in binding the chelate to an Immunoglobulin G antibody (IgG). Among the chelates investigated, only the compounds based on 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid showed the highest labelling yield of the antibody and the best recovery during sample preparation. The use of N-hydroxysulfosuccinimide as linker attached to this compound was described by Lewis *et al.*[106] This group used an excess of 100 molar volumes relative to the protein of interest and performed labelling with radioactive isotopes (^{111}In and ^{90}Y). After optimisation of the labelling procedure the maximal number of labels detected per protein molecule was not more than 3.8 for an antibody and about 9 for c-cytochrome, demonstrating that this value strongly depends on the protein of interest. In all cases this value was much below the theoretical number of binding sites.

The lanthanide ions can be replaced for radioactive metals for conjugation with the DOTA-protein complex so that the proteins can be detected via ICP-MS. Labelling using chelating compound based on 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid has recently been studied by Linscheid and co-workers. They demonstrated that they can reach attomol limits of detection using ICP-MS in a flow injection mode.[107]

4.2 Experimental

4.2.1 Gel Electrophoresis

Vertical electrophoresis was performed as explained in section 2.2.3.1 using a discontinuous buffer system with 10% separating and 6% stacking gels. 1 to 2 µg of protein (unless and otherwise mentioned) from the stock with a final volume of 20 µl (10 µl sample + 10 µl sample buffer) was applied for vertical separation experiements. For the multielemental labelling experiment, horizontal electrophoresis with 10% precast gels was used for separating 1-2 µg of proteins (as explained in section 2.2.3.2). Nitrocellulose membrane was used for the transfer of proteins from gels for all experiment and the semidry blotting procedures was performed as explained in section 2.2.5.

Proteins, Bovine Serum Albumin (66 kDa), CYP1A1 and CYP2C11 antibody (150 kDa) were labelled and the stock solutions with a final concentration of 1 mg/ml were prepared in carbonate-bicarbonate buffer. The final molar amounts of BSA and antibody were 15.1 and 6.6 nmol/ml respectively.

4.2.2 Labelling Protocol for DOTA and DTPA

The protocol was explained in the section 2.2.9.1 for labelling of BSA and antibody and section 2.2.9.2 for labelling of BSA.

4.2.3 Laser Ablation ICP-MS Optimisation

The optimisation of LA-ICP-MS and experiments were carried out as explained in sections 2.2.12 and 3.2.4. For the laser ablation and the ICP-MS measurements, the instrumental parameters were optimised and are shown in Table 4-1. The absolute intensities obtained for different blot membranes could only be compared when they were

measured with similar optimisation conditions or with the help of internal standard. $^{13}C^{+}$ measured at medium mass resolution was used for control of experimental conditions. For this purpose, single line scans were performed at the edges of the blot membranes where there was no electrophoretic spot. Unlike most of the experiments in the chapter 3, a microprocessor control unit was used here for positioning the laser cell and the whole procedure of ablating a full membrane was automated including the triggering of laser.

Table 4-1: Instrumental parameters.

Laser ablation system	
Laser energy	3 mJ
Make-up gas flow rate (Ar)	1 l/min
Carrier gas flow rate (He)	1.6 l/min
Repetition rate, shots per second	15 Hz
Translation velocity	1 mm/s
Distance between line scans	1 mm
Crater diameter	~500 µm
ICP-MS system	**ELEMENT 2**
Incident power	1025 W
Cooling gas flow rate	16 l/min
Auxiliary gas flow rate	1.3 l/min
Ni skimmer cone diameter	1 mm
Ni sampler cone diameter	0.7 mm
Isotopes measured	$^{153}Eu^{+}$, $^{165}Ho^{+}$, $^{159}Tb^{+}$, $^{13}C^{+}$
Resolution setting	400 (LR)
	4,000 (MR)

4.3 Results and Discussions

4.3.1 Indian Ink Staining

Different ways of screening of proteins using LA-ICP-MS and gel electrophoresis had been explored. The common method used in biochemistry for detecting and quantifying proteins in membranes is staining. Indian ink is one of the sensitive staining methods and can be used as alternative method for detection of proteins after separation

whereas labelling can be applied even before protein separation step. Here it was tried for the first time in combination with the LA-ICP-MS to determine the total protein quantification from the blot membranes. In order to be able to detect them with the ICP-MS initially they needed to be screened for elements present in the protein spots after Indian ink staining. For that 7 µl of dual colour marker was used and subjected to SDS-PAGE separations and blotting. After that it was Indian ink stained as explained in the experimental section. The electrophoretic spots were line scanned initially with the following elements at low resolution: $^{60}Ni^{+}$, $^{63}Cu^{+}$, $^{64}Zn^{+}$ and $^{7}Li^{+}$. Only lithium could be found at higher levels and each single band of the marker can clearly be seen from Figure 4.1. In the production process of carbon black (and subsequently Indian ink) the element might have been used which binds to carbon black.

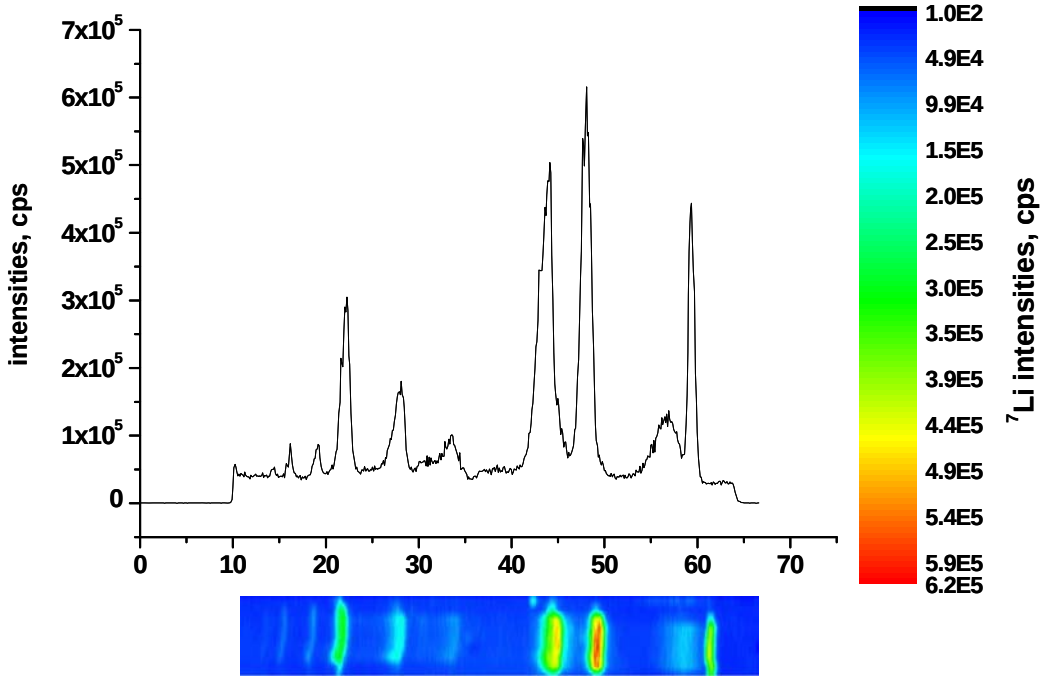
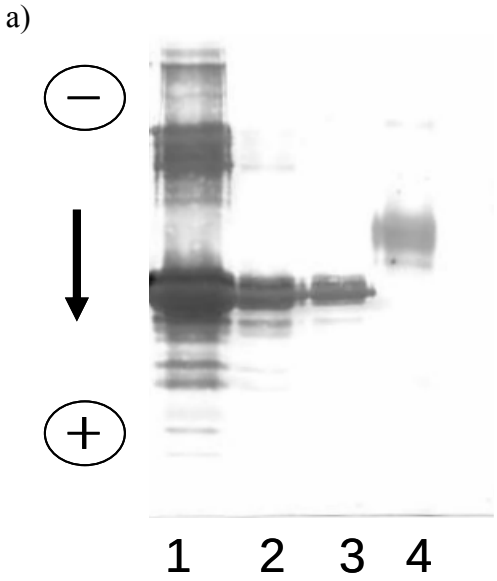


Figure 4.1: LA-ICP-MS surface plot showing the $^{7}Li^{+}$ intensities. Sample: Dual colour marker Indian ink stained, subjected to SDS-PAGE, blotting and measured by LA-ICP-MS.

In order to determine the working linear range for lithium detection by LA-ICP-MS, in the next experiment a blot was prepared with the standard phosphorus proteins β-casein and ovalbumin. Figure 4.2a shows the Indian ink stained blot before laser ablation and Figure 4.2b shows the corresponding lithium intensities after laser ablation of the same blot. The concentrations of proteins used (Figure 4.2a and b) were 1) 500 pmol β-

casein, 2) 50 pmol β-casein, 3) 5 pmol β-casein and 4) 22 pmol ovalbumin. The resulted intensities showed a similar pattern for both the stained blot and the laser ablated lithium intensity blot. For two different proteins the obtained lithium intensities correspond to their binding capacity to the carbon black. In the next step all 3 spots of the protein β-casein were integrated to obtain the calibration blot which is shown in Figure 4.3. A good linear relationship (R^2 = 0.9994) was obtained with the applied amount of protein. The integrated intensity was also obtained for the ovalbumin sample and with the help of the above relation the calculated amount was about 25 pmol which is closer to the applied amount. This method is thus suited as one of the methods for the total protein quantification using LA-ICP-MS. Nevertheless more proteins have to be used in order to validate this method of detection. Multiplexing with simultaneous different protein detection is not possible with this staining method; alternatively with the help of the calibration graph an unknown protein can be estimated. From the ovalbumin spot in Figure 4.2b the detection limit of this method was calculated and it was estimated to be approximately 95 fmol.



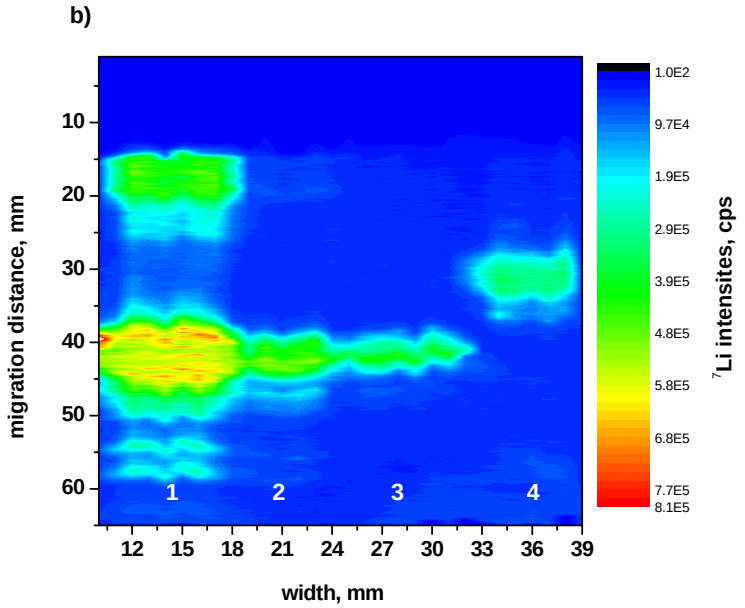


Figure 4.2: (a) Indian ink stained blot after SDS-PAGE and blotting. (b) $^{7}Li^{+}$ intensity distribution measured by LA-ICP-MS of the same blot. Samples β-casein: (1) 500 pmol, (2) 50 pmol, (3) 5 pmol β-casein and (4) 22 pmol ovalbumin.

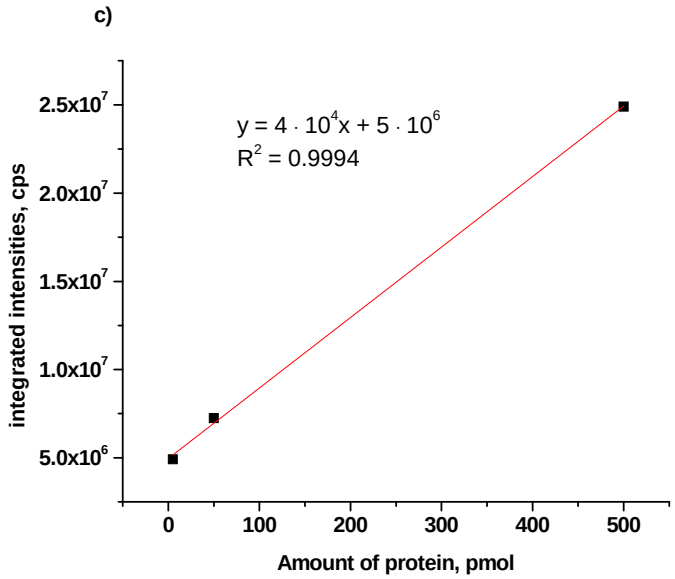


Figure 4.3: Calibration graph obtained after integrating the lanes 1, 2, 3 (β-casein (1) 500 pmol, (2) 50 pmol, and (3) 5 pmol) from Figure 4.2.

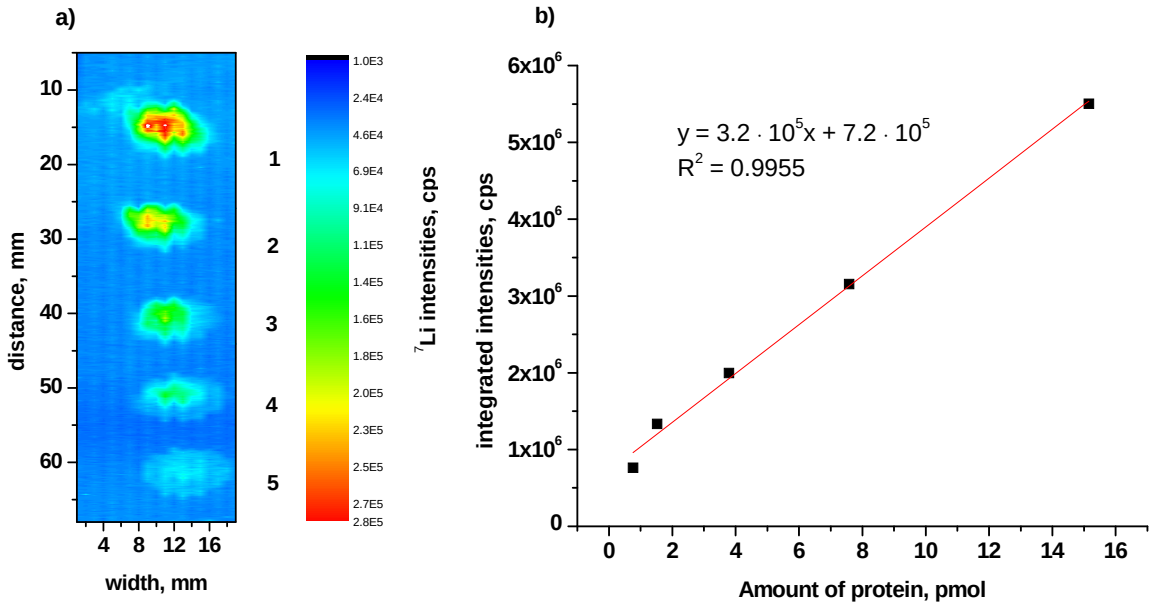


Figure 4.4: (a) $^{7}Li^{+}$ images of dotting experiment measured by LA-ICP-MS after Indian ink staining. Samples: BSA with different concentrations (1) 15.15 pmol, (2) 7.58 pmol, (3) 3.79 pmol, (4) 1.52 pmol, (5) 0.76 pmol, (b) corresponding calibration graph.

As an alternative to the calibration using PAGE separations, dot blotting was used to compare and quantify the protein separated using electrophoresis. For the dotting experiment 2 µl of different concentrations of BSA ranging from 0.76 to 15.15 pmol were dotted directly onto the NC membranes and Indian ink stained overnight. They were then laser ablated after drying. Images of the lithium intensity distribution measured are shown in Figure 4.4a. It can be seen that protein spots are inhomogeneously distributed on the membrane as was also seen in the dotting experiment in chapter 3. The integration of the whole spot was performed and it showed good linearity (R^2 = 0.9955). The SDS-PAGE separated protein ovalbumin from lane 4 of Figure 4.2a was taken as the test protein and it was quantified using the calibration function. About 17 pmol of protein was quantified which was slightly lower than the applied amount (22 pmol). This is comparable to the dotting experiment explained for the phosphorus quantification in section 3.3.2, where higher amounts of protein were used. There the sensitivities for the dotting experiments were higher. Here it was also higher, in spite of lower amounts and different proteins used for the calibration. But here it was also observed that the estimated amount was closer to the applied amount for the PAGE separated calibration method

where protein was loaded together with the sample. Hence both methods are applicable for quantifying total proteins in a given sample.

