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„Elemental labeling of antibody fragments for LC-ICP-MS based immunoassays"

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ABBREVIATIONS

DNA   deoxyribonucleic acid

DOTA-Mal   2,2',2''-(10-(2-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-ethyl) amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid

ECAT   element coded affinity tag

ESI-MS   electrospray-ionization mass spectrometry

Fab   antigen binding fragment

FI   flow injection

ICAT   isotope coded affinity tag

ICP   inductively coupled plasma

ICPL   isotope coded protein label

iTRAQ   Isobaric tags for relative and absolute quantitation

HER2   human epidermal growth factor receptor 2

HPLC   high performance liquid chromatography

HSA   human serum albumin

LC   liquid chromatography

LOD   limit of detection

LOQ   limit of quantification

MALDI-MS   matrix-assisted-laser-desorption-ionization mass spectrometry

MeCAT   metal coded affinity tag

MS   mass spectrometry

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEEK	polyetheretherketone
PFA	perfluoroalkoxy
Protein AQUA	protein absolute quantification
Q-MS	quadrupole mass spectrometry
SEC	size exclusion chromatography
SOD	superoxide dismutase
GSSG	glutathione oxidized
TCEP	tris(2-carboxyethyl)phosphine hydrochloride
Tris	tris(hydroxymethyl)aminomethane

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1. INTRODUCTION

1.1. QUANTIFICATION OF BIOMOLECULES AND ITS CHALLENGES

Biomolecules show a vast diversity in functionality, reactivity and structural complexity. Their comprehensive analysis is playing a crucial role in bio-chemical and medical sciences. Since the quantity or the change of quantity of biologically active molecules is significant regarding the status of a biological system, the global analysis of biomolecules does not only comprise the qualitative information but also calls for quantitative data.

In this context especially proteins are of high interest. The scientific field of proteomics represents the study of the proteins' entirety (the proteome) of a cell or organism (Pandey A and Mann M 2000). Herein, modification and expression patterns as well as their functionality and interaction in metabolic pathways are studied. Quantitative proteome profiling is applied for the functional analysis of biological systems as well as for the identification and detection of clinical, diagnostic or prognostic marker proteins (Zhang H, Yan W and Aebersold R 2004).

However, the quantitative analysis of proteins has to tackle numerous difficulties (Bettmer J et al. 2009): On the one hand, this is due to their different physico-chemical behavior leading to large variations in solubility, stability or isoelectric point. In addition, there is a vast possibility of posttranslational modification. On the other hand, this situation is derived from the complex matrix present in the biological sample as well as the wide dynamic range of possible protein expression extending from one copy to more than a million copies per cell.

All of these challenges taken together indicate that the determination of a reliable and accurate amount of proteins in a biological sample matrix is close to impossible (Zhang H et al. 2004). However, to address this issue innovative quantification strategies are necessary. The large dynamic range asks for suitable detection strategies with similar dynamic ranges (Linscheid MW 2005). In addition, in case of single cell analysis, where the total protein content is approximately 50 picogram, highly sensitive detection is indispensable (Linscheid MW 2005).

For quantitative analysis of proteins several analytical methods are employed, where mass spectrometry (MS) coupled to highly selective separation techniques like liquid chromatography (LC) or capillary electrophoresis, is one of the core techniques (Wu Q et al. 2012). Since, the availability of standards is a pre-requisite for successful quantification of target compounds, inductively coupled plasma mass spectrometry (ICP-MS) showed to be advantageous, as species unspecific calibration can be employed (Szpunar J 2005). In point of fact, ICP-MS based analysis provides elemental instead of molecular information, therefore the combination of molecular and elemental mass spectrometry showed to be beneficial in circumventing restrictions of each technique applied alone (Szpunar J and Lobinski R 2002, Wind M and Lehmann WD 2004, Hann S et al. 2004). Especially the improvements in the detection of sulfur and phosphorus in biological samples (Bandura DR, Baranov V and Tanner SD 2002) paved the way for expanding the tool box for protein analysis. Hence, ICP-MS is not restricted anymore to the field of bioinorganic chemistry measuring only molecules, which ligate or incorporate a metal atom, but is also applied to biomolecules (Baranov VI et al. 2002b).

In general quantification can be performed either on a relative or on an absolute basis. Principally the quantification strategies can be classified into label and label-free methods (Wu Q et al. 2012). Labeling procedures have become an important application for quantitative approaches for protein analysis. They have been introduced into the field of proteomics with the intention either to isolate the analyte from the complex matrix or to improve the protein detection itself (Sanz-Medel A et al. 2008). With the advent of mass spectrometry, gel separation using densitometry or radiolabelling was nearly replaced by the introduction of sophisticated labeling techniques (Lottspeich F and Engels JW 2012). In the following some general aspects of labeling strategies and selected examples will be discussed.

1.2. LABELING OF BIOMOLECULES

The term "labeling" describes a chemical reaction between the analyte and a suitable reagent with formation of a derivate which allows and/ or enhance detection of a biomolecule or a bio-molecular interaction (Hempen C and Karst U 2006).

There are two basic approaches for labeling of biomolecules (Kretschy D, Koellensperger G and Hann S 2012): On the one hand pre-labeling of the target compound can be employed by derivatizing the biomolecule prior to insertion into a biological system and in doing so altering the chemical properties of the analyte. On the other hand post-labeling/ indirect labeling can be applied, where the analyte is labeled in the course of an analytical procedure.

Pre-labeling enables tracking a biomolecule in a biological system (Kretschy D et al. 2012). This strategy has though the major drawback that the labeling may have an impact on the functionality of the biomolecule. Post-labeling, also referred to indirect approach, presents generally a two-step procedure, where first an antibody against a biomarker in a biological system is labeled and subsequently the analyte is detected indirectly *via* immunological reaction system (Kretschy D et al. 2012). Post-labeling strategies are also employed for imaging techniques with fluorescence based markers (Ruedas-Rama MJ et al. 2012). Applying the latter strategy, it is especially important that the labeling procedure does not affect the antibody's functionality (Jakubowski N et al. 2008, Giesen C et al. 2011).

The ideal labeling procedure for quantitative analysis should generate homogenous, well-characterized and completely labeled biomolecules with a well-controlled labeling reaction (Kretschy D et al. 2012). It should not influence the biomolecule functionality in case of pre-labeling or the reactivity of the antibody in case of indirect labeling. Furthermore analytical figures of merit should not be affected negatively and multiplexing should be feasible. Besides the labeling method should allow a less tedious sample preparation and be applicable to every kind of sample (Kretschy D et al. 2012). It has to be pointed out that not all of these characteristics can be fulfilled. Hence, labeling may not provide the ultimate solution to the problems facing when quantitative analysis is strived. Nevertheless, it can be considered as a powerful tool.

1.2.1. Labeling strategies

Numerous different techniques have been developed for the controlled labeling of biomolecules. In general the concept of labeling is commonly employed in bio-medical sciences utilizing fluorescent (Ruedas-Rama MJ et al. 2012), chemiluminescent (Dodeigne C, Thunus L and Lejeune R 2000), radioactive compounds (Coates JE, Lam SF and McGaw WT 1988) or stable isotope as well as elements (Prange A and Pröfrock D 2008). The latter two will be discussed in further detail.

Labeling can be pursued either metabolically, enzymatically or *via* chemical reactions, where reagents target selected functional sites of the different protein species (Prange A and Pröfrock D 2008). In the following the most common strategies will be shortly presented.

In case of metabolic labeling, the labeling takes place *in vivo* and found wide application for relative quantification of protein expression in differently treated cell culture systems (Prange A and Pröfrock D 2008). There are two mainly used methods: In case of the stable isotope labeling by amino acids in cell culture (SILAC) method, selected isotopically-modified amino acids are added to an amino acid deficient cell culture media (Ong SE et al. 2002). These amino acids are subsequently metabolized and incorporated into all proteins synthesized by the cells. For the other method the organisms of interest are grown in media enriched in ^{15}N, as only nitrogen source (Krijgsveld J et al. 2003).

Another approach to introduce stable isotopes into peptides is to perform enzymatic digestion of the sample with $H_2^{18}O$, while the control sample is digested in normal $H_2^{16}O$. This leads to the introduction of up to two ^{18}O into the C-terminal of the carboxylic group formed during digestion (Mirgorodskaya OA et al. 2000).

Depending on the amino acid composition, proteins and peptides carry different functional groups, which can be derivatized (Prange A and Pröfrock D 2008). In this context primary amino groups as well as sulfhydryl groups represent suitable targets

for labeling. The primary amino groups, found at the N-terminus of proteins and peptides and in lysine residues, readily react with succinimide or isothiocyanate. While sulfhydryl groups, found in cysteine residues, are targeted by maleimide or iodoacetamide as possible linkers to form a covalent bound. Depending on the reactive linker employed for the derivatisation reaction different limitations may arise. The applicability of isothiocyanate for example is limited as a basic pH value during reaction is necessary, which may have a negative effect on stability and functionality of the biomolecule (Brinkley M 1992). In case of targeting free thiol groups, in most cases a prior reduction step is required as cysteins are prone to form disulfide bridges. This reduction step may result in a negative impact on the recovery and bio-molecule integrity caused by generating fragments and by-products (Waentig L, Jakubowski N and Roos PH 2011).

Labeling by chemical reaction can be classified into two main approaches either selected amino acid residues are derivatized using isotopically (^{2}H, ^{13}C, ^{15}N, ^{18}O) modified reagents, or they are labeled employing bi-functional chelating agents and certain elements as mass markers. The latter is also known as elemental labeling.

Prominent examples for labeling using isotopically modified reagents are among others isotope-coded affinity tag (ICAT), isotope coded protein label (ICPL), isobaric tag for relative and absolute quantification (iTRAQ) and protein absolute quantification (Protein AQUA) (Prange A and Pröfrock D 2008). The ICAT reagent consists of three components, including an affinity tag (biotin), which is used to isolate ICAT-labeled peptides, a linker that incorporate stable isotopes and a reactive group with specificity toward sulfhydrylgroups (Gygi SP et al. 1999). For labeling *via* ICPL ^{12}C and ^{13}C containing N-nicotinoyloxy-succinimide are employed to target primary amine groups (Schmidt A, Kellermann J and Lottspeich F 2005). In comparison to the ICAT and ICPL, iTRAQ allows a multiplex analysis of up to four samples. The four chemically identical iTRAQ labels consist of a reporter group (N-methylpiperazine moiety), a balancer group (carbonyl moiety) and a reactive group targeting all primary amines (N- nicotinoyloxy-succinimide) (Ross RL et al. 2004). Protein AQUA strategy is actually a variation of isotope dilution mass spectrometry technique and applies a synthetic stable isotope labeled peptide analogue as internal standard. These correspond to selected native peptides formed during enzymatic digestion of the investigated sample (Gerber SA et al. 2003).

For the labeling approaches so far presented, molecular mass spectrometry, either electrospray-ionization-MS (ESI-MS) or matrix-assisted-laser-desorption-ionization MS (MALDI-MS), is employed for detection. However, when employing bifunctional chelating agents and elements as marker, elemental mass spectrometry, namely ICP-MS, is employed.

1.2.2. Elemental labeling

The use of chelating compounds in combination with radioactive tracers was a very common tool for the analysis of proteins and for medical science (Bomke S, Sperling M and Karst U 2010). Especially bi-functional chelating agents are frequently used for various medical application, such as radiopharmaceuticals and contrast improving agents (Jakubowski N et al. 2008). These bi-functional chelators possess a reactive group/ linker for covalent binding to bio-targeting vectors, like antibodies or generally proteins (Waentig L et al. 2012). On account of the development in elemental mass spectrometry chelating compounds with different incorporated metals became applicable for the detection of biomolecules (Bomke S et al. 2010). Beside of the stability against time, temperature and light of elemental label e.g. compared to fluorescence based ones, the advantage is the higher spectral resolution compared to optical methods and therefore the possibility to extend multiplexing (Becker S and Jakubowski N 2009). As a result, they have been also employed in bioanalytical assays (Hempen C and Karst U 2006).

The concept of elemental labeling of bio-molecules by means of attachment of an elemental label either to the target compound directly or to a detection antibody is an emerging field, with the first application in 2002, where gold labels were employed (Quinn ZA et al. 2002, Baranov VI et al. 2002a). Other applications of elemental labeling followed like the element coded affinity tag (ECAT) (Whetstone PA et al. 2004), the metal coded affinity tag (MeCAT) (Krause M et al. 2005) or other labeling attempts (Konz T, Montes-Bayón M and Sanz-Medel A 2012) with flow injections (FI) or LC-MS based analysis of proteins and peptides. MeCAT® and ECAT reagents are based on the macrocycle 1,4,7,10- tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and use lanthanides as chelate ions. Additionally to the functionalization by a maleimid group which targets a cysteine residue, the label features a further position,

which can be modified with an functional group enabling affinity purification and enrichment (Krause M et al. 2005, Whetstone PA et al. 2004).

In general elemental labeling comprises two steps (Kretschy D et al. 2012): First, the elemental label has to be formed by coordination of an element to the internal cavity of a chelating moiety. In the second step the elemental label is attached to the biomolecule. These two steps can be also performed *vice versa*, so that prior to the complexation the functional group of the chelating agent reacts with a targeting group on biomolecules.

Within the coordination step various kinds of chelating moieties can be employed. They are classified into linear chelators, such as diethylenetriaminetetraacetic acid (DTTA) or diethylenetriaminepentaacetic acid (DTPA) (Waentig L et al. 2012), and macrocycles, like DOTA or 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) (Kretschy D, Koellensperger G and Hann S 2011b). A prerequisite for the employed element is that it should not be present in the investigated sample. Therefore many different elements can be used as chelate ions, such as gold (Müller SD et al. 2005), silver (Liu ML and Yan XP 2011), indium (Kretschy D et al. 2011a), gallium (Kubicek V et al. 2010) or lanthanides (Waentig L et al. 2011). In this context especially macrocycles, like DOTA, form strong chelate complexes with trivalent metals, such as lanthanides (Whetstone PA et al. 2004, Ahrends R et al. 2007, Ahrends R et al. 2009). As a result of the similar chemical nature and their low natural abundance in biological samples, lanthanides are particularly suitable for multiplexed labeling of biomolecules (Kretschy D et al. 2011b).

Parameters influencing the stability of the elemental label are e.g. pH value, ionic strength, concentration and temperature, as well as the geometry of the chelator's internal cavity and the ionic radius of the element (Martell A and Hancock R 1996, Kretschy D et al. 2011b). For a reliable application of the elemental label the determination of complex stability is crucial. Kretschy *et al.* assessed the stability of DOTA, NOTA and DTPA under acidic conditions necessary for HPLC separation, where La^{3+}-DOTA showed the highest log K value (Kretschy D et al. 2011b).

In order to further improve the detection sensitivity and detection limit of elemental labeled biomolecules, especially of antibodies, simple signal amplification by attaching more than one label per biomolecule can be achieved. The two main

approaches make use of either multilabel-polymers (Lou X et al. 2007) or metal nanoparticles (Baranov VI et al. 2002a, Quinn ZA et al. 2002, Müller SD et al. 2005).

The detection of elemental labeled compounds is predominantly pursued *via* inorganic mass spectrometry with ICP-MS as main application. This is attributable to its high elemental selectivity and sensitivity as well as its matrix tolerance (Nelms S 2005). Additionally species unspecific calibration (Szpunar J 2005) and isotope dilution analysis is feasible. Besides ICP-MS shows great potential for multiplexed experiments (Tanner SD et al. 2007, Terenghi M et al. 2009, Waentig L et al. 2011). Further details on this technique, including an example for combination, will be discussed in chapter 1.3.