4.3.2 Labelling of Proteins Using DOTA

The optimisation of labelling conditions using chelating compounds based on 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid with SCN as linker was already described in the literature but are by far not optimised for ICP-MS detection.[45,106] All the parameters like pH, reaction time, temperature etc. were taken into consideration for the optimisation procedure except the sequence of the reaction steps. Additionally stable isotopes were used for labelling rather than radioactive, proteins were first reacted with the chelate and then the element was introduced to the complex. Initially, molar ratios of protein to the DOTA compound and the resulting complex with the element were optimised using LA-ICP-MS followed by temperature, reaction time and pH of the reactions. The integrated intensities of labelled proteins were used to compare and draw the conclusions and it was not possible to quantify the intermediate product between the DOTA and the protein. The experiments involving the optimisation of molar ratios between DOTA, protein and lanthanides were also compared by in-solution measurements using ICP-MS.

4.3.2.1 Optimisation of BSA to DOTA Ratio (Step 1)

The initial concentration of the protein amount used was 6 nmol/ml. In addition different amounts of DOTA were mixed. The reaction was accomplished in the carbonate-bicarbonate buffer (pH = 9.0). Initial experiment was done at RT for 24 h. As lanthanide europium (Eu) was selected and the mixture was incubated for 30 min at 37 °C in the acetate buffer (pH = 7.0 - 7.5). The molar ratio of DOTA to europium was maintained at 1:10. Figure 4.5 shows the results from the ICP-MS in-solution measurement. From the results it could be observed that with 20 fold molar excess of DOTA, not even a single label of europium to BSA could be achieved. For 1:30 and 1:80 molar ratios of DOTA to BSA, the approximate europium label to BSA achieved was 1. Only with a surplus of the DOTA to the protein from 1 to 100 a drastic rise of the relationship was observed. This high value of 7.6 can however possibly be attributed to

the free europium that was still present in the mixture. A very high quantity of europium solution had to be used, in order to receive the desired tenfold surplus of europium. It is possible that the surplus free europium with the cleaning steps cannot be removed again.

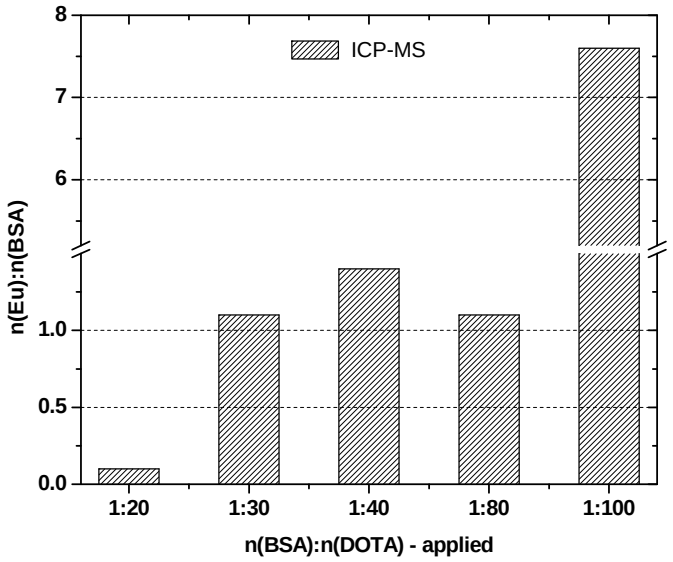


Figure 4.5: Influence of DOTA concentration on the stoichiometric relationship between BSA and europium.

LA-ICP-MS was performed with the samples having different molar ratios between protein and DOTA (n(BSA):n(DOTA) = 1:20 to 1:100). From the in-solution measurement it was observed that there was high amount of europium labels present in the mixture where a molar ratio of 1:100 of BSA to DOTA was present. A similar phenomenon was observed from the surface plot of the LA-ICP-MS results as it is shown in Figure 4.6. In lane 3 in the figure there is a high background of europium ions which comes from the sample and at the end of the migration very intense signals could be spotted. These are the free charged europium ions which migrated together with the sample buffer and could not be removed by the purification steps. At the same time in lane 2 with lower molar ratio used (n(BSA):n(DOTA)-1:40) there is also background but at levels of about one magnitude lower than lane 3. From the integrated intensities, it could be seen that with increasing molar ratios of DOTA to BSA from 20 to 40 the resulted intensities showed a linear increase and it was almost increased twice for 1:100 molar ratio than 1:40. From the experiment it is clear that the intensity is directly related

to excess of DOTA used, but has to be paid by higher background levels. Thus high DOTA amount is desirable to get more intensity and labels but for that more purification steps must be used. In the lane 1 and 2 signals could be detected for samples where there was actually no europium used. There was much of free europium ions present in the wells of the neighbour lane which probably entered into this lane. Another unexplainable result is that for 1:80 molar ratio of BSA to DOTA less signals could be seen which possibly occurred erroneously. The LOD of the method was 0.2 pmol calculated from sample at lane 3 which had a sensitivity of $2.09 \cdot 10^5$ cps/pmol for the 1 µg of protein that was applied.

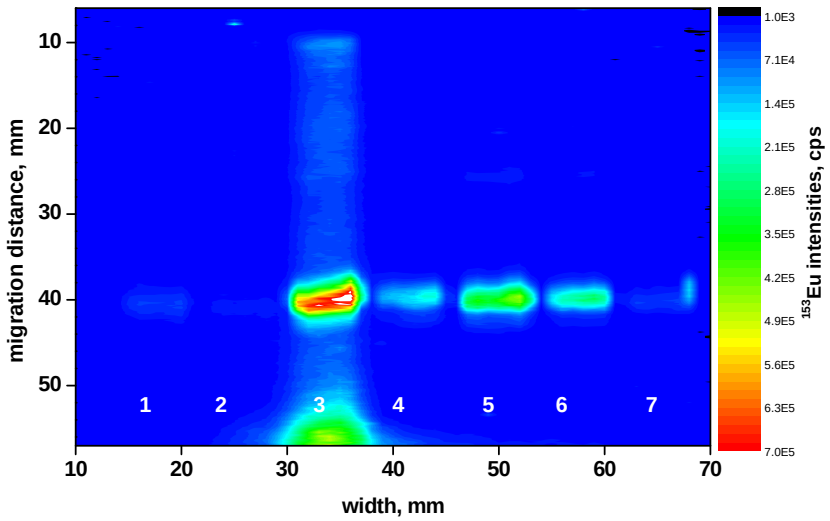


Figure 4.6: LA-ICP-MS surface plot showing the $^{153}Eu^+$ intensities with lanes corresponding to (n(BSA):n(DOTA):n(Eu)): (1) 1:40:0, (2) 1:20:0, (3) 1:100:1,000, (4) 1:80:800 (5) 1:40:400 (6) 1:30:300 and (7) 1:20:200.

4.3.2.2 Optimisation of DOTA to Europium Ratio (Step 2)

The molar ratio of BSA to DOTA was maintained at 1:40 which showed the best signal to background ratio. All the other experimental conditions for the first reaction step between BSA and DOTA were followed as explained in the previous section. In the next step 3 samples were prepared with the chelated product with three different quantities of europium. The molar ratios of DOTA to europium were maintained at 1:2, 1:5 and 1:10. The reaction time for the lanthanide conjugation was 30 min at 37 °C in acetate buffer

(pH = 7.0 - 7.5). The purified samples were measured by means of ICP-MS. From Figure 4.7 it can be seen that for a complete reaction of europium with DOTA in order to achieve a single label, at least a five fold surplus of europium was necessary. A higher amount of europium is recommended in order to achieve more labels which also depend on the protein used and the DOTA label in the step 1. A label of approximately 1.8 could be achieved for a DOTA to europium molar ratio of 1:10. It should also be noted that there was surplus of europium in the final solution which affected mainly the in-solution measurement and it could be removed although not completely by using PD10 columns.

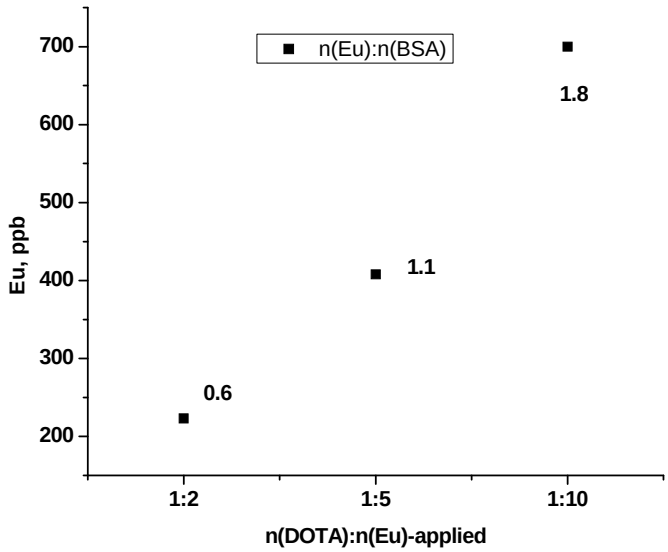


Figure 4.7: Influence of the lanthanide concentration on the stoichiometric relationship between europium and BSA.

LA-ICP-MS experiments were done by changing only the molar ratios of europium to DOTA in the second step. The resulting surface plot is shown in Figure 4.8 and the protein spots were integrated to arrive at the cumulative intensities (lane 1: $5.48 \cdot 10^6$ cps, lane 2: $9.51 \cdot 10^6$ cps, lane 3: $7.77 \cdot 10^6$ cps). The spikes in lane 1 were neglected for calculation. As it was shown with the in-solution experiments, a similar effect on the resulting labelling efficiency was confirmed with the LA-ICP-MS measurement. From the integrated data almost similar intensities could be achieved for the 3 samples according to the europium amount except the sample with DOTA to europium molar ratio of 1:2 which is slightly lower. Here it is confirmed that the added quantity of lanthanide ions was not the crucial factor for the success of the labelling procedure. The

strongest signal intensity under the conditions specified in Figure 4.8 was achieved for lane 2 where a DOTA to europium molar ratio of 1:5 was used although only little higher than 1:10. The reaction time used in the step 1 reaction was longer to achieve this labelling degree. This step was finally dependent on the first reaction between the protein and DOTA and the number of lysine and arginine residues of the protein (BSA has 59 lysine and 23 arginine residues). A molar excess of 10 times of europium to DOTA was further used to achieve the required saturation of DOTA.

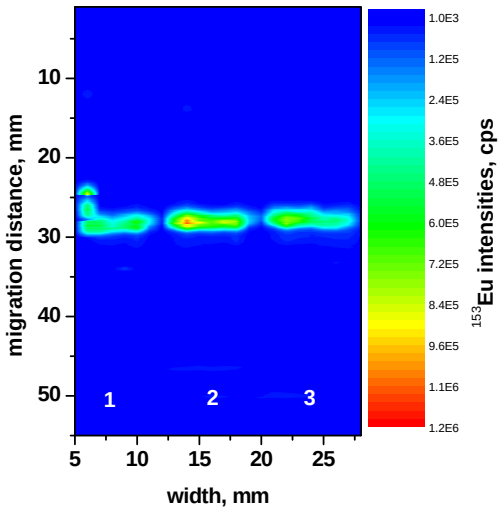


Figure 4.8: LA-ICP-MS surface plot showing the $^{153}Eu^{+}$ intensities from the labelled BSA according to the molar ratios n(BSA):n(DOTA):n(Eu) (1) 1:40:80, (2) 1:40:200 and (3) 1:40:400 with europium reaction at 37°C, 30 min, pH 7-7.5.

4.3.2.3 Optimisation of Temperature and Time

Usually 1 or 2 h of reaction time between the protein and the chelating agent and a very short time of 30 min for the lanthanide labelling are adequate for the complete conjugation. As the labelling efficiency depends mainly on the molar ratio of the protein and chelating agent, longer reaction times are acceptable and need to be proved whether they improve the result. Also temperature needs to be optimised which can influence the reactions in accomplishing the required labels.

A molar ratio of 1:40:400 corresponding to n(BSA):n(DOTA):n(Eu) was used. The reaction was accomplished in the carbonate-bicarbonate buffer (pH = 9.0). Initial experiments were done at RT and the reaction time chosen were varied between 80 min

to 24 h. The step 2 reaction was performed for 30 min at 37 °C in the acetate buffer (pH = 7.0 - 7.5). From the results of the ICP-MS in-solution experiments, for an 80 min reaction time only a small fraction of the BSA was labelled and only after 16 h a clear rise of the europium-labelled to BSA was recognized. After 24 h signals were declining which led to the conclusion that the protein was not only denatured after 24 h, but also already partly destroyed, or that the isothiocyanate group of DOTA was separated again from the protein. In addition to the above experiment a temperature of 4 °C was also examined for the step 1 reaction. But the labelling efficiency was below 1, possibly because at 4 °C reaction between DOTA and the amino groups of the protein hardly took place.

LA-ICP-MS experiment was performed on the samples where the reaction time used was between 1 and 24 h for the step 1 reaction. Figure 4.9 shows the fact that signals could be recognizable for all the reaction times but the highest for 16 h. Although fewer labels shown for the other reaction times used with the in-solution measurements, it was still recognizable with LA-ICP-MS. The spots were integrated and the intensities for 24, 16, 4, 3, 2 and 1 h were found to be $6.22 \cdot 10^6$ cps, $7.13 \cdot 10^6$ cps, $5.9 \cdot 10^6$ cps, $4.55 \cdot 10^6$ cps, $3.89 \cdot 10^6$ cps and $3.76 \cdot 10^6$ cps respectively. It shows an increase from 2 to 16 h reaction time with a little difference between 1 and 2 h. The difference between 1 and 16 h reaction time was about 2 times, but for the in-solution experiments high amounts of labels could be seen which also might have arrived from the free europium ions which were still present after washing steps. With the TXRF measurements also lower labels were obtained when lower reaction times were used.[108] The highest label of 4.1 was obtained for the 16 h reaction time for step 1. From the experiment it could be concluded that reaction time for BSA for which maximum labelling efficiency could be achieved should be around 16 h. This can be different for other proteins considering the binding sites of the DOTA to the protein i.e. the number of accessible amino groups. In case of antibodies discussed in the later section 4.3.2.5 and chapter 5 less reaction time was used, as reaction times can hinder their specificity with their respective antigen.

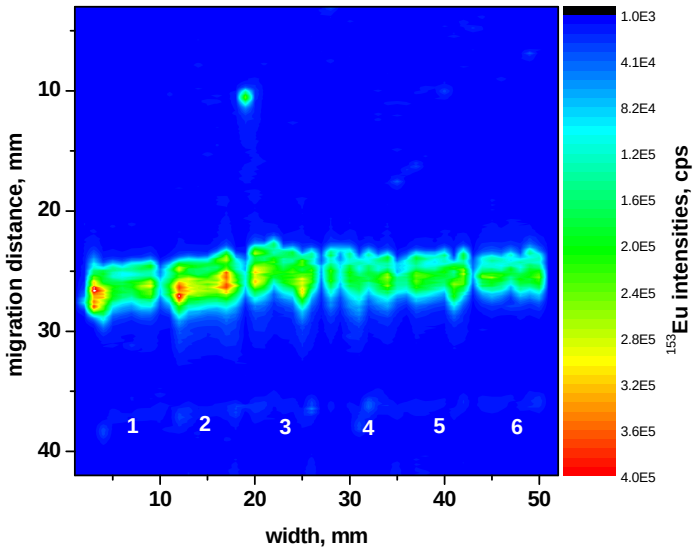


Figure 4.9: LA-ICP-MS surface plot showing the $^{153}Eu^{+}$ intensities with the molar ratio of n(BSA):n(DOTA):n(Eu) maintained at 1:40:400. Reaction time used for samples in lane 1 at 16 h (2) 4 h, (3) 3 h, (4) 2 h and (5) 1 h. Integrated intensities obtained from the respective spots: (1) $6.22 \cdot 10^6$ cps, (2) $7.13 \cdot 10^6$ cps, (3) $5.9 \cdot 10^6$ cps, (4) $4.55 \cdot 10^6$ cps, (5) $3.89 \cdot 10^6$ cps and (6) $3.76 \cdot 10^6$ cps.

The reaction times of 15, 30, 45 and 60 min were tested for the step 2 reaction between the BSA-DOTA complex and europium. The molar ratio of BSA to DOTA to europium was maintained at 1:40:400. The temperature used for the step 2 reaction was maintained at 37 °C and 58 °C (15 and 30 min reaction time only). It can be seen from the surface plot and the corresponding integrated intensities (Figure 4.10a and b) that there was an increase in the final labels achieved for the protein at reaction time of 60 min at 37 °C. But at the same time background increased and high intense signals could be seen at the end of the migration distance. There was an increase in the counts from 15 to 30 min reaction time and an unexpected decrease in integrated counts at 45 min. When the temperature of the reaction increased to 58 °C there was a decrease in the obtained intensities by about half for 15 min reaction time. But for 30 min reaction time at 58 °C it increased but with higher background and intense signals at the end of the migration spot. The reaction between the lanthanide and the DOTA was faster and lower reaction times were sufficient for conjugation reactions to go to completion. Longer reaction times were suitable but with higher background and the degree of labelling was affected by higher

temperatures. The more reactive the DOTA, the shorter the reaction time and a reaction time of 30 min at 37 °C was suitable for the reaction between lanthanide and protein chelate complex.