In order to unfold the full potential of accurate quantification employing elemental labeling combined with ICP-MS detection, the labeling output has to be characterized (Kretschy D et al. 2012). This quality control comprises the determination of labeling sites and yield, bio-molecule functionality and stoichiometry. If applying pre-concentration strategies like nanoparticles, yet again quality control in terms of size control of the nanoparticle as well as stoichiometry of protein to nanoparticle has to be assessed (Bettmer J, Jakubowski N and Prange A 2006). These steps are critical for further applications of e.g. elemental labeled antibodies in an immunoassay for straight-forward quantification of a biomarker in biological samples.

1.2.3. Immunoassay based on ICP-MS detection

The combination of the analytical characteristics of ICP-MS with the specificity of immunoreaction offers a new approach for the challenging task of quantification of biomolecules on an absolute basis.

The term "immunoassay" describes an affinity assay based on the biomolecular interaction of an antigen with a corresponding antibody (Quinn ZA et al. 2002). The strong affinity of antigen-antibody interactions is mainly based on non-covalent bonds and conformational fit. The high selectivity and reactivity of the antibody-antigen recognition is utilized for various biotechniques including: separation and purification steps, imaging techniques aiming at the visualization of specific cellular proteins and structures through labeled antibodies and immunoassays for quantitation.

Immunoassays find widespread application in several fields, such as drug analysis, food analysis, environmental chemistry and clinical chemistry (Hempen C and Karst U 2006). Predominantly immunoassays rely on indirect approaches based on post-labeling strategies. Though different labels can be applied (fluorophores, radioisotopes or enzymes), the main focus of the present work was put on element labeling. In comparison to other labeling strategies, the application of elemental labels allows the direct analysis of the element (Baranov VI et al. 2002b). In addition impurities have generally less impact, as they normally do not contain the target element.

For elemental labeling of antibodies often chelators, which feature a cysteine reactive maleimide group are applied (Terenghi M et al. 2009, Waentig L et al. 2012). This is because maleimid groups employed as linkers yield to high labeling degrees and additionally they are more selective than e.g. iodoacetamides, as they do not react with histidine or methionine (Brinkley M 1992). However, the limitation to this linking reaction is the necessary reduction of the antibody prior to labeling in order to generate free sulfhydryl groups on cysteine residues. Consequently this reduction leads to a fragmentation of the antibody. As shown in Figure 1, there may be various disulfide bridges ready to be reduced. But since the labeled antibody still shows antigen selectivity in the immune reaction, it is assumed that preferentially the Hinge region of the antibody is reduced (Waentig L et al. 2012, Liu H and May K 2012, Ornatsky OI et al. 2008).

A possible way to overcome this uncertainty of fragmentation and being independent of a harsh reduction step is the use of a genetically engineered antigen binding fragment (Fab) instead of a complete antibody. In Figure 1 the Fab is highlighted. To this Fab an additional cysteine group can be introduced by point mutation (Junutula JR et al. 2008). Due to this introduced cysteine it is not necessary to reduce disulfide bridges of the protein for labeling and therefore a straight-forward and stoichiometric labeling is realizable.

Figure 1: Scheme of an immuneglobin G antibody structure presenting inter- and intrachain disulfide bridges
modified from (Thermo Scientific Pierce Protein Biology Products)

The first report on employing elemental labels for immunoreaction was published by Zhang *et al* (Zhang C et al. 2001). They described a quantitative method for determination of thyroid stimulating hormone in human serum employing europium labeled antibodies in an immune reaction and ICP-MS detection. Based on the work of Zhang *et al.* several concepts and application have been established for the multiplexed protein determination *via* immunoreaction.

Immunoassays with ICP-MS based detection can be combined manifold: Careri *et al.* developed a flow injection ICP-MS method for detection of peanut allergens in food (Careri M et al. 2007). Besides elemental labels were applied in protein analysis using laser ablation ICP-MS in combination with blotting procedures (Waentig L et al. 2011), or in imaging experiments for detection of tumor markers in breast cancer tissue (Giesen C et al. 2011) and for research related to neurodegenerative diseases in brain tissue (Becker JS et al. 2010).

Another trend address the design of liquid based immunoassays combined with LC-ICP-MS detection. Herein, the key advantage can be seen in overcoming the problems arising in quantitative analysis by means of Western blots and laser ablation ICP-MS (e.g. losses during blotting procedures, time consuming sample separation and laser ablation process or the quantification problems of laser ablation

technology) (Hann S, Boeck K and Koellensperger G 2010). Furthermore the formation of the antibody-antigen conjugate is kinetically favored in solution. As a result the analysis time is significantly shortened. Therefore liquid chromatography-ICP-MS allows the development of a high-throughput system.

The proof of principle study has been published by Terenghi *et al.*, who employed metal labeled antibodies for multiplexed determination of proteins (Terenghi M et al. 2009). Besides Hann *et al.* developed a solution based immunoassay using indium labeled antibodies and LC-ICP-MS detection for quantification of an anti-inflammatory candidate drug in cellular samples (Hann S et al. 2010). Both approaches made use of size exclusion chromatography as separation technique.

The possibility of multiplexed analysis was demonstrated e.g. by Giesen *et al.* employing labeled antibodies in imaging experiments using laser ablation–ICP-MS detection (Giesen C et al. 2011) and could be also proved by Terenghi *et al.*, using LC-ICP-MS detection (Terenghi M et al. 2009). Though, maybe the most prominent example for multiplexed application is the concept of flow cytometry with ICP-MS detection published by Tanner *et al.* (Tanner SD et al. 2007).

As quantification of biomolecules calls for accuracy, sensitivity and high selectivity, liquid chromatography coupled to ICP-MS provides a straight-forward solution, by separating elemental labeled antigen-antibody conjugate in a liquid based immunoassay.

1.3. INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY

The analysis *via* ICP-MS started in the early 1980's, where ICP-MS detection was developed primarily for liquid samples. Today the field of application has extended to the analysis of gaseous as well as solid samples (Thomas R 2001b). ICP-MS, as a highly sensitive (pg L^{-1} – fg L^{-1}) (Aamann AA 2007) and isotope specific detector for nearly all elements present in the periodic table (Houk RS et al. 1980), is nowadays routinely employed in various research fields, such as geology, nuclear and life science (Aamann AA 2007). Using ICP-MS, rapid multi-element analysis is possible by means of quasi-simultaneously detection of analytes in a very short time (Nelms S 2005, Bettmer J 2010, Kretschy D et al. 2012). Hence, this technique shows great potential of multiplexed application (Tanner SD et al. 2007). Other advantages are the wide linear dynamic range with up to nine orders of magnitude and the applicability of isotope dilution mass spectrometry (IDMS) strategies, allowing species specific and unspecific calibration and therefore addressing the problem of suitable commercially available standards (Nelms S 2005, Bettmer J 2010, Kretschy D et al. 2012). Due to the harsh conditions of generating ions, ICP-MS is generally less prone to matrix effects (Aamann AA 2007). Particularly the possibility of combination to different separation techniques, like liquid chromatography or capillary electrophoresis makes ICP-MS a versatile tool for quantitative bio-analysis (Kretschy D et al. 2012).

As a basic principle of ICP-MS detection, high-temperature plasma discharge is employed to generate positively charged ions. Roughly outlined, a liquid sample is first transferred into the sample introduction system, consisting of a spray chamber and a nebulizer. Emerging as an aerosol, it is led to the base of the argon plasma *via* the sample injector. In doing so, the sample changes its state of matter by passing different heating zones of the plasma torch, being dried, vaporized, atomized and finally ionized. In the analytical zone of the plasma, where temperatures around 6000- 7000 K are achieved, the sample only exists as excited atoms and ions. In the following the ions are transferred into the mass analyzer, separated according to their mass to charge ratio and finally detected by an appropriate detector (e.g. secondary electron multiplier, Faraday cup, photoplate or ioncounter) (Nelms S 2005, Thomas R 2001a).
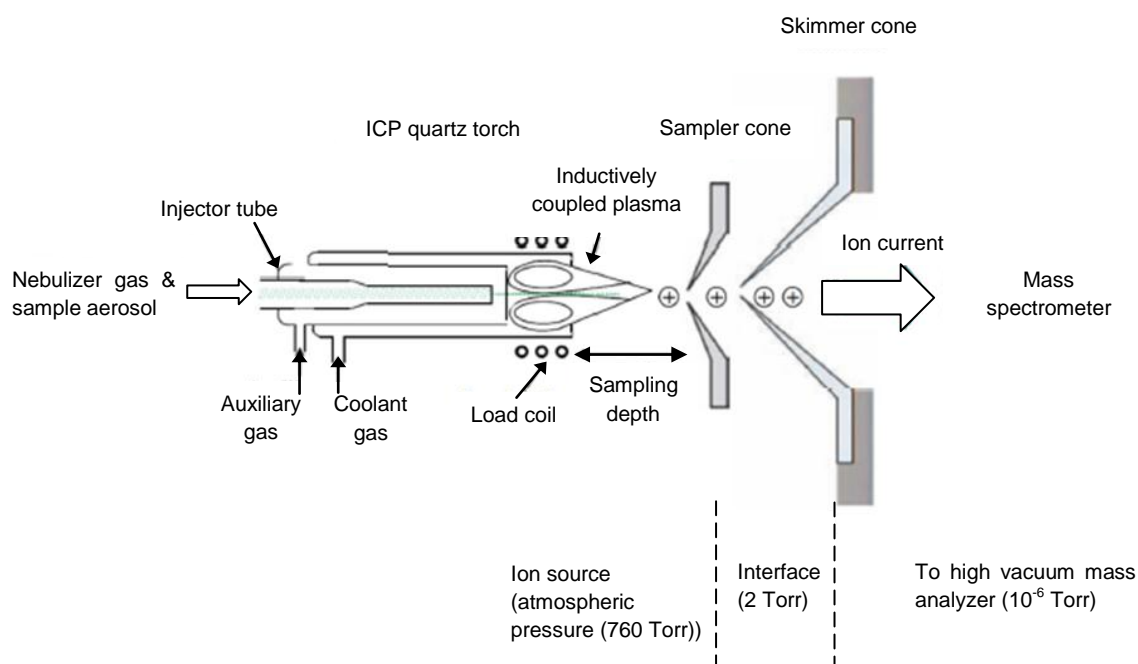
Figure 2: Schematic diagram of an interface region of an inductively coupled plasma mass spectrometer
modified from (Becker S 2007)

Figure 2 shows a schematic diagram of an interface region of an ICP-MS. In the following the sample introduction system, the plasma source, the mass analyzer as well as the detector will be discussed. Additionally a short overview to liquid chromatography will be given.

1.3.1. The sample introduction system

Generally a sample introduction system consists of a spray chamber and a nebulizer. It is followed by a plasma torch comprising three concentric tubes with different diameters, for the carrier, the auxiliary and the coolant gas (Thomas R 2001c). Between the outer and the middle tubes, the coolant gas is employed to form the plasma and to prevent overheating. The auxiliary gas is used to change the position of the base of the plasma relative to the tube and the injector and to isolate the plasma from the injector tube. The third gas, the carrier gas, transfers the sample aerosol from the sample introduction system to the plasma.

The main purpose of the sample introduction system is to generate a fine aerosol of the sample (Thomas R 2001b). Sample introduction in ICP-MS can be seen as two separated events, where a nebulizer is generating an aerosol and a spray chamber is selecting droplets (Thomas R 2001b). Depending on the combined system, there are different ways for sample introduction, though all resulting in a fine droplet aerosol suitable for being ionized in the plasma (Thomas R 2001b). For liquid samples either continuous flow aspiration or the combination of separation techniques like liquid chromatography (LC) or capillary electrophoresis (CE) to ICP-MS, can be employed. Apart from liquid also gas chromatography can be coupled to ICP-MS. Solid samples are often analyzed using laser ablation (LA) (Nelms S 2005).

In the following the introduction systems for liquid samples will be discussed in further detail, as in the course of this master thesis only this type of samples was analyzed.

Aerosol generation is achieved *via* a nebulizer (e.g. concentric or cross-flow design) combined with a spray chamber (e.g. cyclonic or double pass design) (Thomas R 2001b). The liquid sample is pumped into the nebulizer either *via* a peristaltic pump or sucked into the nebulizer in case of a pneumatic device (concentric nebulizer). When the liquid enters the nebulizer, it is broken up into a fine aerosol by being exposed to a gas flow smashing the liquid into an aerosol consisting of larger and smaller droplets. Since the plasma discharge is ineffective regarding ionization of elements present in larger droplets, the spray chamber's main task is selecting only droplets in a size range approximately below 10 µm (depending on the design of the respective spray chamber) and transferring them into the plasma. The larger droplets are separated by gravity and exit through the drain tube at the bottom of the spray chamber. Beside of the droplet selection, the spray chamber damps pulses occurring during the nebulization process due to the peristaltic pump for example.

Since only 1- 2% of the sample are transferred into the plasma, the sample introduction system is considered as the component, which has the highest impact on transmission efficiency of the entire ICP-MS system. (Browner RA and Boorn AW 1984).

In the following, the selected droplets are ionized in the plasma.

1.3.2. The plasma source

The basic components of an ICP ionization source are a plasma torch out of quartz glass and a radio frequency (RF) coil, usually out of copper, located around the quartz torch connected to a RF power supply (Thomas R 2001c).

By applying a RF power to the coil, an alternating current oscillates within the coil at a rate corresponding to the frequency of the generator (Thomas R 2001c). This oscillation leads to an intense electromagnetic field in the area at the top of the torch. As argon gas is flowing through the torch, a high-voltage spark is applied to the gas, causing some electrons to be stripped off the argon atoms. These electrons are accelerated in the magnetic field and collide with other argon atoms, stripping off more electrons. This collision induced ionization leads to a chain reaction breaking down the gas into argon atoms, ions and electrons, creating thereby the inductively coupled plasma discharge.

This ICP discharge is preserved within the torch and load coil, as RF energy is persistently transferred to it through the inductively coupled process (Thomas R 2001c).

After the sample aerosol traveled through the torch and gets during its way dried, vaporized, atomized and finally ionized. It is transferred *via* the interface region into the mass analyzer, where separation by mass to charge ratio takes place.

1.3.3. The mass spectrometer

Prior to the mass analyzer an interface region and ion optics are put in front. The interface transports the ions efficiently, consistently and with electrical integrity from the plasma, which is at atmospheric pressure (760 Torr), to the mass analyzer (at approximately 10^{-6} Torr) (Thomas R 2001d).

The interface region, where a pre-vacuum of approximately 2 Torr exists, comprises two metallic cones with small orifices, the sampler and the skimmer cone. After the sample is ionized in the plasma, it first passes the sampler cone, followed by the skimmer cone, which has in contrast to the first cone a much smaller orifice. Subsequently the ions are focused in the ion optics. The ion focusing system

consisting of one or more electrostatically controlled lens components, transfers the maximum number of analyte ions from the interface region to the mass analyzer and at the same time rejecting as many of the matrix components and non-analyte based species as possible (Thomas R 2001e).

Predominantly quadrupole (Q), magnetic sector field and time of flight mass analyzers are employed for mass to charge (m/z) separation in ICP-MS (Thomas R 2001f). Besides, combinations of magnetic and electric sector field mass analyzers can be used. As in the course of the master thesis ICP-QMS were employed, only this mass analyzer will be shortly described.

The quadrupole mass filter is the most common mass analyzer employed for ICP-MS, consisting of four cylindrical or hyperbolic metallic rods of the same length and diameter (Thomas R 2001f). The main principle behind this mass analyzer is that a certain direct current (DC) field is placed on one pair of rods and a radio frequency field on the opposite pair. Due to that only ions of selected mass to charge ratio are deflected resulting in a stable trajectory, which allows them to pass through the rods to the detector. The other ions with different mass to charge ratios are ejected from the quadrupole. As the RF-DC voltage for a specific m/z ratio is repeatedly scanned, the ions are stored as electrical pulses and counted by a multichannel data acquisition system (Thomas R 2001f). The quadrupole is employed as a mass scanning device, as the described process is repeated for other m/z ratios applying different RF-DC voltages.