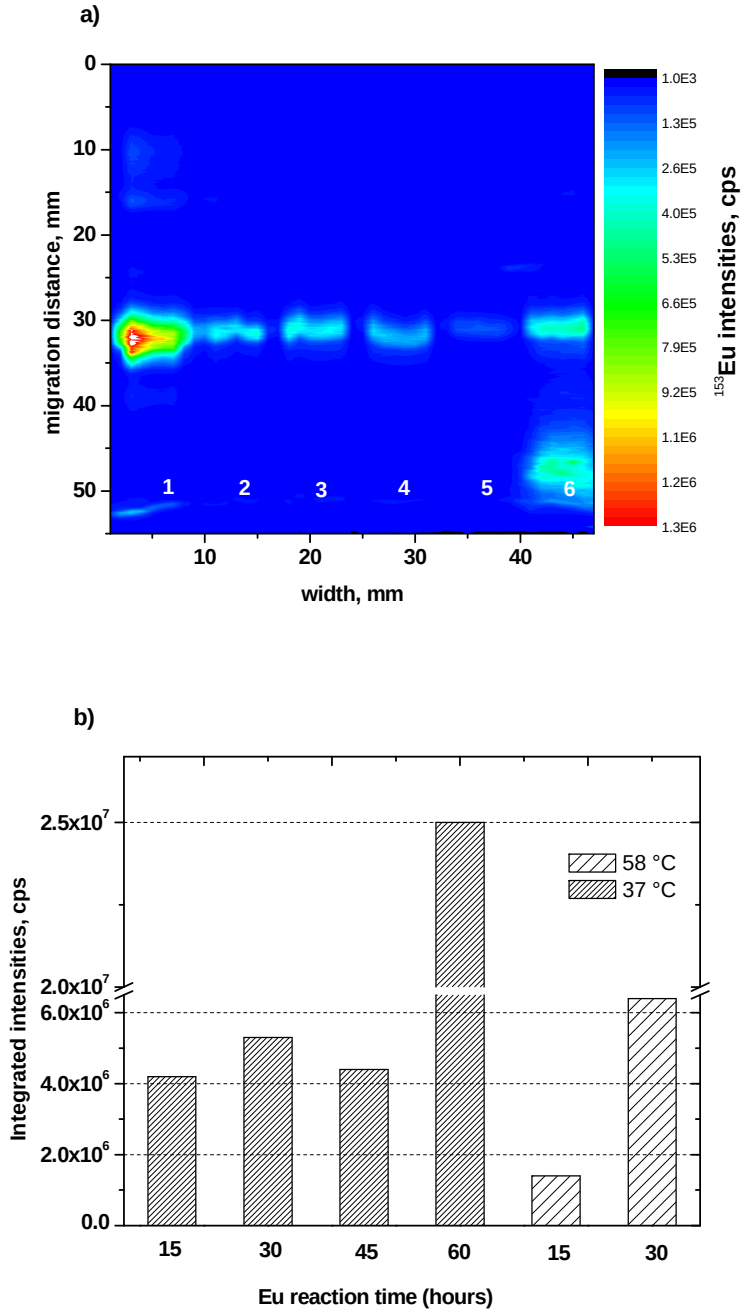


Figure 4.10: (a) LA-ICP-MS surface plot showing the $^{153}Eu^{+}$ intensities with the molar ratio of n(BSA):n(DOTA):n(Eu) maintained at 1:40:400. Europium reaction time used for samples in lane 1 at 60 min (2) 45 min, (3) 30 min and (4) 15 min performed at 37 °C, (5) 15 min and (6) 30 min at 58 °C. (b) Integrated intensities obtained from the respective spots as a function of europium reaction time.

4.3.2.4 Buffer Conditions

In the literature many different buffer systems are used for labelling of antibodies and proteins.[45] But it depends on the type of chelating agent used. The manufacturer of DOTA recommends carbonate-bicarbonate buffer for the reaction between proteins and the chelating agent. All the experiments explained in the above sections were performed with this buffer. There was no problem in using this buffer for the LA-ICP-MS measurements. In addition other buffers were also investigated to see if they are suitable. It should be noted that the amino group of the commonly used buffer can likewise react with the active group of the DOTA. Therefore trichloroethylene HCl is used for stopping the reaction. For the step 1 reaction different buffers are applicable, depending mainly on the type of chelating agent. Theoretically only the terminal amino group of the protein should react with the DOTA at a pH value of 7.5. In this range at the best phosphate buffers are used. Citrate buffers are less suitable since they are complexing agents and also could bind to the lanthanide, if the intermediate product is not properly buffer exchanged. This can again lead to a high background in the LA-ICP-MS. At a pH value of 9 and more highly above all the lysine groups of the protein are labelled since at this pH lysine amines are good nucleophiles. Ammonium acetate was used at pH 7.0 to 7.5 as buffer during the step 2 reaction. Different buffer systems were tested for the first step. HEPES buffer showed no signal, Citrate phosphate buffer was tested at pH 7.7 and 8.6 and carbonate-bicarbonate buffer at pH 9.0. A molar ratio of 1:40:400 corresponding to n(BSA):n(DOTA):n(Eu) was used. The surface plots of the europium intensities from europium-labelled BSA for both carbonate-bicarbonate and citrate phosphate buffers are shown in Figure 4.11a and b, respectively. It can be seen from lane 1 of Figure 4.11b, that there was a high background possibly due to the complex building of the citrate buffer. Additionally in step 2 it could react with the lanthanide. Therefore more purification and buffer changing steps between step 1 and step 2 were needed in this case. From Figure 4.11b it also can be seen that when the pH decreased there was loss in the signal intensity and therefore in the labelling efficiency. But with carbonate-bicarbonate buffer there was lower background and comparable intensities could be obtained. So as previously performed it was further used for the step 1 reaction at pH 9.

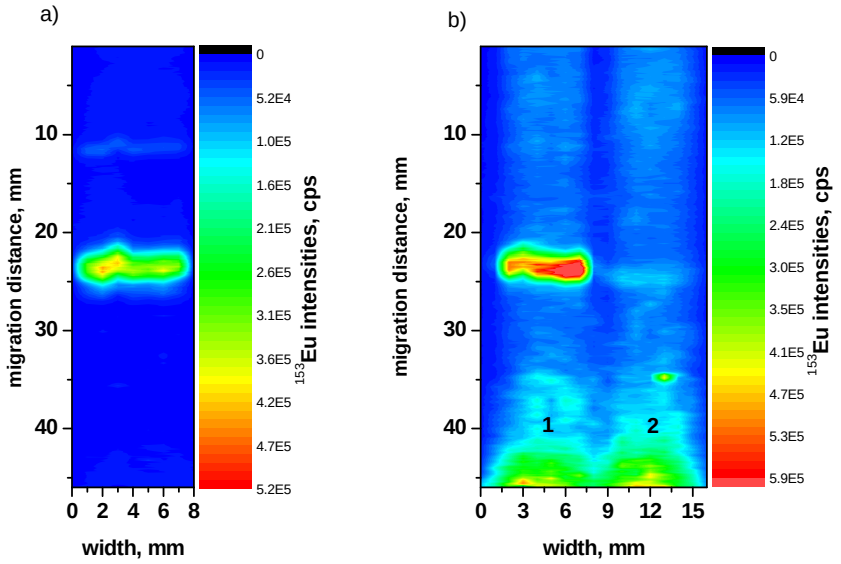


Figure 4.11: $^{153}Eu^{+}$ signals measured by LA-ICP-MS on a blot membrane; a) carbonate-bicarbonate buffer at pH 9 and b) citrate phosphate buffer used for the reaction between DOTA and the BSA, lane 1: pH 8.6 and lane 2: pH 7.7. (n(BSA):n(DOTA):n(Eu)-1:40:400).

4.3.2.5 Labelling of Antibody CYP1A1

In the next step it was investigated if the conditions optimised for proteins can also be applied for antibodies. Such an approach can be used as basis for multielemental labelling of antibody for detection of multiple antigen samples which will be explained in detail in chapter 5. Since an antibody should maintain its antigen specificity, a lower molar ratio of DOTA to antibody was initially used and checked whether this was enough to get a similar sensitivity achieved for experiments involving BSA. CYP1A1 was used as the antibody and it will be explained in detail in chapter 5. The reaction conditions such as buffer and pH from the above mentioned steps were used; the reaction time for the step 1 reaction was maintained for 80 min as longer reaction times could influence the antigen specificity and step 2 reaction was performed similar to section 4.3.2.2; the molar ratio of n(Ab):n(DOTA):n(Eu) was maintained at 1:20:200. The resulting LA-ICP-MS surface plot for the 8 pmol antibody sample is shown in Figure 4.12. Two bands corresponding to heavy and light chains were visible because of the denaturing conditions used during the SDS-PAGE. The sensitivity was higher in the heavy chain (around 50 kDa) when compared to the light chain (around 20 kDa) which relied on the amount of protein at each spot. The sensitivity of the method with respect to the heavy chain of the

antibody was found to be $2.9 \cdot 10^6$ cps/pmol which was higher than those of the respective BSA sample used in section 4.3.2.1. Thus a molar ratio of 1:20 for antibody to DOTA was enough to achieve better sensitivity and loading more of chelating agent could lead to the antigen unspecificity. In addition a LOD of about 30 fmol could be achieved for the antibody which was better than BSA prepared with similar reaction conditions.

For the quantification of antigen samples (which will be explained in detail in chapter 5) using these antibodies, a calibration was needed with the labelled antibody. Therefore an experiment was performed by labelling antibody CYP2C11 with holmium using the conditions explained in section 4.2.2. Holmium was chosen to compare the sensitivity of the method with other lanthanides. A calibration was performed with four different amounts of one and the same labelled antibody. 4-10 pmol of antibody were loaded for separation onto the gel and the resulting LA-ICP-MS surface plot is shown in Figure 4.13. The observed intensities were proportional to the loaded amounts. Here approximately 8 to 9 line scans representing each electrophoretic lane for each amount were added to obtain a single electropherogram. Two peaks were seen, one high and another low molecular weight as it was the case also for the europium-labelled antibody. The protein spots were integrated, and is shown in Figure 4.14 as a function of scanning distance. Even a low amount of 4 pmol of antibody gave a signal above the blank value. A sensitivity of $9.65 \cdot 10^4$ cps/pmol could be obtained with respect to the heavy chain which was lower but after normalization with the signals obtained for the internal standard $^{13}C^+$ in comparison to measurements measured during the same time, the resulted sensitivity should be almost two orders of magnitudes higher which is comparable to europium-labelled antibody.

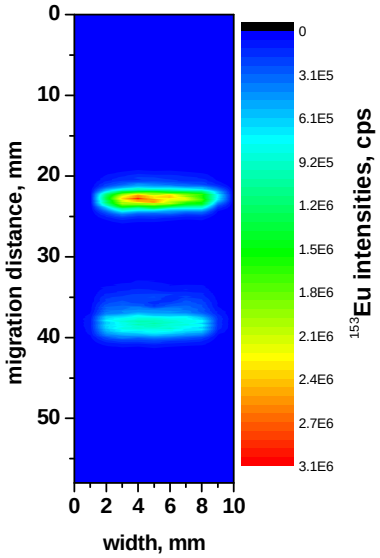


Figure 4.12: $^{153}Eu^{+}$ signals measured by LA-ICP-MS on a blot membrane; samples: 8 pmol europium-labelled antibody CYP1A1 with DOTA reaction at 80 min, RT, pH 9.0 and europium reaction at 37 °C, 30 min, pH 7-7.5.

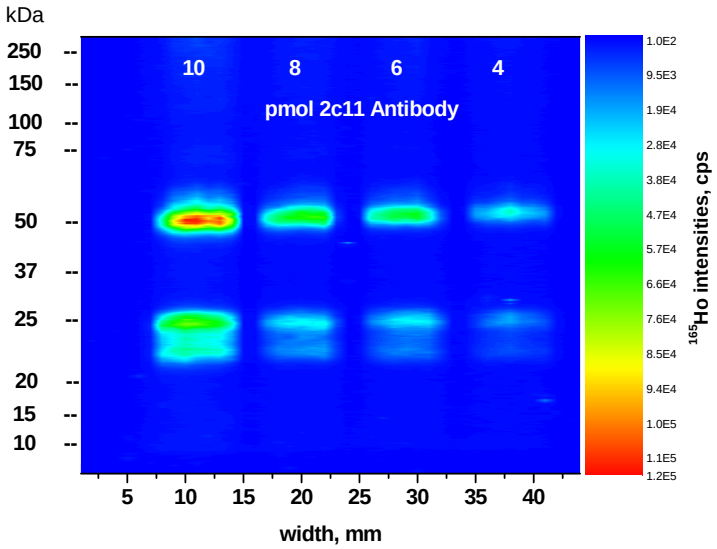


Figure 4.13: $^{165}Ho^{+}$ signals measured by LA-ICP-MS on a blot membrane; Samples: holmium-labelled antibody CYP2C11 with DOTA reaction at 80 min, RT, pH 9.0 and europium reaction at 37 °C, 30 min, pH 7-7.5.

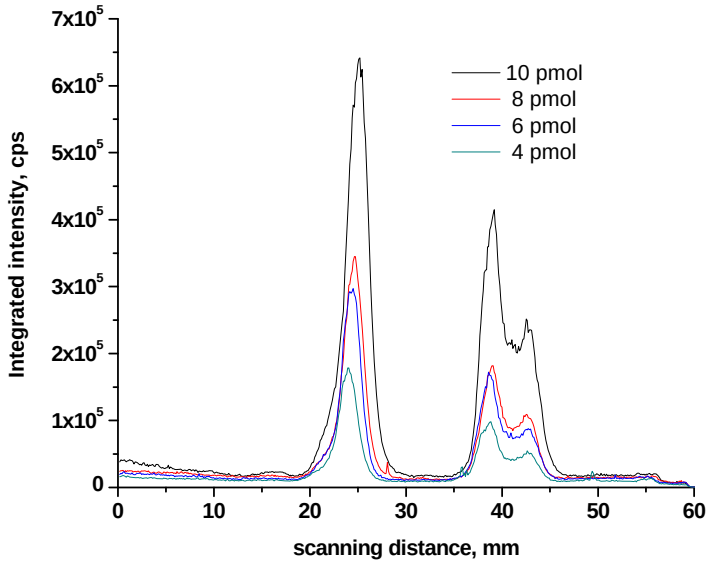


Figure 4.14: Sum of intensities of single laser line scans per each electrophoretic lane; black line: 10 pmol; red line: 8 pmol; blue line: 6 pmol; green line: 4 pmol were loaded onto a gel. Labelling conditions: 80 min at RT with a molar ratio of 1:20:200 (n(Ab):n(DOTA):n(Ho)).

4.3.3 Comparison of DTPA with DOTA

The SCN-DOTA is quite expensive and therefore a much cheaper alternative using chelating complex DTPA was also investigated. A detailed study of the optimising procedures is already available in literature[55] but not with respect to ICP-MS based detection. In most cases it has been used for experiments with radioactive measurements. For both, DTPA and DOTA experiments, BSA was chosen as the model protein for comparison. Two types of molar ratios between BSA and chelating agents, DTPA and DOTA were used. The molar ratio for BSA to DTPA was chosen to be 1:2 and 1:4 but for DOTA it was 1:10 and 1:20. In case of DTPA the molar excess europium used was only 4 times higher than the BSA molar amount. But for DOTA it was used 100 and 200 times higher as explained before in the section 4.3.2. After that 1 µg of protein with 2 different ratios from each set of samples was used for separation and the surface plot is shown in Figure 4.15 and Figure 4.16 for DTPA and DOTA experiments, respectively.

When DTPA was used as the chelating agent 2 bands were visible where it was expected to have only one around 66 kDa. In all previous experiments with BSA and DOTA and also as shown here in Figure 4.16 only one protein band is visible when

DOTA was used. As the two experiments were done on the same blot the intensities can be compared. And that the integrated intensities were approximately one order of magnitude higher in case of DTPA labelled protein than DOTA when taken into consideration only one band at 66 kDa (Table 4-2). Also the sensitivity was one order of magnitude higher and LODs were better in case of DTPA. The LODs for labelling experiments using DOTA were higher probably due to the lower ratios of the chelating agent to protein used and that they are not sufficiently linked. Despite the better sensitivity and detection limits, DTPA showed 2 bands possibly due to the dimer formation by linking 2 protein molecules. This chelator needs to be further studied in detail as it showed good detection levels.

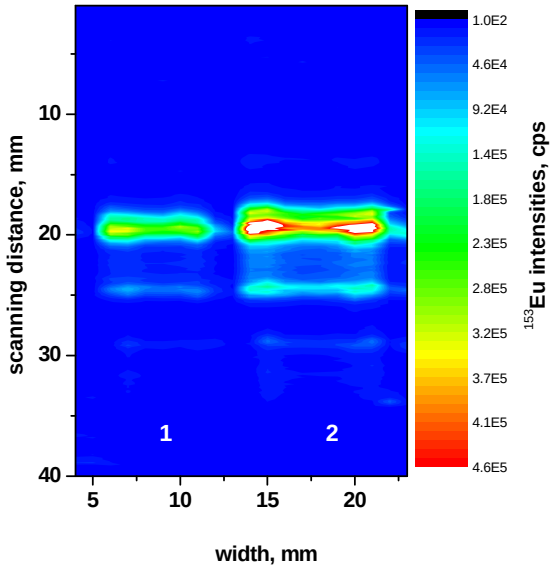


Figure 4.15: $^{153}Eu^{+}$ intensities measured by LA-ICP-MS; samples: labelled BSA lane 1: molar ratio of n(BSA):n(DTPA) was maintained at 1:2 and in lane 2 at 1:4. For both samples in the step 2 reaction the ratio of europium to BSA was maintained at 1:4.