When employing a quadrupole as mass analyzer, the detection capability for certain elements is compromised due to the formation of polyatomic spectral interferences caused by either argon, solvent or sample based ionic species (Thomas R 2002, Nelms S 2005). To overcome this problem different approaches exist, one of them is the collision reaction cell technology. In this case a collision or reaction gas is introduced into a cell, which consists of a multipole operated usually in the RF-only mode (Thomas R 2002). The applied RF-only mode focuses the ions, which consequently collide or react with molecules of the present reaction/ collision gas. In order to either convert the interference ions to non-interfering species or by converting the analyte ions to other ions, which are not interfered, different ion-molecule collision and reaction mechanism take place (Sakata K and Kawabata K 1994). Within this process secondary reactions occur forming many other undesired

interfering species, which need to be eliminated or rejected (Thomas R 2002). For efficient removal two approaches exist: either discrimination by kinetic energy or by a mass shift or interference elimination *via* gas phase reaction can be employed. The principle of kinetic energy discrimination is based on the loss of kinetic energy when ions collide. So when placing a potential barrier downstream of the cell, only ions with higher kinetic energy (usually the analyte ion) can overcome this barrier. Ions with lower kinetic energy (usually the polyatomic interference ion) are hence discriminated. The second approach employs gas phase reactions utilizing highly reactive gases, which tend to be more efficient at interference reduction. They can be employed and chosen according to the reactivity of the analyte and the interference ions with the gas. Possible reaction gases are ammonia, methane, oxygen or hydrogen, whereas for collision gas helium or xenon is used (Thomas R 2002). In this context, a method for detection of ultra-trace sulfur and phosphorus with the DRC technology employing oxygen as reaction gas, was described by Bandura *et al.* (Bandura DR et al. 2002) and led to low detection limits due to the reduced polyatomic isobaric interferences, when detecting the two atoms as $^{31}P^{16}O^+$ at m/z 47 and $^{32}S^{16}O^+$ at m/z 48, respectively.

1.3.4. Combination with liquid chromatography

High performance liquid chromatography (HPLC) is one of the key techniques for separation, including purification of all kinds of samples and as such has been established as the major technique for the analysis of peptides and proteins. Good reproducibility, high recoveries, extended variation in selectivity manipulation, among other benefits triggered the success of this technique in the last decade.

The chromatographic separation of the analytes present in a flowing mobile phase is based on the different strength of interaction with a stationary phase (Mikkelsen SR and Corton E 2004). These interactions depend on the molecular composition of the biomolecule's surface and together with other properties lead to different partition in mobile and stationary phase and consequently different retention times. Typically reversed phase chromatography (RPC), ion exchange chromatography (IEC), hydrophilic interaction chromatography (HIC) separate a mixture of biomolecules based on differences in surface charge or hydrophobicity, whereas size exclusion

chromatography (SEC) is employed to separate according to their molecular size. Additionally also affinity chromatography is applied in this field. As harsh elution conditions of RPC can influence the 3D structure of proteins, separation techniques like SEC or IEC are most commonly used for analysis on a native level (Lottspeich F and Engels JW 2012).

As in the present work SEC was the applied for separation, only the principles of this technique are discussed further.

Size exclusion chromatography is also known as gel permeation chromatography or gel filtration chromatography as the stationary phase consists of a gel media (Mikkelsen SR and Corton E 2004). This gel media is composed of spherical porous particles with controlled pore size, through which biomolecules can diffuse to different extent depending on the molecular size or more precisely the hydrodynamic radius. Small macromolecules can diffuse deeper into the pores and are temporarily retained, whereas larger molecules are too big and remain in the interstitial space and therefore elute earlier (Wu CS 1995). The fundamental principle of SEC is the separation in order of decreasing hydrodynamic radii. The separation conditions are selected to avoid both specific and non-specific adsorption (Mikkelsen SR and Corton E 2004).

As stationary phase the gels used for bio-analytical SEC need to have hydrophilic properties (Mikkelsen SR and Corton E 2004). Polysaccharides, such as dextran and agarose, as well as synthetic polymers like polyacrylamide are often used in this context (Mikkelsen SR and Corton E 2004) Besides, for higher pressure application, there is also the possibility of silica based media (Wu CS 1995).

Since the composition of the mobile phase is only weakly influencing the chromatographic selectivity of size exclusion based chromatographic techniques, the mobile phase may be chosen to suit the species being separated, for stability of the analytes (Mikkelsen SR and Corton E 2004).

One disadvantage is that the recoveries of this separation technique are quite low. Nevertheless, one main advantage of SEC is that it can be performed under native non-denaturing conditions.

2. STUDY DESIGN AND EXPERIMENTAL

2.1. STUDY DESIGN

The objective of this master thesis was the development of a labeling procedure for a genetically modified antigen binding fragment (Fab), so-called ThioFab (Junutula JR et al. 2008), with rare earth element label macrocycles and its analysis *via* liquid chromatography inductively coupled plasma mass spectrometry (LC-ICP-MS).

Compared to a complete antibody, a Fab shows similar antigen binding properties and its molecular weight is approximately one third. Most importantly, the use of this ThioFab, where an additional cysteine was introduced recombinantly, allows a straight-forward labeling with only a mild reduction step and therefore no fragmentation is obtained.

As a proof-of-concept the formation of the final ThioFab/ antigen conjugate employing Anti-HER2/ HER2 (human epidermal growth factor receptor 2) as model compound was demonstrated in a solution based approach using LC-ICP-MS.

In Figure 3 the workflow of the master thesis is presented (adapted from Kretschy (Kretschy D 2012)).
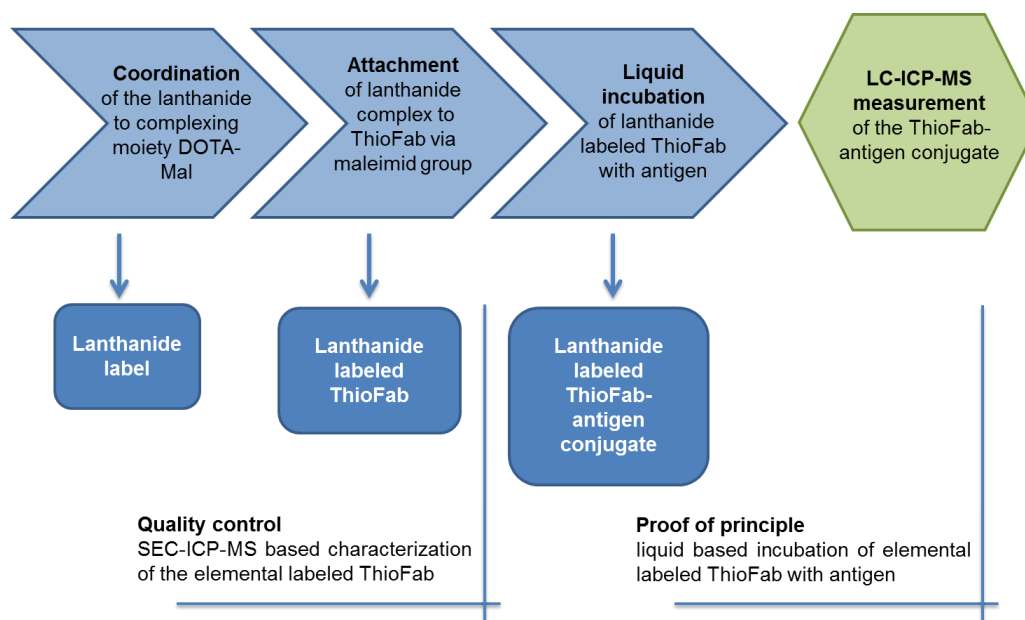

Figure 3: Workflow- liquid based immunoassay for quantification of biomolecules utilizing LC-ICP-MS detection

2.1.1. Labeling Procedure

The derivatisation of antibody fragments with elemental labels and their ICP-MS based detection offers the possibility for indirect quantification of various targets (e.g. cancer markers or cellular metabolites). This straight-forward technique is applicable in the clinical field for imaging *via* LA-ICP-MS (Giesen C et al. 2011) or for immunoassays *via* ICP-MS based absolute quantification of the targets in biological samples.

For the elemental labeling of biomolecules in bio-analytics and medicine, predominantly bi-functional chelators are used. In this context especially the macrocycle DOTA proved to be advantageous as it forms strong chelate complexes with trivalent metals, such as lanthanides. (Whetstone PA et al. 2004, Ahrends R et al. 2007, Ahrends R et al. 2009)

As depicted in Figure 3, the elemental labeling is generally a two-step procedure, where in the first step the lanthanide label is formed by coordinating the lanthanide to the chelating moiety DOTA, which was employed in the course of this master thesis. The second step comprises the attachment of the label to the ThioFab *via* a linker. As the employed ThioFab carries a free sulfhydryl residue the chelating moiety, DOTA, is functionalized with a maleimid group.

The major part of the present work dealt with the optimization of these two steps, as the comprehensive characterization of the labeled ThioFab is crucial for the later immunoassay.

2.1.2. Analysis *via* LC-ICP-MS

As biological samples are generally characterized by a complex matrix, limited sample volume and low concentrations of the analyte (e.g. a biomarker), quantification requires highly sensitive, selective and robust strategies. These prerequisites can be fulfilled by analysis *via* LC-ICP-MS.

Mostly ICP-MS is used for detection of lanthanide labeled biomolecules (Kretschy D et al. 2012). This is due to the fact, that especially for lanthanides, a high signal response with hardly any interferences is obtained (Nelms S 2005), together with the

low background levels in biological samples, superior limits of detection can be achieved.

For a successful application of the elemental labeled ThioFab in an immunoassay, characterization as quality control is required and was accomplished by size exclusion chromatography coupled to ICP-MS. Identification of ThioFab/ antigen conjugate could be also realized *via* SEC due to the resulting mass shift. Species unspecific quantification was performed after calibration *via* flow injection coupled to ICP-MS.

2.1.3. Proof of concept: Immunoassay Anti-HER2-ThioFab/ HER2

In the course of this master thesis, a ThioFab targeting the extra cellular domain of HER2 (human epidermal growth factor receptor 2) was used as a model system. HER2 is localized on the cell membrane of most tumors, including breast cancer, and triggers a network of signaling pathways leading to rapid uncontrolled growth of cancer cells (Abd El-Rehim DM et al. 2004). An overexpression of this receptor can be related to breast cancer (Asgeirsson KS et al. 2007) and can be therefore used as biomarker.

In order to demonstrate that the labeling procedure did not affect the functionality of the ThioFab, the formation of the final ThioFab/ antigen conjugate employing Anti-HER2/ HER2 was shown.

2.2. EXPERIMENTAL

2.2.1. Chemicals

For the production of the elemental label, the functionalized chelating moiety DOTA-Mal (2,2',2''-(10-(2-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid) was purchased at CheMatech (Dijon, France) and the lanthanides thulium and holmium, both as chlorides ($TmCl_3$, $HoCl_3$), were obtained from Sigma Aldrich (St.Louis, MO). For the mild reduction step of the genetically engineered ThioFab TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) was purchased at Sigma Aldrich (St.Louis, MO). ErbB2/ HER2 Protein employed as antigen in the immunoassay was purchased at Sino Biological Inc. (P.R.China).

In order to obtain a size ladder for the employed size exclusion chromatography column human serum albumin (HSA), superoxide dismutase (SOD) from bovine erythrocytes and L-Glutathione oxidized disodium salt (GSSG) were used and purchased at Sigma Aldrich (St.Louis, MO). Depending on the employed elemental label either holmium in 2% nitric acid (High Purity Standards, Charleston, PO) or thulium standards in 5% nitric acid (inorganic ventures, Christiansburg, VA) were used for species unspecific quantification *via* flow injection.

The other chemicals used in the labeling procedure and the analysis *via* LC-ICP-MS and FI-ICP-MS are listed in Table 1.

Table 1: Chemicals and suppliers

Chemical	Supplier
Deionized water	SG Ultra Clear Basic, SG Wasseraufbereitung und Regeneration GmbH, Barsbüttel, Germany
Suprapure ammonia solution	Merck, Darmstadt, Germany
Suprapure acetic acid	Merck, Darmstadt, Germany
Hydorchloric acid, in-house subboiled	Merck, Darmstadt, Germany
Nitric acid, 65% in-house subboiled	Merck, Darmstadt, Germany
Tris(hydroxymethyl)aminomethane (Tris)	Merck, Darmstadt, Germany
Sodium chloride	Merck, Darmstadt, Germany
Potassium chloride	Riedel-de Haën, Germany
Sodium dihydrogen phosphate dihydrate	Sigma Aldrich, St.Louis, MO
Potassium dihydrogen phosphate	Fluka, Germany
L-Ascorbic acid	Sigma Aldrich, St.Louis, MO

2.2.2. Production of ThioFab

For a selective and stoichiometric labeling, an antigen binding fragment with a free sulfhydryl group in form of an additional unpaired cysteine residue was produced recombinantly. The production of this genetically modified ThioFab, which targets the cancer biomarker HER2, was based on a research paper of Jununtula and his working group (Junutula JR et al. 2008).

The production of this genetically modified protein was achieved by introducing a point mutation A121C on the heavy chain of the 4D5 Fab (QuikChange II site-directed mutagenesis kit, Agilent Technologies, Inc.). After DNA amplification *via* polymerase chain reaction (PCR) and restriction enzyme digestion, competent cells (Novablue Singles) were transformed. In order to proof the cysteine substitution in the heavy chain of the Fab, the obtained plasmid clones were subsequently screened by DNA sequencing.

For expression *Pichia pastoris X33* strain was used. The yeast was transformed with pPICZαA vector (Invitrogen by life technologies™) containing the Fab expression cassettes and transformants were selected on zeocine-containing medium. Secretion of the recombinant protein into the medium was induced with methanol and

continued for 5 days. Culture supernatant was harvested and purified *via* two-step affinity chromatography (Anti-κ and Protein-G). The 4D5_A121C Fab was eluted with 0.1 M glycine, pH 2.5 and immediately neutralized by Tris buffer. After dialysis against phosphate buffered saline (PBS), the concentration of the purified ThioFab was assessed photometrically. A concentration of 80 µg mL^{-1}, corresponding to 1.7 µM, was achieved.
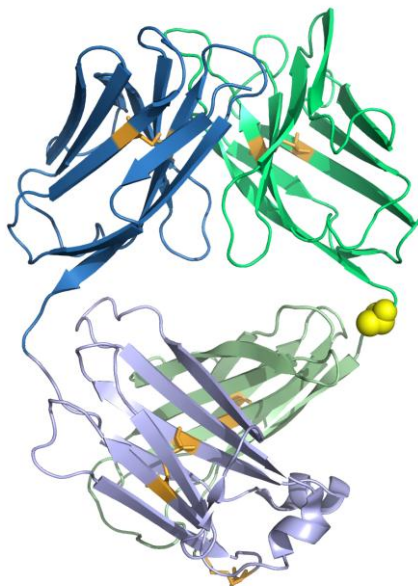

Figure 4: Structure of 4D5 A121C ThioFab

A representation of the 3D-structure of the genetically modified ThioFab in form of a ribbon diagram is shown in Figure 4. The blue ribbons represent the light chain- dark blue indicates the constant domain, light blue the variable domain, respectively. The heavy chain of the ThioFab is shown in green, whereas the constant domain is presented in dark green and the variable domain in light green. Disulfide bridges are shown in orange. The *de novo* cysteine is highlighted in yellow.