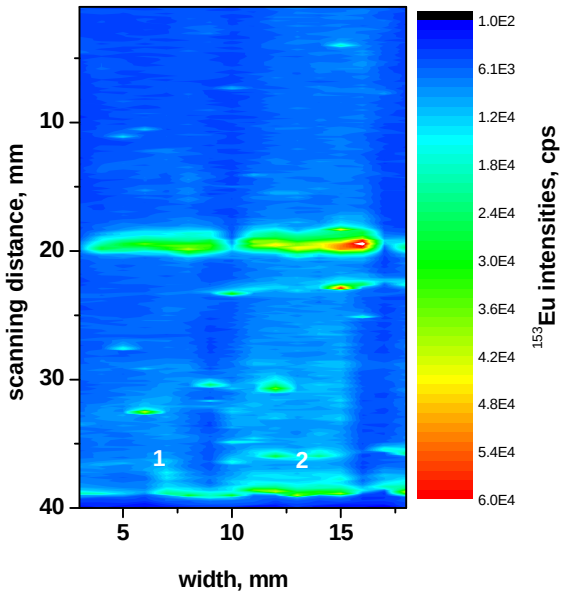


Figure 4.16: $^{153}Eu^{+}$ intensities measured by LA-ICP-MS; samples: labelled BSA lane 1: molar ratio of n(BSA):n(DOTA) was maintained at 1:10 and in lane 2 at 1:20. For both samples in the step 2 reaction the molar ratio of europium to DOTA was maintained at 1:10.

Table 4-2: Sensitivity and LOD of the method with respect to europium-labelled BSA via DTPA and DOTA.

	DTPA		DOTA	
Parameters	**Lane 1**	**Lane 2**	**Lane 1**	**Lane 2**
Peak height (cps)	$4.07 \cdot 10^6$	$1.84 \cdot 10^6$	$1.85 \cdot 10^5$	$2.78 \cdot 10^5$
Sensitivity (cps/pmol)	$2.72 \cdot 10^5$	$1.23 \cdot 10^5$	$1.23 \cdot 10^4$	$1.86 \cdot 10^4$
Integrated intensity (cps)	$8.8 \cdot 10^5$ (low mw) $7.3 \cdot 10^6$	$3.37 \cdot 10^5$ (low mw) $2.96 \cdot 10^6$	$2.1 \cdot 10^5$	$3.1 \cdot 10^5$
LOD (fmol)	15	37	300	150

4.4 Concluding Remarks

In this chapter, 2 different labelling methods and one staining method were investigated for the quantification of proteins and antibodies.

Indian ink staining was performed overnight on blots. The screenings of different elements using LA-ICP-MS were performed and only lithium was detected at high levels. Two types of calibration methods were studied using dotting and PAGE separated protein standards and as test sample ovalbumin was quantified. The value obtained was close to the theoretical applied on the blots for PAGE separated quantification method and slightly lower when dot blotting was used. Indian ink stained blots can be used for the total protein quantification. The LOD of this method was estimated to be approximately 95 fmol for lithium in proteins.

The next two methods were based on the bioconjugation chemistry. The one using DTPA dianhydride showed good sensitivities even with lower ratios between protein and the chelating agent. But in the experiments performed always more than one band was detected for a single protein possibly due to the dimer formation of the protein molecules. Nevertheless labelling based on this chelator should be investigated in detail in future.

The other chelating agent p-SCN-Bn-DOTA was used which specifically target amino groups of the proteins. The labelling conditions using this chelating agent were optimised here for multielemental detection of proteins. The procedure consists of 2 parts: binding of the protein to the chelate (step 1) and reaction of the first step product with lanthanides (step 2). The results obtained from in-solution and LA-ICP-MS experiments show that reaction times have to be optimised for each protein separately. For BSA higher labelling efficiency could be seen when a longer reaction time of 16 h and RT were chosen between protein and DOTA in step 1. BSA was stable after this long reaction time and could be detected by LA-ICP-MS. The molar amount of DOTA should be used 40 times higher than those of the BSA to achieve a final labelling degree of 2. For the step 1 reaction carbonate-bicarbonate buffer at pH 9.0 was ideal when compared with the other buffer systems where high background could be seen.

For the step 2 reactions 10 times higher amounts of lanthanide in relation to DOTA was enough for the complete saturation. The ideal temperature to achieve this was

37 °C, the reaction time was 30 min and the reaction was carried out in acetate buffer at pH 7.0 to 7.5.

With higher molar ratios between DOTA and BSA, higher background could be seen with LA-ICP-MS experiments. This was largely due to the free lanthanide ions present in the sample. Better purification steps will be needed to avoid this contamination. Beyond 16 h reaction time between DOTA and BSA signals were declining probably due to denaturation of proteins. Since BSA has more binding sites it is expected to have more labels. But it also depends on the quaternary structure of the protein and the available amino groups for the linking.

The same set of experimental conditions was also applied for two antibodies (CYP1A1 and CYP2C11). Both were labelled with europium and holmium simultaneously. The ratio between DOTA and protein in the step 1 reaction differed for the antibody where even with a 20 fold molar excess of DOTA to antibody similar sensitivities could be achieved. A reaction time of 80 min between antibody and DOTA was enough to have better signals than with BSA. Two bands were visible for both denatured antibodies from the surface plot which shows that both the heavy and light chains were labelled. Nevertheless higher labelling degree was seen for the heavy chain. The sensitivity obtained was higher for the europium-labelled antibody with respect to the heavy chain and the LOD was estimated to about 30 fmol whereas for the holmium-labelled antibody it was about 40 fmol.

In conclusion, a simple and optimised method was described for covalently attaching the chelator DOTA to proteins and antibodies and for labelling of the coupled proteins with the lanthanides. The antibody labelling experiments showed that the antibodies can be labelled and detected by the LA-ICP-MS method but after labelling their specificity for antigen has to be checked which will be shown in the next chapter.

Chapter 5

LA-ICP-MS based Detection of Multiple Cytochromes P450 by Element-Labelled Monoclonal Antibodies

The ICP-MS is a multielement detector but has been applied as single element detector in most of the previous applications. In this chapter multielement capabilities should be explored at hand of a selected application. The example chosen here was based on Cytochromes P450 (CYP) detection. CYPs are groups of enzymes which are structurally very similar and difficult to study simultaneously with standard biochemical methods. But very specific monoclonal antibodies were available for some of the enzymes and provided by PD. Dr. Roos. It is the idea of this investigation to use antibodies differentially labelled with different elements so that different proteins can be detected concomitantly. Two differentially labelled antibodies one with iodination and the other using DOTA will be investigated for the immunoblotting step for detection of the respective CYP enzymes on the blots. From these blots the different CYP enzymes will be semi-quantitatively detected using LA-ICP-MS.

5.1 Cytochromes P450

Cytochromes P450 belonging to the group of heme proteins are enzymes which are involved in a variety of metabolic and biosynthetic processes. The 450 in this name is attributed to the position of the Soret maximum in the absorption spectrum of the reduced Fe^{2+}-CYP carbon monoxide complex at 450 nm.[109] The human genome encodes about 60 Cytochrome P450 proteins, but the number of known CYP enzymes exceeds 1,000 considering also invertebrate, plant and bacterial enzymes. All CYP enzymes exhibit similarity in their structure and general mechanism of action; however, there are significant differences in the detailed function of individual enzymes as well as in the structures and properties of their active sites.

In mammalian species including man, CYPs are the most important class of enzymes involved in the metabolism of xenobiotics and are, thus, part of the detoxification machinery of an organism. There are over 200,000 chemicals which are

metabolized by these CYPs. Their primary task is to introduce new functional groups into a molecule, thereby facilitating the subsequent work of phase II-enzymes which attach hydrophilic groups such as glutathione or sulphate to the CYP-derivatized xenobiotics. This concerted action of enzymes aims to increase the water-solubility of compounds to achieve their efficient excretion.

5.1.1 Cytochromes P450 Enzymes

There are approximately 20 CYP isoforms among the CYP 1, 2, 3 and 4 subfamilies present which metabolise drugs. In humans, CYPs comprise about 57 different enzymes within this enzyme superfamily.[110] Roughly, a half of them recognise xenobiotic substrates. These xenobiotics metabolizing CYPs are membrane bound enzymes which are predominantly localized in the endoplasmic reticulum. The different CYP enzymes are structurally and in some cases also with respect to substrate specificity very similar so that distinction by electrophoretic behaviour, enzymatic reactivity and spectroscopic properties can be a problem, but highly specific structural features of individual CYP enzymes can be recognized by antibodies, in particular by monoclonal antibodies.[110] However, cross-reactivities of antibodies recognizing more than one CYP enzyme occur frequently.[111]

It is a great challenge to identify and quantify the numerous different Cytochrome P450 proteins of microsomal fractions. CYP profiles differ between species, between individuals, between tissues and as a function of age. Furthermore, the profiles are adaptively modulated by xenobiotics which may induce or suppress a set of CYP enzymes dependent on the compound.

5.1.2 Expressions of Cytochromes P450 Enzymes

CYP1A1 (enzyme no. 1 in subfamily A of family 1) is not present or found at low levels only in several tissues under normal conditions. The enzyme can be induced, however, via a receptor dependent process by certain xenobiotics, such as polycyclic aromatic hydrocarbons (PAH), dioxin and coplanar polychlorinated biphenyls (PCB).[112,113] Polycyclic aromatic hydrocarbons are a group of over 200 different chemicals formed when coal, wood, gasoline, oil, tobacco or other organic materials are

burned. And humans are exposed to these chemicals in their day to day activity. These chemicals are identified to cause mammary cancer in rats and mice. It was reported that the PAH benzo[a]pyrene causes breast cancer in rats when given in high doses for a longer period of time. These chemicals are metabolized in the human body through CYP isozymes. So once exposed to these chemicals CYP isozymes are induced. As these PAHs are involved in causing various diseases particularly tumours it is important to study their levels in our body. Their impact on animals could be studied through their metabolizing enzyme levels and their expressions. It was shown that CYP1A1 expression increases upon oral PAH exposure in many tissues including small intestine, liver, kidney, lung and spleen of rats and minipigs[114,115] enabling the respective tissues to metabolize just the inducing PAH compounds. This CYP1A1-dependent PAH metabolism leads to chemically reactive intermediates[116] and is considered as one of the initial steps in chemically induced carcinogenesis by PAH.[117,118] Induction of CYP1A1 and of some further CYP enzymes (CYP1A2, CYP1B1, CYP2S1) is controlled by the aryl hydrocarbon receptor, a ligand activated transcription factor.[119,120] Cigarette smoking, barbecue food and omeprazole are the main inducers of CYP1A1 enzyme. In association with further proteins, the receptor is primarily located in the cytoplasm. Upon ligand binding (PAH, dioxin), some proteins dissociate from the receptor complex which is subsequently translocated to the nucleus. Here, the aryl hydrocarbon receptor becomes active as transcription factor in association with the ARNT protein thereby initiating the transcription of the aforementioned CYP genes and other genes.[121] In contrast to CYP1A1, the CYP isoenzyme CYP2E1 metabolizes smaller and also hydrophilic molecules. Among these are industrial bulk materials such as benzene, toluene, vinyl chloride and trichloroethylene.[122] Further CYP2E1 substrates are ethanol and nitrosamines. As in the case of CYP1A1-dependent PAH metabolism, the latter are converted to chemically reactive compounds by CYP2E1.[123] It is only mentioned that DNA-methylation in the CYP2E1 promoter region, ligand dependent mRNA and protein stabilization as well as protein kinase A-dependent phosphorylation of the CYP2E1 protein play a role besides transcriptional regulation via cis-acting elements.[110,124,125,126,127] CYP2E1 is also mainly induced by ethanol, chloral hydrate and isoniazid.[128,129,130]

Isoniazid is one of the safe drugs used to treat tuberculosis.[131] As it is more and more used in the post HIV era, it also increases the adverse drug-drug interactions. Isoniazid is known to be involved in various drug-drug interactions including with phenytoin which decreases its release from humans as well as from animals. So an optimum dose of isoniazid is required for the tuberculosis treatment which involves less or no side effects. Isoniazid is known to be involved in the inhibition of various CYP isozymes, thereby makes the process difficult for metabolizing various other drugs which are co-administered together with isoniazid. In contrary it is also involved in the induction of various enzymes including CYP2E1. It is known that isoniazid is a weak noncompetitive inhibitor of CYP2E1.[132] The isoform CYP2E1 was studied in detail in human and animal models after isoniazid administration and the inhibition and induction effects of this enzyme were studied in rat liver microsomes.

5.2 Experimental

5.2.1 Antigen and Monoclonal Antibodies

The purified monoclonal antibodies and microsomal samples were provided by PD Dr. P. H. Roos from IfADo, Dortmund. They were isolated and purified as explained in the literature.[133]

5.2.2 Protein Quantification

The concentrations of proteins and antibodies were determined according to the method of Lowry explained in section 2.2.2.3 with bovine serum albumin as a standard. For quantification of antibodies, a Nanodrop instrument was used and the experiment was performed as explained in section 2.2.2.2.

5.2.3 Antibody Labelling with Europium and DOTA

The protocol was explained in the section 2.2.9.1 for labelling of the monoclonal CYP1A1 antibody with europium using 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid. The molar ratio of n(Ab):n(DOTA):n(Eu) was maintained at 1:20:200.

5.2.4 Antibody Labelling with Iodine

The method for iodination was very well described in the papers of Markwell,[46] Jakubowski[49] and by the manufacturer of the Iodo-beads and therefore only a brief description follows here focusing on practical aspects.

Buffer 1 consisted of 0.1 M Tris (12.11 g/l) and with HCl optimised for a pH range from 6.5 to 7. The iodination buffer (buffer 2) consisted of 100 ml buffer 1 to which 0.01 M NaI (Merck, Darmstadt, German) equivalent to 0.15 g was added and stored in the refrigerator. Aliquots of 250 µl were used for the iodination procedure only.

On the day of use, for cleaning, the bead(s) were washed for 30 s in 500 µl of the buffer solution 1 in Eppendorf tubes and dried on filter paper. After this the beads were loaded into a new Eppendorf tube together with 250 µl of the iodination buffer 2. The manufacturer recommends applying up to 6 beads in a single reaction vessel, but this depends on the total amount of protein and buffer used and the protein itself. Incubation took place for 5 min usually at room temperature. The production of free iodine was observed by the change of the colourless solution to brown in less than 15 s. This part of the experiment should be performed in a fume hood due the presence of volatile iodine. In the next step a fixed amount of antibody of 500 µg (30 µl dual colour marker in case of iodination of marker) was dissolved in 250 µl of buffer 1 and added to the reaction vessel. The total reaction volume of 0.5 ml was always kept constant. If not mentioned otherwise the reaction was stopped after 4 min. Antibodies were always iodinated for not longer than 4 min in order not to loose the binding capability by oxidation of the binding sites. For stopping the reaction the solution was removed from the Iodo-bead. Each bead was used only once. In case of the dual colour protein weight standard the complete loss of all colours was observed shortly after starting the iodination procedure due to oxidation.

5.2.5 Gel Electrophoresis and Blotting

Horizontal SDS-PAGE with 10% precast gels was used for separating proteins as explained in section 2.2.3.2 as gels of large size can be used. Nitrocellulose membrane was used for the transfer of proteins from gels for all experiments and the semidry blotting procedure was performed as explained in section 2.2.5. This was

followed by immunoblotting method as explained in section 2.2.7. The membrane was air-dried and then used for laser ablation.

5.2.6 Luminescence-based Antibody Detection

After blotting, the nitrocellulose sheets were incubated over night in PBS (9.5 mM sodium phosphate buffer, pH 6.9, 137 mM NaCl, 2.7 mM KCl), 0.1% Tween 20, 10% milk powder followed by 3 washing steps with PBS/0.1% Tween 20 for 10 min each. The blot membrane was then exposed for 1 h to the first antibody (anti-CYP1A1 or anti-CYP2E1) diluted in PBS at a concentration of 1 µg/ml. After 5 washing steps with PBS/0.1% Tween 20, incubation with the peroxidase coupled secondary goat anti-mouse IgG was performed for 90 min. Non-bound antibodies were removed by 5 washing steps with PBS/0.1% Tween 20 (10 min each).

5.2.7 Laser Ablation ICP-MS Optimisation

The optimisation of LA-ICP-MS and measurements were carried out as explained in section 2.2.12 and 3.2.4. The isotopes $^{53}Eu^{+}$ and $^{127}I^{+}$ were measured at low resolution mode and $^{13}C^{+}$ was measured at medium mass resolution. As before $^{13}C^{+}$ measured at medium mass resolution was used for control of experimental conditions. For the laser ablation and the ICP-MS measurements, the instrumental parameters were optimised and are shown in Table 5-1.

The antibody-treated nitrocellulose blot membranes were rastered and measured simultaneously in a single run using the updated high electric scan speed system as explained in section 4.2.3.

Table 5-1: Instrumental parameters.

Laser ablation system	
Laser energy	3 mJ
Make-up gas flow rate (Ar)	1 l/min
Carrier gas flow rate (He)	1.05 l/min
Repetition rate, shots per second	15 Hz
Translation velocity	1 mm/s
Distance between line scans	1 mm

Crater diameter	~500 µm
ICP-MS system	
Incident power	1350 W
Cooling gas flow rate	16 l/min
Auxiliary gas flow rate	1.3 l/min
Ni skimmer cone diameter	1 mm
Ni sampler cone diameter	0.7 mm
Isotopes measured	$^{153}Eu^{+}$, $^{127}I^{+}$, $^{13}C^{+}$
Resolution setting	400 (LR), 4000 (MR)

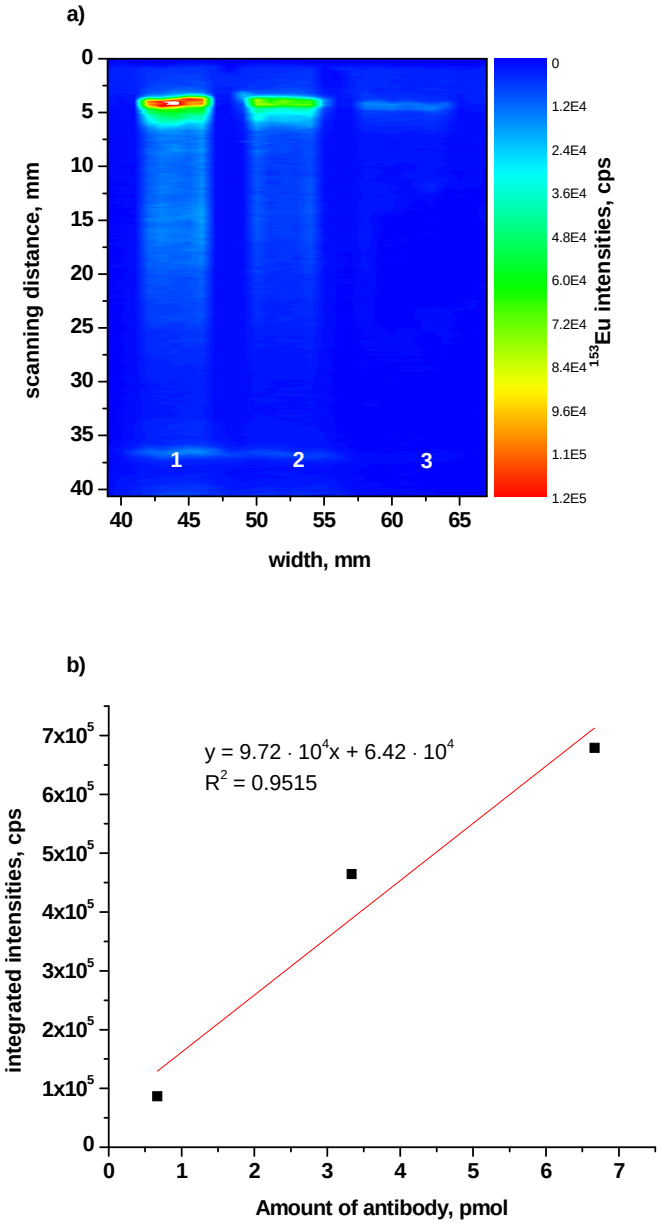
5.3 Results and Discussion

5.3.1 Analysis of Labelled Antibodies

The chelating agent DOTA specifically targets the amino groups present in the antibody. For instance IgG1 type of antibody has 64 lysine residues present in the 2 heavy chains which are targeted by DOTA. To check the labelling procedure and also the binding capacity of the europium and iodine to the heavy and light chains, an experiment with denaturated conditions was performed and is explained as follows.

For evaluating the success of labelling procedure and of maintenance of antibody integrity, the labelled antibodies anti-CYP1A1 and anti-CYP2E1 were analysed by SDS-PAGE, blotting and subsequent LA-ICP-MS. Electrophoresis was performed for the europium labelled CYP1A1 antibody in presence or absence of disulfide reducing agents, DTT, resulting either in dissociation of the antibody molecule into heavy and light chains or in keeping the quaternary structure intact. The result is shown for the europium-labelled anti-CYP1A1 antibody under non reducing conditions in Figure 5.1a. Only one $^{153}Eu^{+}$ band is seen just in the molecular weight range of an intact antibody molecule indicating that 1) the antibody was successfully labelled with europium, 2) that the antibody was pure and 3) that the antibody was not degraded by or during the labelling procedure. Application of different amounts of labelled antibodies showed that at least 0.1 µg antibody corresponding to about 0.66 pmol antibody can be detected by LA-ICP-MS. The areas of the antibody spot were integrated and plotted against the antibody

concentration. The resulting calibration is shown in Figure 5.1b and a linear dependence of applied antibody concentration with respect to the intensity was found.



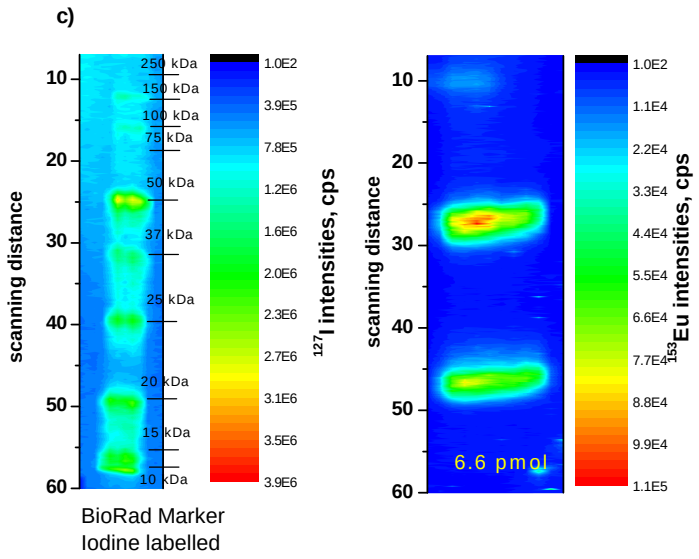


Figure 5.1: (a) $^{153}Eu^{+}$ intensity distribution of different amounts of the non denaturated europium-labelled CYP1A1 antibody measured by LA-ICP-MS after SDS-PAGE and transfer to an NC membrane (1) 6.6 pmol, (2) 3.3 pmol, (3) 0.66 pmol. (b) Integrated total europium intensities of 3 different concentrations of anti-CYP1A1 antibody obtained by SDS-PAGE. (c) $^{153}Eu^{+}$ intensity distribution of 6.6 pmol amount of the denaturated europium-labelled CYP1A1 antibody measured by LA-ICP-MS after SDS-PAGE and transfer to an NC membrane. Left figure shows the $^{127}I^{+}$ intensities from the iodinated Bio-Rad marker. [133]

In the presence of DTT for 6.6 pmol of antibody as shown in Figure 5.1c, two clear bands are visible which correspond to the heavy and light chains of the antibody due to the cleavage of the antibody into heavy and light chains. The molecular weight was checked by running the iodine labelled Bio-Rad marker simultaneously with the sample. Both spots were integrated and the resulted intensities were used for quantifying the CYP samples. The sensitivity was higher in the heavy chain which migrated at around 50 kDa when compared to the light chain around 20 kDa. The obtained sensitivity reflected the binding sites (amino groups) available for the chelating compound, i.e. more chelating compound binding to the heavy chain and less to the light chain. But the light chain was more efficiently labelled than the heavy chain. While molecular weights of heavy and light chains differed by a factor of about 2 the $^{153}Eu^{+}$ intensities differed only by 1.4. The intensities obtained with the present labelling conditions were enough for the LA-ICP-MS detection. The sensitivity with respect to the heavy chain and LOD of the method was estimated to be about $1.46 \cdot 10^{5}$ cps/pmol and 40 fmol respectively. It should

be noted that for the immunoblot preparations the europium labelled antibody was faced with milder conditions than for the electrophoretic separation in the presence of SDS. The same antibodies were used for the immunoblotting experiments for the detection of the respective CYP enzymes, thus quantification of the sample was possible.

In order to make the quantification process comparable, the iodine labelled CYP2E1 antibody was also analysed by SDS-PAGE only in the presence of the reagent DTT. Six different concentrations of iodine-labelled antibodies were applied. The result presented in Figure 5.2a shows iodine-labelled bands corresponding to the separated heavy and light chains. An additional band above the heavy chain can be interpreted as un-cleaved dimers consisting of one heavy and one light chain. Nevertheless, the pattern shows that both chains were labelled with iodine which involved tyrosine and possibly histidine residues of the protein. The resulted intensities of both chains were integrated and plotted against the antibody amount applied. As shown in Figure 5.2b it is linear over the concentration investigated (R^2=0.993). The LOD was calculated and found to be 10 fmol which is better than that of the europium-labelled antibody detection. The sensitivity was higher and estimated to be $5.83 \cdot 10^7$ cps/pmol.

There are many types of calibration and quantification approaches used in ICP-MS. Some of them were already discussed in chapter 2 and 3. In chapter 3 standard phosphorus proteins, with known amount of phosphorus were used to quantify unknown phosphorus proteins either in the same blot after separation or after dot blotting. With the results shown in chapter 3, calibration using standard proteins subjected to PAGE separation similar to sample was worked well for quantification. One approach of quantifying CYP proteins from the different microsomal preparations is via purified CYP enzymes. But such purified standards are not available and therefore an alternative approach for CYP protein quantification has to be elaborated using LA-ICP-MS via absolute quantitation of the label elements, here europium and iodine from the calibrations using the labelled antibodies. This was tried here for the initial study. The same labelled antibodies which were used for the immunoblotting experiments were PAGE separated and blotted under similar conditions to the microsomal preparations and used as calibration for simultaneously quantifying multiple antigens, thus avoiding the knowledge of the labelling degree of antibody. Nevertheless a theoretical 1:1

stoichiometry between monoclonal antibodies and monomer CYPs was taken into account. Thus here only a preliminary semiquantitative detection of CYPs was possible which required additional methods for the validation.

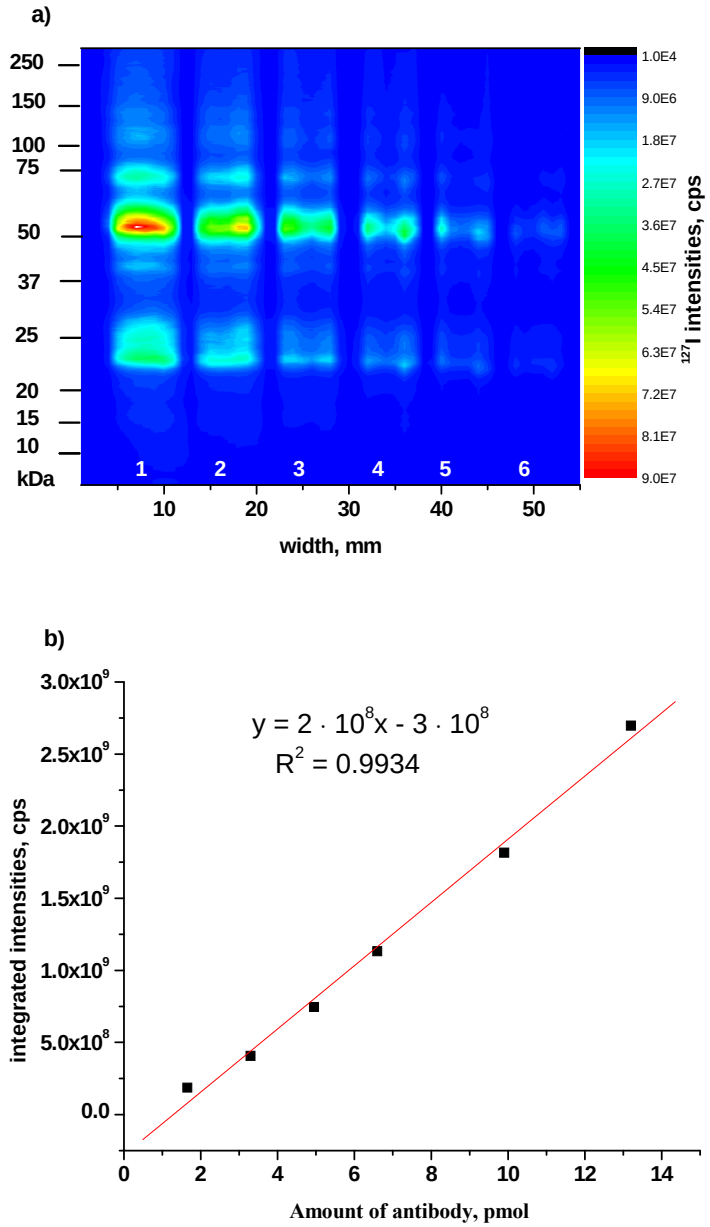


Figure 5.2: (a) $^{127}I^+$ intensity distribution of different amounts of the denaturated iodine-labelled CYP2E1 antibody analysed by LA-ICP-MS after SDS-PAGE and transfer to an NC membrane (1) 13 pmol, (2) 10 pmol, (3) 6.6 pmol, (4) 5 pmol, (5) 3.3 pmol and (6) 1.66 pmol. (b) $^{127}I^+$ integrated signal intensities from Figure 5.2a as a function of applied antibody amounts.[133]

The calibration functions from the denaturated iodine from Figure 5.2b and corresponding intensities from the europium-labelled antibody from Figure 5.1c were used for quantification of the CYPs in this chapter. But before quantification of the CYPs could be done, it was required to first detect the CYPs and check whether the antibodies are still binding after labelling. Therefore 2 separate experiments were performed with the two labelled antibodies separately which will be explained in the following sections.

5.3.2 Detection of CYP1A1 with a Europium-Labelled Antibody

For assessing the binding activity of the europium-labelled anti-CYP1A1 antibody and its suitability for CYP1A1 quantitation by LA-ICP-MS, it was applied in an immunoblotting experiment. As a first application liver microsomes of rats which were treated with specific inducers for CYP enzymes, namely 3-methylcholanthrene for CYP1A1 were used.[134] Their spectrophotometrically determined microsomal CYP content amounts to 2.08 nmol CYP per milligram of protein. Thus CYPs made up about 10% of the total microsomal protein, calculated from an average CYP molecular mass of 50 kDa. Specific monoclonal antibodies for both CYP enzymes were already developed.[135] One of the affinity-purified antibodies for CYP1A1 was labelled with europium via the DOTA chelating agent by the procedure described in section 5.2.3.

0.1 µg to 5 µg of liver microsomal proteins raised in rats treated with CYP1A1-inductor 3-MC were PAGE-separated. After blotting, the membrane was incubated in europium-labelled anti-CYP1A1-antibody. The CYP enzyme CYP1A1 in liver microsomes of 3MC treated rats was detected with the specific europium labelled monoclonal antibody by means of LA-ICP-MS. The $^{153}Eu^{+}$ band pattern shows that the antibody specifically recognizes CYP1A1 in the relevant molecular weight range of around 50 kDa (Figure 5.3).

The intensity obtained corresponds to the amount of applied microsomal protein. The detection limit for CYP1A1 was close to 0.1 µg of applied microsomal protein. Thus it is estimated that about 140 fmol of CYP1A1 can be detected with this method taking into consideration that CYP1A1 can make up to about 70% of the total CYPs in liver microsomes of 3-MC treated rats.[134]

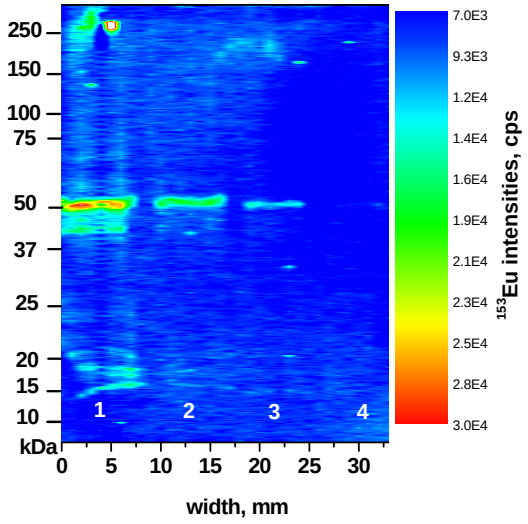


Figure 5.3: $^{153}Eu^{+}$ intensity distribution of different amounts of the rat liver microsomal protein samples (isolated from rats after 3MC treatment): (1) 5 µg, (2) 1 µg, (3) 0.5 µg, (4) 0.1 µg, measured by LA-ICP-MS after SDS-PAGE, transfer to an NC membrane and immunoblotted with europium-labelled CYP1A1 antibody.[133]

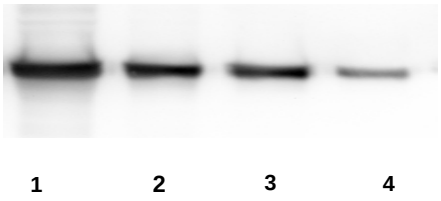


Figure 5.4: Detection of SDS PAGE separated and blotted CYP1A1 from rat liver microsomes of 3-methylcholanthrene treated rats. Amount of microsomal protein applied: (1) 5, (2) 1, (3) 0.5 and (4) 0.1 µg. Primary antibody: anti-CYP1A1. Secondary antibody: goat anti-mouse IgG, peroxidase coupled. Detection: luminescence.[133]

Parallel to detection by LA-ICP-MS, conventional luminescence detection was performed to compare the sensitivity with the LA-ICP-MS method and validate the results. In this method initially the microsomal samples from rats after 3-methylcholanthrene treatment were subjected to SDS-PAGE and Western blotting. Then the membrane was treated with respective primary monoclonal antibody followed by a peroxidase-labelled secondary antibody directed against mouse immunoglobulin G. The result obtained from the method is shown in Figure 5.4. The sensitivity obtained through

luminescence based procedure was slightly higher than the LA-ICP-MS based method using DOTA-chelated europium. This method allowed the readout of a single CYP protein in a 1D separation. But concomitant detection of proteins was not possible when compared with the LA-ICP-MS method.