2.2.3. Standard solutions

Standard solution for protein quantification:

As the number of sulfur containing amino acids of the ThioFab was known (11 cysteins and 3 methionins per mol), the concentration of the protein was determined by measuring sulfur as sulfur oxide *via* flow injection coupled to ICP-QMS using the reaction cell. For this purpose human serum albumin, which comprises 41 mol sulfur per mol protein, was employed as standard. Approximately 1 mg of the protein was weighed into an Eppendorf tube and dissolved in 1 mL PBS buffer (137.0 mM NaCl, 2.7 mM KCl, 10.0 mM $Na_2HPO_4•2H_2O$, 2.0 mM KH_2PO_4 (Dulbecco and Vogt 1954)). HSA standards with concentrations of 0.5, 1.0, 2.5 and 5.0 µM (corresponding to 20.5, 41.0, 102.5 and 205 µM sulfur) were prepared by appropriate dilution of the 1 mg mL^{-1} solution with PBS buffer.
The HSA standards were employed for a maximum of two weeks and were stored at +6 °C.

Standard solution for the rare earth elements:

Depending on the coordinated lanthanide in the elemental label, either thulium or holmium was employed as standard. Rare earth element standards with concentrations of 0.0625, 0.125, 0.250, 0.500 and 1.0 µM were prepared by appropriate dilution of a 1 g L^{-1} thulium or holmium ICP-MS standard with 2% nitric acid. The thulium and holmium standards were stored at +6 °C.

2.2.4. Elemental labeling of ThioFab

Coordination reaction of the lanthanide to DOTA-maleimid:

The procedure for the preparation of the elemental label was adapted from the work of Kretschy and Ahlgren (Kretschy D et al. 2011b, Ahlgren S et al. 2008).

Two elemental labels, differing in the employed rare earth element, were assessed. As the ionic radius plays a crucial role in complex stability and the advantage of employing a monoisotopic rare earth element (Kretschy D et al. 2011b), either thulium or holmium was used for complexation.

In order to produce the lanthanide label La^{3+}-DOTA-Mal, the chelating moiety functionalized with a maleimid group, DOTA-Mal, was weighed into an Eppendorf tube and dissolved in degassed 200 mM ammonium acetate buffer, pH 6.0. Besides the chloride salt of the employed rare earth element was weighed into an Eppendorf tube as well and dissolved in water.

After appropriate dilution of the chelating agent and the lanthanidechloride solution, the coordination reaction was carried out under equimolar conditions for 1 h at 37 °C under argon. The reaction equation is depicted below. The elemental labels were always produced freshly.

DOTA-Mal [804 µM] + TmCl$_3$ [804 µM] → Tm-DOTA-Mal [402 µM]
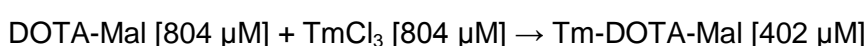
The coordination reaction for the rare earth element thulium is depicted in Figure 5.

DOTA-Mal
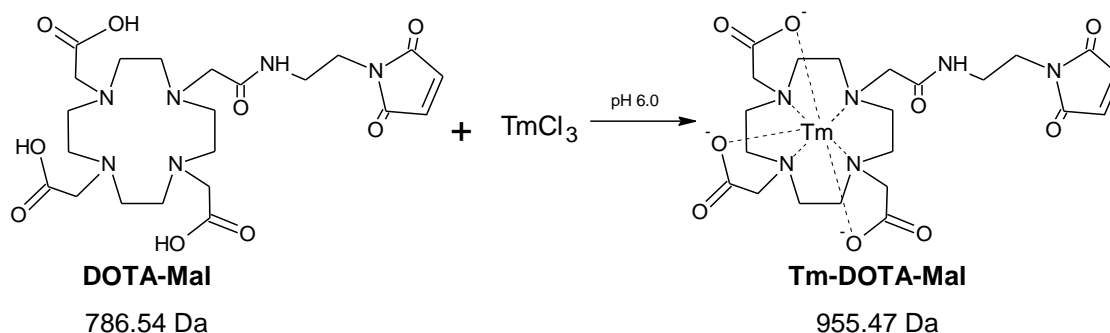786.54 Da

Tm-DOTA-Mal
955.47 Da

Figure 5: Coordination reaction of Tm to DOTA-maleimid

Linking reaction of the elemental label with the ThioFab:

As the maleimid group on the rare earth elemental label targets sulfhydryl groups, prior to the linking reaction of label with the ThioFab, a mild reduction step with TCEP was performed. In order to reduce the sulfhydryl group of the recombinantly introduced cysteine on ThioFab, TCEP was weighed in and dissolved in 100 mM TrisAcetate buffer, pH 7.0 and applied in a 50 fold molar excess to the ThioFab. The TCEP solution was always prepared freshly. The reagent mixture was incubated for 45 min on a shaker at room temperature. Subsequently for labeling of the ThioFab, a 10-fold molar excess of lanthanide-DOTA-Mal was employed.

The linking reaction, which is depicted in Figure 6 for Tm-DOTA-Mal as elemental label, was performed under oxygen exclusion, overnight on a shaker at room

temperature. In order to keep the dilution of the ThioFab to a minimum, a volume of only 10 µL of TCEP solution and elemental label, respectively were added. The reaction equation is depicted below.
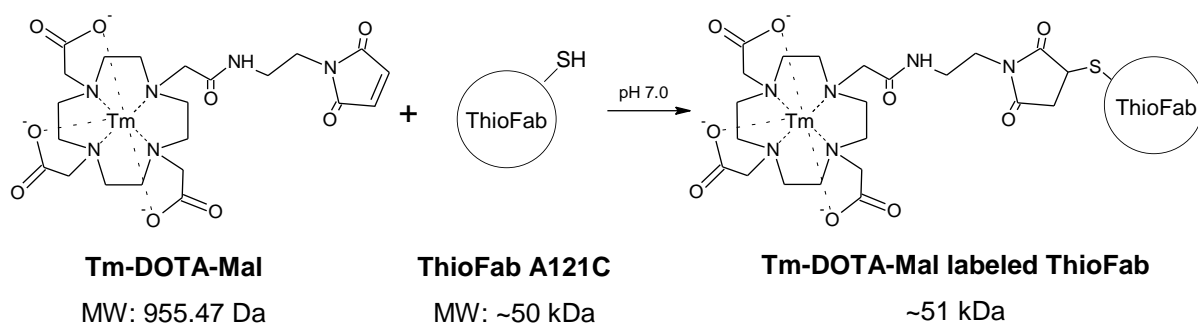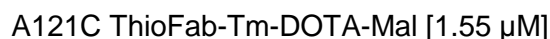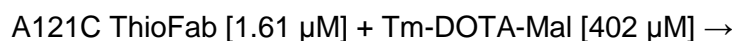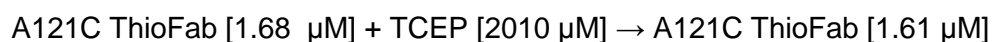
A121C ThioFab [1.68 µM] + TCEP [2010 µM] → A121C ThioFab [1.61 µM]

A121C ThioFab [1.61 µM] + Tm-DOTA-Mal [402 µM] →
A121C ThioFab-Tm-DOTA-Mal [1.55 µM]

Tm-DOTA-Mal   **ThioFab A121C**   **Tm-DOTA-Mal labeled ThioFab**
MW: 955.47 Da   MW: ~50 kDa   ~51 kDa

Figure 6: Linking reaction of the elemental label Tm-DOTA-Mal to the ThioFab

In the next step, the unreacted label was removed by employing centrifugal filter devices with a 30 kDa nominal molecular weight limit membrane (Ultrafree-0.5 centrifugal filter & tube, 0.5 mL, 30 K NMWL Biomax high-flux polyethersulfone membrane, Millipore Co, Billerica, MA, USA). For centrifugation a Mini Spin plus centrifuge (Eppendorf AG, Hamburg, Germany), which was located in a cooling chamber (20 °C) was used. The clean-up procedure was modified based on the work for MeCAT-labeling from Waentig *et al.* (Waentig L et al. 2012).

After conditioning the molecular weight cut-off filters with PBS by centrifuging the device at 14 000 x g, the labeled ThioFab solution and an appropriate volume of PBS to attain a final volume of 400 µL, was added and spun at 7500 x g until the holdup volume was reached (approximately 20 min). This clean-up procedure was performed twice. Finally, the molecular weight cut-off filter was spun reversely at 1000 x g to recover the labeled ThioFab.

The elemental labeled ThioFab was subsequently transferred into polypropylene microvials for LC-ICP-MS measurement.