5.3.3 Detection of Blotted CYP2E1 with an Iodine-Labelled Antibody

An antibody corresponding to CYP2E1 was labelled by iodine using iodination. Liver microsomes of rats treated with isoniazid was used which is known to increase CYP2E1 levels.[136] Liver microsomal protein of isoniazid treated rats was applied to SDS-PAGE in amounts of 0.125 to 5.0 µg. LA-ICP-MS of the blotted samples for $^{127}I^{+}$ revealed a clear band in the 50 kDa molecular weight range (Figure 5.5). The intensity obtained corresponds to the amount of applied microsomal protein. The estimated detection limit for CYP2E1 was close to 0.25 µg of applied microsomal protein which corresponds to about 70 fmol CYP2E1. The calculation was based on the specific CYP-content of the microsomes which amounted to about 1 pmol total CYPs/µg microsomal protein and the fact that CYP2E1 constituted about 30% of total CYPs in liver microsomes of isoniazid-treated rats.[134] A parallel analysis of the samples by means of a secondary antibody and luminescence detection showed that both methods have comparable sensitivity (Figure 5.6).

The above 2 experiments (section 5.3.2 and 5.3.3) were performed on separate membranes with the liver microsomal samples of differentially treated rats. There was a difference in the maximum intensities achieved for the 2 different blots which was due to the different labelling procedures. Higher intensities and sensitivities could be seen for CYP2E1 detection via iodinated antibody. The difference in intensities was not important because for quantification the same labelled antibodies were taken into account which was PAGE separated and blotted. Until now only one antibody was applied during the immunoblotting procedure in the examples presented so far. In order to save time and reduce errors, a new method was tried to identify proteins in a single experiment.

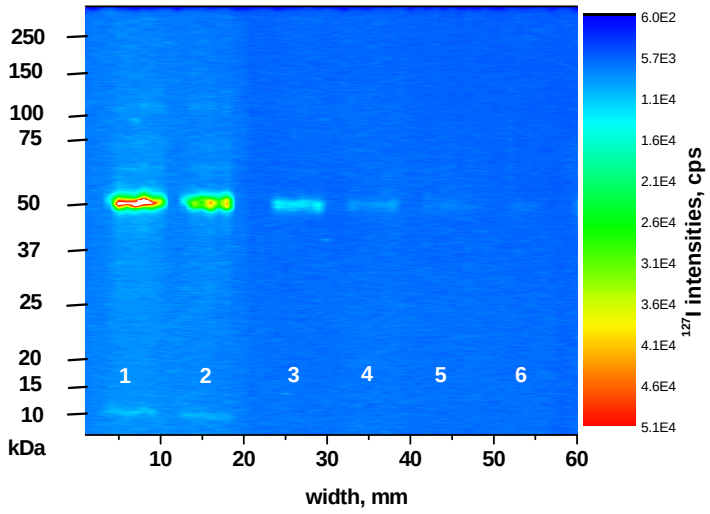


Figure 5.5: $^{127}I^{+}$ intensity distribution of different amounts of the rat liver microsomal protein samples (isolated from rats after isoniazid treatment): (1) 5 µg, (2) 3 µg, (3) 1 µg, (4) 0.5 µg, (5) 0.25 µg and (6) 0.125 µg, measured by LA-ICP-MS after SDS-PAGE, transfer to an NC membrane and immunoblotted with iodine-labelled CYP2E1 antibody.[133]

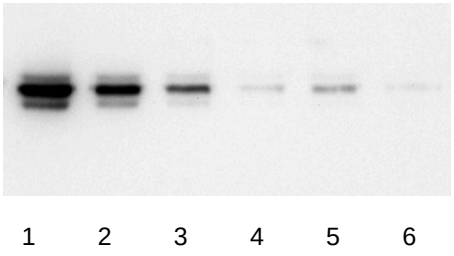


Figure 5.6: Detection of CYP2E1 in liver microsomes of isoniazid-treated rats with luminescence based detection using monoclonal antibody specific for CYP2E1 and peroxidase coupled secondary antibody. Microsomal protein in the amounts of (1) 5, (2) 3, (3) 1, (4) 0.5, (5) 0.25 and (6) 0.125 µg were separated by SDS-PAGE and subsequently transferred to a nitrocellulose membrane by electro-blotting.[133]

5.3.4 Simultaneous Detection of 2 CYPs in One Western Blot Experiment

In the next experiments, simultaneous detection of CYPs was investigated. In order to perform this experiment, 2 differentially labelled monoclonal antibodies (section

5.3.2 and 5.3.3) were mixed together during the immunoblotting procedure. The blots were then analysed by LA-ICP-MS to visualize the proteins from each sample on the same membrane. This way of simultaneous detection of various biological parameters at the same time can be seen as a proof of principle for future multiplexing experiments. Multiplexing of samples reduces the number of gels required for an experiment, minimizes quantitative and qualitative experimental errors from inter-gel or inter-membrane comparisons. The same microsome samples were applied as before after treating rats with 3MC and isoniazid. A third microsomal sample used was obtained from minipigs which were subjected to oral intake of a PAH mixture. PAH was shown to induce CYP1A1-dependent enzymatic activity in cells of the minipig duodenum. Little is known on the presence and the level of CYP2E1 in these cells.[115] Thus experiments to measure the levels of CYP1A1 and CYP2E1 in the microsomal protein samples will provide new insights in protein expression studies simultaneously.

5.3.4.1 Liver Microsomes of 3-Methylcholanthrene-treated Rats

0.5 µg to 10 µg of microsomal proteins raised in rats treated with CYP1A1-inductor 3-MC were PAGE-separated. After blotting, the membrane was concomitantly incubated with the europium-labelled CYP1A1 antibody and iodine-labelled CYP2E1-antibody. The CYP enzyme CYP1A1 in liver microsomes of 3MC treated rats was detected with a specific monoclonal europium-labelled antibody CYP1A1 (Figure 5.7a). LA-ICP-MS of the blotted samples for $^{153}Eu^{+}$ revealed a clear band in the 50 kDa molecular weight range. The acquired intensity is proportional to the amount of microsomal protein applied.

The $^{153}Eu^{+}$ intensities were then used for quantifying the respective CYP1A1 samples by using the corresponding europium-labelled intensities from Figure 5.1c. The respective CYP1A1 concentration is shown in Table 5-2. The antigen amount was calculated by taking into account the complete integrated signals belonging to CYP1A1. For a 10 µg total microsomal protein 281 ng CYP1A1 was calculated, and the CYP1A1 expression increased with the microsomal sample after 3MC treatment.

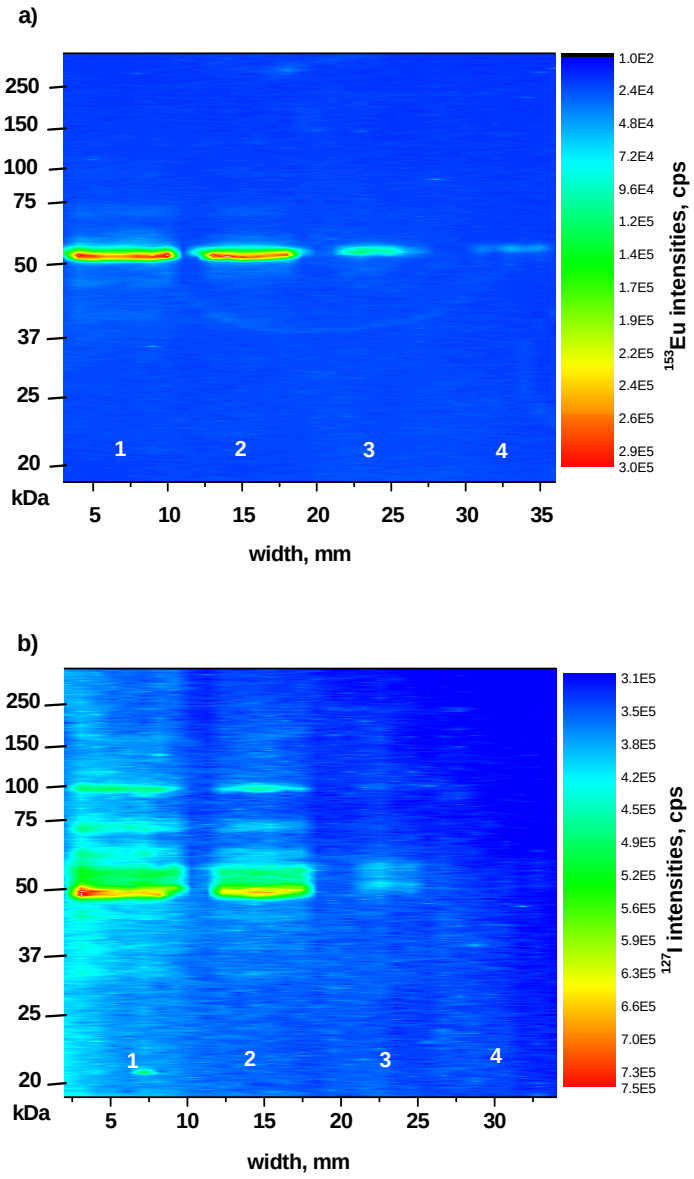


Figure 5.7: Concomitant detection of (a) CYP1A1 and (b) CYP2E1 with a europium-labelled and iodine-labelled antibody, respectively. Blot membranes were scanned and analysed by LA-ICP-MS for $^{153}Eu^{+}$ and $^{127}I^{+}$. Samples: liver microsomes of 3-methylcholanthrene treated rats. The amounts of microsomal proteins applied for SDS-PAGE separation were (1) 10 µg, (2) 5 µg, (3) 1 µg and (4) 0.5 µg.[133]

$^{127}I^{+}$ measurement of the same blots simultaneously shows specific signals for CYP2E1 in the same lane (Figure 5.7b). $^{127}I^{+}$ intensities increased linearly with the amount of applied microsomal protein but could not be detected below 1 µg. In addition to the specific band around 50 kDa 3 more bands were visible. The origin of these bands was not clear but it could be assumed that they represented ubiquitous CYP2E1-protein

still recognized by the antibody. These bands were also detected by luminescence-based detection and confirmed that they were not artefacts of the LA-ICP-MS method.

Table 5-2: Table shows the amounts of CYP1A1 present in the sample from the total microsomal proteins which were loaded on the gel and separated using SDS-PAGE.

3MC microsomes (µg)	CYP1A1 antigen (ng)
10	281.0392996
5	226.5154669
1	49.36132296
0.5	17.25437743

By using the calibration graph from Figure 5.2b, the estimated amount of CYP2E1 present in the 10 µg sample was calculated and estimated to be 70 ng. The amount obtained was very low when compared to those of the CYP1A1 which was induced upon stimulation by 3MC. This showed that the 3MC treatment did not or very mildly induce CYP2E1 expression.

The protein spots of 10 µg microsomal protein for CYP1A1 and CYP2E1 were integrated and the line scan of both is shown in black and pink colour, respectively. It should be noted here that the molecular weight of rat CYP1A1 was 59393 Da and of rat CYP2E1 was 56627 Da.[137] The small difference of less than about 3000 Da protein could be differentiated by the element labels and electrophoretic mobility which then was detected by LA-ICP-MS. In Figure 5.8 the obtained $^{127}I^{+}$ intensities for CYP2E1 were background corrected in order to show them together with the $^{153}Eu^{+}$ intensities.

The increase in the CYP1A1 expression was also observed in literature from the Western blot analysis where the levels of this enzyme in addition to CYP1A2 increased in the liver microsomes of 3MC treated guinea pigs.[138] This study was done to compare the induction of CYP1A1 and CYP1A2 in guinea pig liver after 3MC treatment. Only the levels of CYP1A1 were induced both in liver of the guinea pig after treatment. A similar result was also observed in a study conducted on transgenic rats with insufficient blood growth hormone.[139] The expressions of CYP1A1 and CYP2B using the enzyme activity assays and immunohistochemistry were compared. 3MC administration induced CYP1A1 in both wistar and mini rats. But the induction was higher in the mini rat liver than in the wistar rat liver. Tani *et al.* investigated the induction of CYPs by their EROD

activity. The EROD activity (7-ethoxyresorufin O-deethylase activity) test showed that about 2458+/-1058 pmol/min/mg protein were found for the CYP1A1 enzyme after 3MC treatment. CYP1A1 associated enzyme activity could be studied by using 7-ethoxyresorufin as substrate. This EROD activity is the most sensitive and reliable method for determining the induction of CYPs.

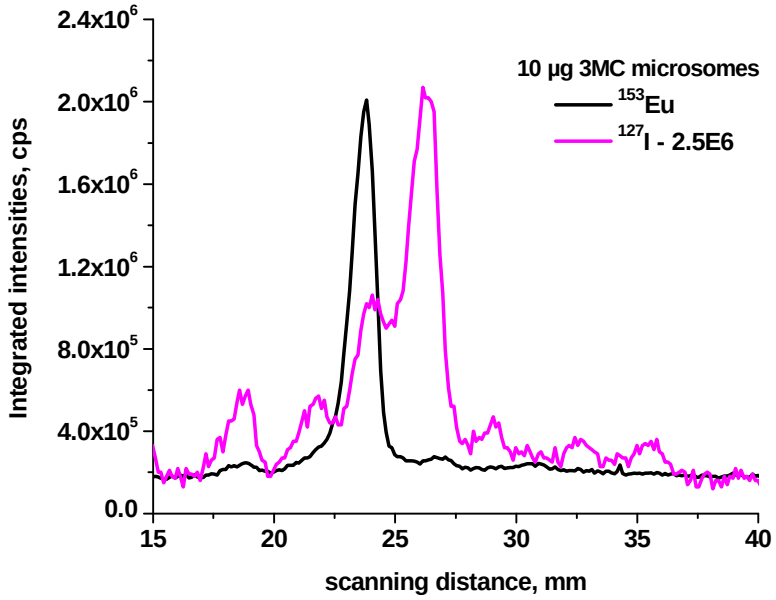


Figure 5.8: Superimposed line scans for $^{153}Eu^{+}$ and $^{127}I^{+}$ intensities of the 10 µg samples of Figure 5.7 corresponding to CYP1A1 and CYP2E1, respectively. The main protein peaks were electrophoretically separated. A CYP2E1 rider peak overlaps with the CYP1A1 peak clearly distinguished by the different labels (3MC 3-methylcholanthrene).[133]

5.3.4.2 Liver Microsomes of Isoniazid-treated Rats

0.5 to 2 µg of microsomal protein samples after isoniazid treatment were SDS PAGE separated and immunoblotted. From Figure 5.9a, it can be inferred that a CYP1A1-associated $^{153}Eu^{+}$ signal is not or is only slightly seen even at 2 µg microsomal sample.

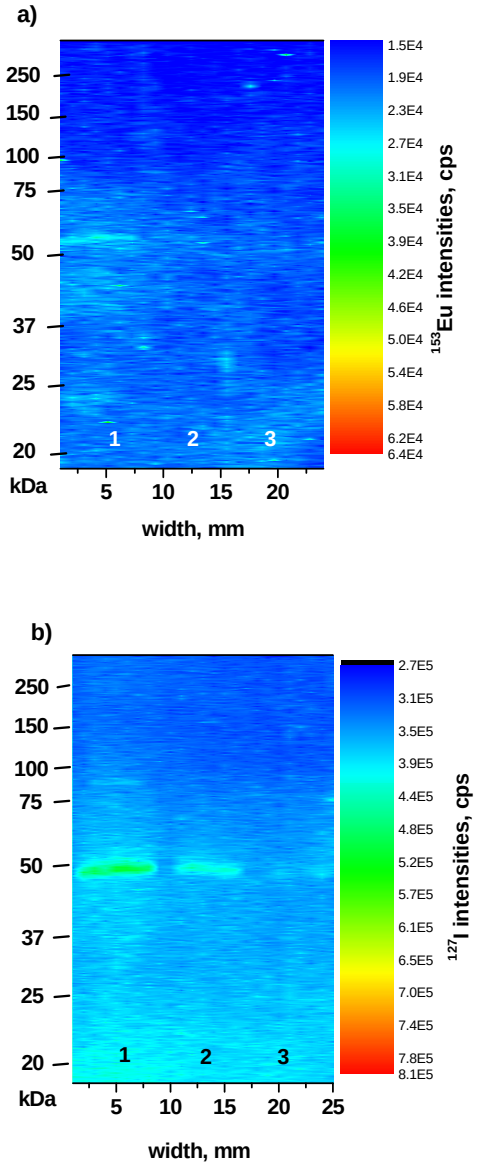


Figure 5.9: Concomitant detection of (a) CYP1A1 and (b) CYP2E1 with a europium-labelled and iodine-labelled antibody. Blot membranes were scanned and analysed by LA-ICP-MS for $^{153}Eu^{+}$ and $^{127}I^{+}$. The samples were liver microsomes from isoniazid-treated rats. The amounts of protein applied for SDS-PAGE separation were (1) 2 µg, (2) 1 µg and (3) 0.5 µg.[133]

But for the same concentration of the protein sample significant intensities could be obtained for CYP2E1 down to 1 µg using the iodine-labelled CYP2E1. CYP2E1 expression was detectable in the liver microsomes of isoniazid-treated rats of around 0.5 µg of microsomal proteins (Figure 5.9b) which corresponded to about 160 fmol of CYP2E1 taking into consideration that CYP2E1 made up about 1.6% of total liver microsomal protein in isoniazid-treated rats.[134] The protein spots of 2 µg microsomal

protein for CYP1A1 and CYP2E1 were integrated and the line scan of both is shown in black and pink colour, respectively, in Figure 5.10. The background of the iodine intensities for CYP2E1 was subtracted in order to show them together with the europium intensities corresponding to CYP1A1. It is obvious from Figure 5.10 that CYP2E1 was present in higher amounts when compared to those of the CYP1A1 which was completely absent or present at low levels after treatment with isoniazid. In conclusion, the results confirmed what was known about the CYP expression profile in liver microsomes of isoniazid-treated rats.

After applying the same calibration function from Figure 5.2b, the amount of CYP2E1 was estimated to be 70 ng for a 2 µg rat liver microsome after isoniazid treatment. A very low amount of CYP2E1 could be observed here, but we are not sure whether it was a weak inhibition or induction of it by isoniazid in vitro. Some studies suggest that there are both effects of isoniazid.[140] In fact some of the substrates could be cleared because of the binding of isoniazid to the active sites of CYP2E1 leading to its metabolic inactivity. To conclude it, a detailed study is needed with more concentration ranges and also studying in vivo will help in determining the drug-drug interactions involving isoniazid.

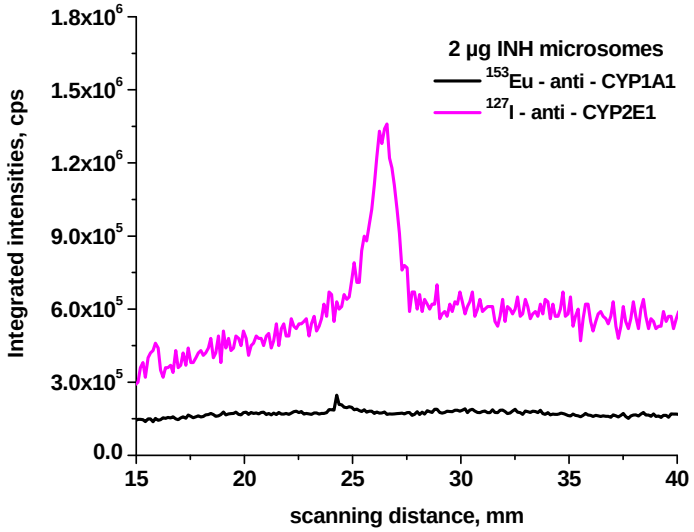
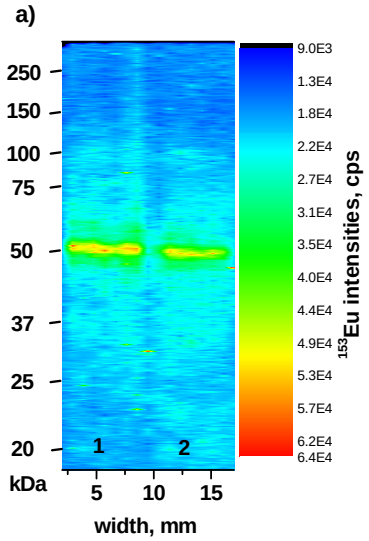


Figure 5.10: Superimposed line scans for $^{153}Eu^{+}$ and $^{127}I^{+}$ of the 2 µg samples of Figure 5.9 corresponding to CYP1A1 and CYP2E1, respectively.[133]

5.3.4.3 Duodenal Microsomes of Minipigs Orally Exposed to Polycyclic Aromatic Hydrocarbons

Two different concentrations of 10 and 20 µg of duodenal microsomes from minipigs after oral intake of a polycyclic aromatic hydrocarbon mixture were subjected to SDS PAGE and immunoblotting with 2 differentially labelled antibodies as explained before. The amounts of microsomal protein used were high because of low CYP P450 levels in microsomes of the gastro-intestinal tract. After laser ablation, from the corresponding europium intensities (Figure 5.11a) it was found that microsomal CYP1A1 was expressed in duodenal cells of minipigs after treatment with polycyclic aromatic hydrocarbon which was in contrast to liver microsomes of isoniazid-treated rats. With the corresponding intensities from the europium-labelled antibody from Figure 5.1c the respective amounts of CYP1A1 expression corresponding to the amount of total microsomal proteins were calculated and shown in Table 5-3. 40.5 ng of CYP1A1 was found for a 20 µg applied microsomal sample. A slightly higher amount than the half of the CYP1A1 was observed for the 10 µg microsomal sample. As no other specific bands were visible it was clear that only CYP1A1 was expressed which was also observed in the studies of Roos *et al.* who obtained the highest induction of CYP1A1 with a sample containing 274 mg 5- and 6- ring PAH/kg soil, resulting in a 360 fold increase in the EROD activity.[141] These experiments showed that the labelled antibodies could very well be used for simultaneous detection and quantification of proteins.



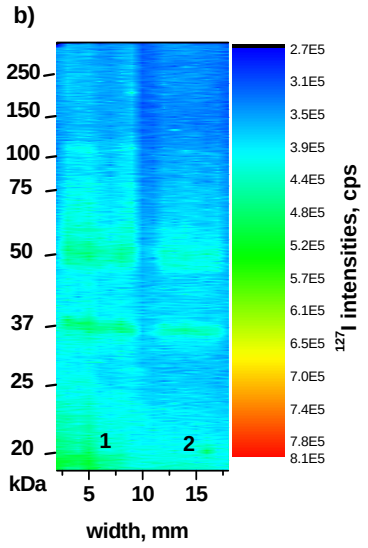


Figure 5.11: Concomitant detection of (a) CYP1A1 and (b) CYP2E1 with a europium-labelled and an iodine-labelled antibody, respectively. The samples were duodenal microsomes of minipigs orally exposed to polycyclic aromatic hydrocarbons. Blot membranes were scanned and analysed by LA-ICP-MS for $^{127}I^+$ and $^{153}Eu^+$. The amounts of microsomal proteins applied for SDS-PAGE separation were (1) 20 µg and (2) 10 µg.[133]

The $^{127}I^+$ distribution is shown in Figure 5.11b for the 10 and 20 µg samples. CYP2E1 was also detectable but at low levels. Again with the iodine-labelled CYP2E1 antibody 2 bands were seen. A new band at lower molecular weight range in addition to the normal band at 50 kDa was detected and its identity is not clear. In addition higher background levels could be seen for $^{127}I^+$. The superimposed line scans corresponding to the 20 µg microsomal sample are shown in Figure 5.12. After integration of the spot, $5.7 \cdot 10^5$ cps could be achieved for a 10 µg CYP2E1 duodenal microsomal sample but the levels were lower than in liver microsomes of 3MC treated rats (Figure 5.7) ($3.07 \cdot 10^6$ cps – 10 µg). The total amount of CYP2E1 present was calculated from the calibration function (Figure 5.2b) and found to be 70 ng for the total of 20 µg microsomal protein, which was low compared to the CYP1A1 expression (Table 5-3).

Two blot membranes developed by means of the primary antibodies and a peroxidase-coupled secondary antibody were used for separate detection of CYP1A1 and CYP2E1 by luminescence. In contrast to liver microsomes of isoniazid-treated rats, CYP1A1 was clearly expressed in duodenal minipig microsomes after PAH exposure

(Figure 5.13a). CYP2E1 was also detectable but only at low levels (Figure 5.13b). The results shown in Figure 5.13a and b are in accordance with the LA-ICP-MS data.

The expression of the liver microsomal Cytochrome P450 enzymes CYP1A1 and CYP2E1 which were induced by PAH in exposed rats were studied. The presence of CYP2E1 protein in minipig duodenal cells is shown here for the first time. The expression of 2 CYP isozymes were shown in this study which can be further continued for more CYP enzymes once the improvement in the labelling procedure was done.

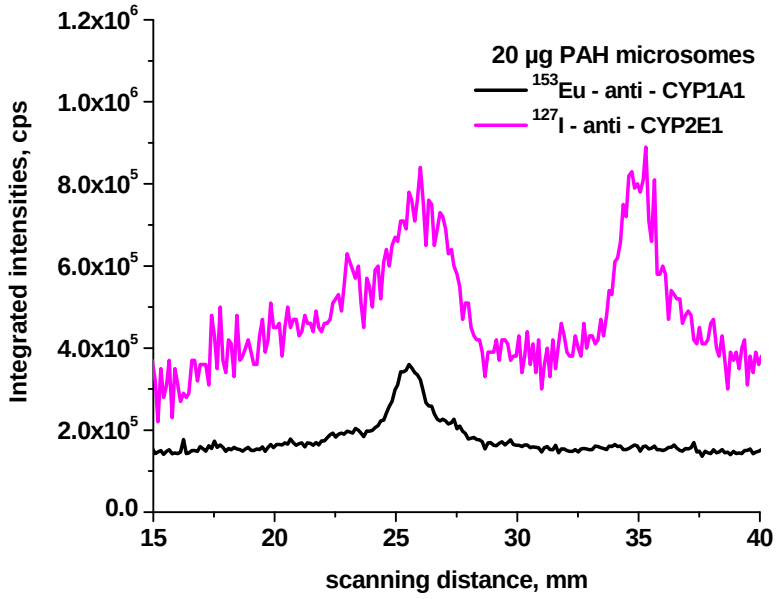
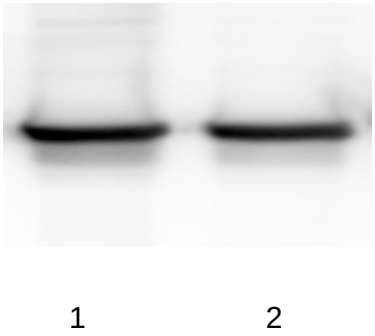


Figure 5.12: Superimposed line scans for $^{153}Eu^{+}$ and $^{127}I^{+}$ of the 20 µg samples of Figure 5.11 corresponding to CYP1A1 and CYP2E1, respectively.

Table 5-3: Amount of CYP1A1 with respect to the total microsomal protein after PAH exposure.

Microsomal protein after exposure to PAH (µg)	CYP1A1 (ng)
20	40.51327821
10	29.62231518

a)



b)

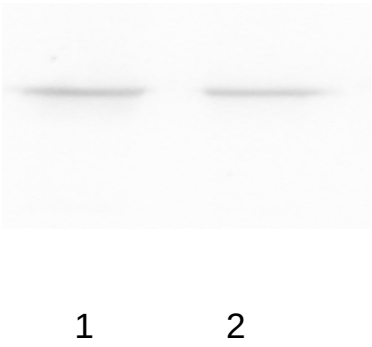


Figure 5.13: Detection of CYP1A1 and CYP2E1 with their specific antibodies. Samples: duodenal microsomes of minipigs orally exposed to polycyclic aromatic hydrocarbons (applied amounts of proteins (1) 20 and (2) 10 µg). A set of blots was treated with a primary anti-CYP1A1 antibody (a) or an anti-CYP2E1 antibody (b) and a secondary peroxidase coupled goat anti-mouse IgG antibody. The bands were visualized by chemiluminescence detection.[133]

5.4 Concluding Remarks

A novel approach to detect, identify and semi quantify different CYPs simultaneously in a single step by means of co-incubation with iodinated and lanthanide-labelled antibodies using LA-ICP-MS was demonstrated. For this purpose, the CYP1A1 antibody was labelled with europium via DOTA and CYP2E1 was iodinated. Under denaturating conditions 2 bands were visible for europium and iodine-labelled antibody whereas only one band was visible without denaturation. The LOD for iodine-labelled antibody with respect to the heavy chain was calculated and found to be 10 fmol whereas

for europium-labelled antibody it was 40 fmol. Initially these labelled antibodies were individually probed on Western blots with microsomal samples of rat liver and minipig duodenum to check their specificity for antigens. They recognised their respective CYP enzymes and by this method about 140 fmol of CYP1A1 and 70 fmol of CYP2E1 could be detected from their respective microsomal samples.

In the next step, both the iodine and europium-labelled antibodies were mixed together during the immunoblot procedure and then the blot was subjected to LA-ICP-MS. By this way 2 different CYPs, CYP1A1 and CYP2E1 in microsomal samples of differentially treated rats and minipigs, were simultaneously detected. In combination with the electrophoretic separation, a small difference of less than about 3000 Da protein could be differentiated using LA-ICP-MS method.

The calibrations obtained from surface plot of different concentrations of labelled antibody were used to simultaneously quantify the CYPs. About 281 ng and 70 ng of CYP1A1 and CYP2E1 were quantified respectively for a 10 µg total microsomal protein sample from 3-MC treated rats. The CYP1A1 levels were completely absent in liver microsomal samples of isoniazid-treated rats compared to CYP2E1. The quantified amounts of CYP1A1 and CYP2E1 were about 30 ng and 70 ng respectively for a 10 µg microsomal sample obtained from duodenum of minipigs which were orally exposed to a PAH mixture.

The LODs were better for iodine-labelled antibody compared to europium-labelled antibody. At the same time higher background levels were observed for iodine-labelled antibody. With the optimised laser ablation sampling conditions, the LOD for the europium-labelled antibody was improved.

For the analysis, only ‘primary’ antibodies were necessary. For comparison and validation, chemiluminescence detection was applied which showed a comparable sensitivity. But for this method a peroxidase or otherwise labelled secondary antibody is required in addition to the primary antibody.

The levels of CYP2E1 present in the microsomal samples obtained from duodenum of minipigs which were orally exposed to a PAH mixture were shown here for the first time. As shown here, the LA-ICP-MS method is useful to measure multiple CYPs simultaneously. In future, the number of enzymes and parameters to be analysed

can be extended further with the help of more than 2 differentially labelled antibodies. Labelling of antibody with more elements such as lanthanides or iodine will allow then to develop multiplexing approaches for the LA-ICP-MS method. Thus many proteins can be simultaneously detected and quantified which is not possible with methods such as 2-dimensional difference gel electrophoresis (DIGE) where up to only 5 different protein samples can be compared and analysed simultaneously.

Chapter 6

Conclusions and Future Work

In this work 2 different strategies for detection and quantification of proteins using LA-ICP-MS in combination with SDS-PAGE and blotting were developed and presented. One is based upon direct detection of hetero-elements especially phosphorus and the second was done by using metal containing stains and through labelling of proteins and antibodies via chelating agents. All the approaches were optimised here for ICP-MS based applications. For this purpose a LA-ICP-MS cell was developed and optimised for sample introduction of blot membranes into an ICP-MS. The procedures developed were tested at hand of 2 applications; phosphoproteomics and cytochrome expressions.

The optimisation of LA parameters such as carrier and make-up gas flow rates, scan velocity and laser energy in addition to ICP-MS generator forward power, torch positions was performed to achieve better detection limits and sensitivity for the detection of phospho-proteins on blot membranes. With the laser setup used throughout this investigation NC membrane was found to be advantageous compared to PVDF membrane and semidry blotting method was suitable. With this method, good linearity could be achieved in the range from about 20 to 380 pmol phosphorus in proteins and a LOD of about 1.5 pmol of phosphorus in β-casein was calculated when measured at medium mass resolution. In addition comparable sensitivities could be achieved for different phospho-proteins. The experiments showed good reproducibility with relative standard deviation between 6% and 10% if the whole protein spot area was taken into account. $^{13}C^{+}$ signals from the ablated membrane material was found out to be suited as internal standard for drift correction.

Two calibration procedures 1) Blotting and 2) Dotting calibration for quantification of unknown amounts of phosphorus containing proteins were investigated and compared. Better accuracy was achieved using the first method which led to the conclusion that not only the standards were ablated and transported in the same way as the test sample, but also that possible blotting losses were compensated if proteins were

chosen as standards. Therefore protein standards have always been used for internal calibration and quantification.

With the internal PAGE calibration approach the changes in the phosphoproteome of a single cancer cell line 5637 after application of hormone (EGF) and oxidative stress stimuli by H_2O_2 were measured quantitatively. It could be shown that H_2O_2 (100 µM) induced an increase in total phosphorylation by a factor of 1.3 after treatment of cells for 7.5 min and caused more phosphorylation than EGF (8.1 nM) in the high molecular weight range above 90 kDa.

Indian ink staining was used to visualize the protein spots on membranes and a contaminating element (Li) was applied for ICP-MS detection as well resulting in moderate limits of detection. Significant improvements have been achieved with a method based on bioconjugation chemistry. For this purpose two different labelling strategies based on chelation of lanthanide elements with 1) DTPA dianhydride and 2) DOTA were investigated here for detection of proteins by use of LA-ICP-MS. The strategy based on chelation with DTPA dianhydride showed the best sensitivity but was hampered possibly due to dimer formation of the protein molecules. Nevertheless this method looks very promising for future applications with mono-valent DTPA with specific target linkers, e.g. targeting amino or sulphur groups of the proteins.

The other labelling strategy was based on the commercially available chelating agent DOTA and the reaction of the protein and Eu as one typical lanthanide element with this chelating agent was optimised for detection by LA-ICP-MS. The labelling procedure consisted of two steps: a slow reaction of DOTA with a protein (4 to 24 h reaction time) and a faster second one (30 min) where DOTA was binding the Eu(III) cation. The model protein BSA was analysed with ICP-MS in-solution and by means of LA-ICP-MS after SDS-PAGE separation and blotting onto membranes. Different reaction parameters were studied such as pH, temperature, reaction time and also molar excess ratios of DOTA which were varied for both reaction steps. The optimised labelling conditions were slightly different for proteins such as BSA and for antibodies. It could be shown that the labelling efficiency was strongly depend on the protein structure and the amino groups being available for labelling. LODs of about 30 fmol and 200 fmol for an europium-labelled antibody and for BSA were calculated, respectively.

The labelling procedure developed using DOTA in combination with Eu was applied to monoclonal antibodies. Additionally iodination of antibodies following standard protocols was applied for simultaneous detection and quantification of 2 different CYPs (CYP1A1 and CYP2E1) using LA-ICP-MS and Western blotting in complex biological samples. For this purpose rats and minipigs have been exposed to several CYP P450 inducers and liver microsomes of the rats and duodendum of the minipigs have been used to study CYP P450 induction by different pharmaceutical and cancerogenic chemical compounds.

First results for the 2 enzymes CYP1A1 and CYP2E1 show that the antibodies maintained their antigen binding properties after labelling as demonstrated by LA-ICP-MS analysed immunoblots. In the animal studies, a strong CYP1A1 signal was found in liver microsomes of 3-methylcholanthrene treated rats while it was (nearly) absent in rats treated with isoniazid. The constitutively expressed CYP2E1 was found in microsomes of both treatment groups. Duodenal microsomes of minipigs orally exposed to polycyclic aromatic hydrocarbons showed a clear CYP1A1 signal with low levels of CYP2E1. From these results, it can be estimated that about 140 fmol of CYP1A1 and 70 fmol CYP2E1 are still detectable with this method taking into consideration that CYP1A1 can make up to about 70% of the total CYPs in liver microsomes of 3-MC treated rats and CYP2E1 constitutes about 30% of the total CYPs in liver microsomes of isoniazid-treated rats.

From all these results it can be concluded that detection of proteins and antibodies by use of natural or artificial hetero-elements and detection by LA-ICP-MS looks already very promising. Nevertheless, instrumental and procedural improvements are required and are still possible. For instance, analysis of one blot membrane by LA-ICP-MS is time consuming. One analysis of a conventional blot membrane can take a few hours. In order to speed up the measurement time instrumental improvements for the laser ablation system are needed. The present instrumental software of the ICP-MS system used can not handle easily the huge amounts of data generated nor is directly able to support data handling. Here the manufacturers are challenged.

Concerning procedural improvements, we have measured $^{31}P^{+}$ intensity distributions on blot membranes and thus can quantify phosphorylated proteins but were not able to investigate unphosphorylated or dephosphorylated proteins. In principle this

problem can be solved by detection of sulphur isotopes being present in most proteins, but this could not be applied here due to high sulphur blank from the SDS chemical. This problem can be partially overcome with a native PAGE separation for instance of metalloproteins or alternatively by the methods developed here just by labelling a whole proteome, but the latter has to be investigated in more detail. Alternatively new staining methods should be elaborated for this purpose. In this work it was shown that Indian ink staining of protein spots on blot membranes can already be used for the determination of the total amount of a protein. In future, other staining techniques based on Au or Ag colloids look more promising due to the very high number of atoms being present in the colloids, which can be used to improve the sensitivity of the ICP-MS detection significantly.

So far, limits of detection at pmol levels have been realized in this work for phospho-proteins. Further improvements can be expected either by application of selective phospho-protein preconcentration techniques or alternatively by reduction of the limiting blank value in cell culture experiments by use of improved sample preparation methods.

As already mentioned in one of the previous chapters, postulating the changes in the phosphoproteome without identification of the specific protein involved has little meaning. Once a phosphorylated protein is detected, purified and quantified by the ICP-MS methods described in this work, identification of the peptide pattern after a tryptic digest and separation by HPLC can be performed. Thus the phospho-protein studies of a cancer cell line discussed in this work can now in a next step be used to select the most highly phosphorylated proteins in a signalling pathway for identification by use of organic mass spectrometry such as ESI-MS and MALDI-MS.[94,142] Of course, the developed labelling methods can be easily extended to protein digests and labelling of polypeptides. LC-ICP-MS can then be used for detection and quantification to complement organic mass spectrometric techniques as it was discussed recently by Navaza *et al*.[143]

After the experimental work of this thesis was finished, ESI-MS measurements of the intact labelled protein were performed additionally to better understand the reaction chemistry.[108] It could be validated that labelling of amino-groups in particular of lysine

residues mainly take place, which was used here already for amplification of the hetero-element signal of a protein, because most proteins contain many lysine residues. The resulting labelling degree was much lower than the expected number of lysine residues from which it can be concluded that surface lysines are labelled in case of BSA mainly. For quantitative proteomics calibration of proteins by standards is required, if the stoichiometry of the protein of interest is not known. Alternatively, the labelling of N-terminal amino groups looks more advantageous, because each protein molecule can bind only one elemental label. In this case, amplification of the sensitivity can be gained by use of labelling with nano-particles instead of single elements, but such strategies have to be elaborated in future work.

The results obtained in this work were validated with Coomassie staining and chemiluminescence (CL) methods. The LA-ICP-MS method allows concomitant determination of CYPs via labelled antibodies thereby exhibiting similar sensitivity as conventional CL detection via peroxidase labelled secondary antibodies. For validation of our quantification results of phospho-proteins still alternative methods have to be tested such as phospho-staining or labelling of phospho-specific antibodies.

Although results are presented here only for labelling by use of two elements, iodine and europium, the same strategy can be extended in principle for many more lanthanides or for enriched isotopes of these elements, because of an identical chelation chemistry. In such a way LA-ICP-MS detection of proteins by immunostaining offers a new capability to elaborate highly multiplexed assays for CYP determinations via labelled antibodies. And maybe in future all CYP isozymes and their phosphorylation status can be detected simultaneously, which is still a vision of this work. Such a work, of course, can also be extended to other protein signalling processes or expression studies.

In this investigation two dimensional intensity distributions have been measured with a very good local resolution which is nothing else than imaging of elemental distributions. Quantitative elemental bio-imaging for the determination of the distribution of metals and metallo-drugs is a very new and promising application area of LA-ICP-MS as it has been shown already for instance in investigations of brain tissues samples to understand the biological processes occurring in certain diseases such as Parkinson's or Alzheimer's disease. Such studies combined with the immunostaining staining developed

in this work look very promising for cancer detection in tissue sections by use of highly specific labelled biomarkers.[144] Indirectly, cancer detection might become possible, if labelled antibody arrays are designed for LA-ICP-MS detection.[145]

In conclusion this work employing ICP-MS in combination with gel electrophoresis offers a new valuable, versatile and complementary tool for novel applications in quantitative proteomics.

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Appendix

Chemicals, Reagents and materials	Manufacturer
3-methylcholanthrene	Fluka, Buchs, Switzerland
Acetic acid	Sigma Aldrich, Taufkirchen, Germany
Acrylamide / Bis	Bio-Rad, Munich, Germany
Ammonia	Merck, Darmstadt, Germany
Ammonium acetate	Merck, Darmstadt, Germany
Ammonium peroxodisulphate	Carl Roth, Karlsruhe, Germany
Benzene	Merck, Darmstadt, Germany
Bovine Serum Albumin	Sigma Aldrich, Taufkirchen, Germany
Bromophenol blue	Merck, Darmstadt, Germany
Centriprep ultra filtration column	Millipore, Schwalbach, Germany
Colloidal Coomassie	Fluka, Buchs, Switzerland; Carl Roth, Karlsruhe, Germany
Coomassie Roti Blue	Carl Roth, Karlsruhe, Germany
Copper sulphate	Merck, Darmstadt, Germany
Diethylenediaminepentaacetic Acid	Sigma Aldrich, Taufkirchen, Germany
Dithio-DL-threitol	Carl Roth, Karlsruhe, Germany
Dual colour molecular weight marker	Bio-Rad, Munich, Germany
Ethanol	Carl Roth, Karlsruhe, Germany
Europium chloride	Sigma-Aldrich, Taufkirchen, Germany
Folin-Ciocalteu's phenol reagent (FCP)	Sigma Aldrich, Taufkirchen, Germany
Fountain pen ink	Pelikan, Hannover, Germany
Glycerol	Carl Roth, Karlsruhe, Germany
Glycine	Applichem, Darmstadt, Germany
Holmium chloride	Acros-Organics BVBA, Geel, Belgium
Hydrogen chloride	Merck, Darmstadt, Germany
Hydrogen peroxide	Sigma Aldrich, Taufkirchen, Germany
Immobilon membrane	Millipore, Schwalbach, Germany
Iodo-beads	Perbio Science, Niederkassel, Germany
Isonicotinic acid hydrazide (isoniazid)	Sigma-Aldrich, Taufkirchen, Germany
Long epidermal growth factor	IBT, Reutlingen, Germany
Lysozyme	Sigma Aldrich, Taufkirchen, Germany
Methanol	Carl Roth, Karlsruhe, Germany
Milk powder	GE, Munich, Germany
N,N,N',N'-tetramethylethylene diamine	Riedel-de Haen, Seelze, Germany
N'N'-bis-methylene-acrylamide	Merck, Darmstadt, Germany
Nitric acid	Merck, Darmstadt, Germany
Nitro cellulose membrane	Schleicher and Schuell, Dassel, Germany
PD-10 column	GE Healthcare, Munich, Germany
Pencillin/streptomycin	Invitrogen, Karlsruhe, Germany
Pepsin	Dunn Labortechnik, Asbach, Germany

Peroxide coupled secondary goat anti-mouse IgG	Sigma Aldrich, Taufkirchen, Germany
Phosphoric acid	Sigma-Aldrich, Taufkirchen, Germany
Potassium hydrogen phosphate	Merck, Darmstadt, Germany
Precast CleanGels	ETC, Kirchentellinsfurt, Germany
p-SCN-Bn-DOTA (2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic	Macrocyclics, Dallas,USA
Rhodium and cerium	VWR International, Langenfeld, Germany
Roti-Blue	Carl Roth, Karlsruhe, Germany
RPMI 1640	Invitrogen, Karlsruhe, Germany
Sodium azide	Serva, Heidelberg, Germany
Sodium carbonate	Merck, Darmstadt, Germany
Sodium chloride	Merck, Darmstadt, Germany
Sodium Dodecyl Sulphate	Applichem, Darmstadt, Germany
Sodium hydrogen carbonate	Merck, Darmstadt, Germany
Sodium iodide	Fluka, Buchs, Switzerland
TEMED	Riedel de Haën AG, Seelze, Germany
Terbium chloride	Acros-Organics BVBA, Geel, Belgium
Tris (hydroxymethyl) amino-methane (Tris) hydrochloride	Applichem, Darmstadt, Germany
Tris-HCl (Tris(hydroxymethyl)-aminomethane)	Applichem, Darmstadt, Germany
TritonX	Acros-Organics BVBA, Geel, Belgium
Tween 20	Carl Roth, Karlsruhe, Germany
ultrapure water	Synergy and Milli-Q water purification systems, Millipore, Bedford, MA
Western Lightning Chemiluminescence Reagent Plus	Perkin Elmer, Waltham, USA
α-casein, β-casein, ovalbumin	Sigma Aldrich, Taufkirchen, Germany

Publications

1. A. Venkatachalam, C.U. Köhler, I. Feldmann, P. Lampen, A. Manz, P. H. Roos and N. Jakubowski: Detection of phosphorylated proteins blotted onto membranes using laser ablation inductively coupled plasma mass spectrometry, Part I: Optimization of a calibration procedure, *J. Anal. At. Spectrom.,* 2007, 22, 1023-1032.

2. A. Venkatachalam, C.U. Köhler, I. Feldmann, A. Dörrenhaus, A. Manz, P. H. Roos and N. Jakubowski: Detection of phosphorylated proteins blotted onto membranes using laser ablation inductively coupled plasma mass spectrometry, Part II: Influence of hormonal and stress stimuli on protein phosphorylation in the human urothelial carcinoma cell line 5637, *submitted to J. Anal. At. Spectrom.*(under review)

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4. P. H. Roos, A. Venkatachalam, C.U. Köhler, L. Wäntig, A. Manz, and N. Jakubowski: Detection of electrophoretically separated cytochromes P450 by element-labelled monoclonal antibodies via laser ablation inductively coupled plasma mass spectrometry, *Anal. Bioanal. Chem.*, 392, 1135-1147.

Monographs

1. A. Venkatachalam, C.U. Köhler, I. Feldmann, J. Messerschmidt, A. Manz, N. Jakubowski and P.H. Roos: Multiplexed probing of cytochromes P450 using Inductively Coupled Plasma Mass spectrometry (ICP-MS), *Naunyn-Schmiedeberg's Arch. Pharmacol.,* 375 (Suppl 1), Abstr. No. 460 (2007).

2. A. Venkatachalam, C.U. Köhler, I. Feldmann, A. Manz, N. Jakubowski and P. H. Roos: Quantification of protein modifications by use of elemental tags and laser ablation ICP-MS, *Naunyn-Schmiedeberg's Arch. Pharmacol.* 372 (Suppl 1), Abstr. No. 464 (2006).

Presentations

1. A. Venkatachalam, J. Messerschmidt, C.U. Köhler, I. Feldmann, A. Manz, P. H. Roos and N. Jakubowski, "Multiplexed probing of cytochromes P450 using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS)"; **Poster** presentation at 2008 Winter Plasma Conference, 7-14-January-2008; Temecula, California, U.S.A.

2. A. Venkatachalam, J. Messerschmidt, C.U. Köhler, I. Feldmann, A. Manz, P. H. Roos and N. Jakubowski, "Multiplexed probing of cytochromes P450 using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS)"; **Poster** presentation at 2007 Tracespec Conference, 11th Workshop on progress in Analytical Methodologies for Trace metal speciation, 4-7-September-2007; Münster, Germany.

3. A. Venkatachalam, A. Dörrenhaus, C.U. Köhler, I. Feldmann, A. Manz, P. H. Roos and N. Jakubowski, "Monitoring the cell signaling events using ICP-MS"; **Oral** presentation at 2007 European Winter Plasma Conference, 20-Feb-2007; Taormina, Italy.

4. A. Venkatachalam, C.U. Köhler, I. Feldmann, J. Messerschmidt, A. Manz, N. Jakubowski and P.H. Roos, "Multiplexed probing of cytochromes P450 using Inductively Coupled Plasma Mass spectrometry (ICP-MS)"; **Poster** presentation at 2007 Deutsche Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie (DGPT), 14-March-2007; Mainz, Germany.

5. A. Venkatachalam, C.U. Köhler, I. Feldmann, A. Manz, N. Jakubowski and P.H. Roos, "Improved quantification approaches for protein analysis"; **Poster** presentation at 2006 Kolloquium des Forschungsbandes Chemische Biologie und Biotechnologie, University of Dortmund, Dortmund, Germany.

6. A. Venkatachalam, C.U. Köhler, I. Feldmann, A. Manz, N. Jakubowski and P. H. Roos, "Quantification of protein modifications by use of elemental tags and laser ablation ICP-MS"; **Poster** presentation at 2006 DGPT, 5-April-2006; Mainz, Germany.

Curriculum Vitae

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