2.2.5. Analysis by LC-ICP-MS

The analysis of the labeled ThioFab was performed on an HPLC system (ICS-3000DP dual pump system in combination with a CTC PAL autosampler (including a temperature-control device), Thermo (Sunnyvale, CA, USA)) coupled to ICP-QMS (Elan 6100 DRC II; Perkin Elmer Sciex, Ontario, Canada). The coupling of the HPLC system to the ICP-MS was achieved by connecting the HPLC-effluent to a PFA-nebulizer (Elemental Scientific Inc., Omaha, NE, USA) positioned in a cyclonic spray chamber (PE-SCIEX). For all connections PEEK capillaries (IDEX) with an internal diameter of 0.127 mm were used.

Chromatographic separation was performed on a size exclusion chromatography column Waters Biosuite 125, 4µm UHR SEC, 4.6 x 300 mm with a Waters BioSuite SEC Guard Column, 4 µm, 4.6 mm x 35 mm, using 100 mM ammonium acetate buffer, pH 6.5 as eluent. Sulfur and the lanthanides thulium or holmium, depending on the employed elemental label, were measured as $^{32}S^{16}O^{+}$ at m/z 48, $^{165}Ho^{16}O^{+}$ at m/z 181 and $^{169}Tm^{16}O^{+}$ at m/z 185, respectively, by using the dynamic reaction cell (DRC) technique with oxygen. Beside of the lanthanide oxides, holmium and thulium ions, $^{165}Ho^{+}$ and $^{169}Tm^{+}$, were measured as well. The ICP-QMS measurement was performed applying a daily tuning procedure for optimization of the nebulizer gas flow, auxiliary gas flow and the ICP-RF-power.

For external calibration and species unspecific quantification of the ThioFab labeled with lanthanide-DOTA-Mal as well as for the ThioFab/ antigen conjugate, flow injection (FI) analysis was performed with the same instrument setup, though instead of the separation column a PEEK capillary (i.d. 0.025 mm) was used and the eluent comprised additionally 1% nitric acid (w/w).

For the quantification of the lanthanides a 5-point calibration curve ranging from 0.0625 µM to 1 µM was obtained and for monitoring the sulfur concentration a 4-point calibration ranging from 20.5 to 205 µM sulfur was employed.

In Table 2 the operation parameters for the analysis of the labeled ThioFab are shown.

Table 2: Operation parameters for LC-ICP-MS and FI-ICP-MS

Carrier flow	100 mM Ammonium acetate, pH 6.5 for LC 100 mM Ammonium acetate, 1% HNO_3, pH 1.5 for FI
Flow rate	0.3 mL min^{-1}
Injection loop	10 µL
Injection volume	15 µL
Tray temperature	6 °C
LC-Column	Waters Biosuite 125, 4µm UHR SEC, 4.6 x 300 mm with a Waters BioSuite SEC Guard Column, 4 µm, 4.6 x 35 mm
Nebulizer	PFA-ST microconcentric
Spray Chamber	Cyclonic
Plasma gas	16.0 L min^{-1}
DRC mode	Oxygen as collision gas
Cell gas flow	0.8 L min^{-1}
m/z measured	$^{32}S^{16}O^+$, $^{165}Ho^{16}O^+$, $^{165}Ho^+$, $^{169}Tm^{16}O^+$, $^{169}Tm^+$

Generation and export of HPLC-ICP-QMS chromatograms was performed employing Chromlink (Version 2.1, Perkin Elmer SCIEX) in combination with Totalchrom (Version 6.2, Perkin Elmer SCIEX). Chromeleon software (Version 6.80, Dionex, Sunnyvale, CA, USA) was used for integration and evaluation of all chromatographic data acquired by ICP-MS detection.

3. RESULTS AND DISCUSSION

For quality control and as criteria for the labeling output the labeling degree was assessed. The labeling degree was calculated by applying Equation 1, shown below. The sensitivity factor was reassessed in every analysis and is determined as the ratio of the peak areas of 1 µmol lanthanide and 1 µmol sulfur.

Equation 1: Calculation of the labeling degree

$$\left(\frac{\frac{peak\ area\ [lanthanide\ oxide]}{(Mol\ lanthanide\ per\ Mol\ protein)}}{\left(\frac{peak\ area\ [sulfur\ oxide]}{(Mol\ sulfur\ per\ Mol\ protein)} \right) * sensitivity\ factor} \right) * 100$$

As starting point for the production of the elemental label the work of Kretschy *et al.* (Kretschy D et al. 2011b) was used and adapted. The reaction conditions were the following: around 1 mg of DOTA-Mal (corresponds to approximately 1.3 mM) was dissolved in 20 mM ammonium acetate, pH 6.0 and incubated equimolarly for 1 h at 37 °C with a lanthanide chloride, which was dissolved in water.

For the labeling of the ThioFab, the freshly prepared lanthanide-DOTA-Mal was added in 20 fold molar excess to the protein and incubated for 1 h at 37 °C. In doing so, the dilution of the ThioFab was kept as low as possible.

Analysis was performed on a BioSuite size exclusion chromatography column from Waters (see chapter 2.2.5.). In order to assess the separation of the applied SEC-ICP-MS setup a protein/ peptide mix was prepared and analyzed under the same conditions as described in the experimental. The mix contained HSA, SOD, GSSG and inorganic sulfur. As HSA forms dimers the mass of the dimer was also taken into account. In Figure 7 the chromatogram and isotherm of the size ladder is depicted, Table 3 shows the retentions times as well as the molecular weight of the chosen compounds.
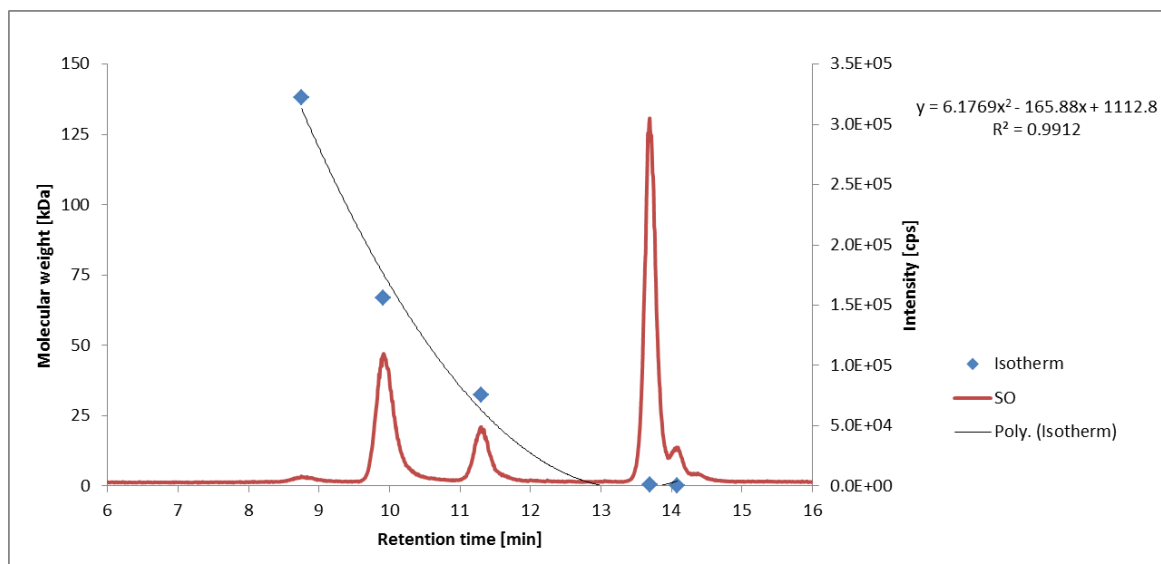
Figure 7: Size ladder of the employed SEC column

Table 3: Retention time and molecular weight of the compounds chosen for the size ladder

Compound	Retention time [min]	Molecular weight [kDa]
Albumin, dimere	8.8	138
Albumin, monomer	9.9	67
SOD	11.3	32.5
GSSG	13.7	0.6
S inorganic	14.1	0.032

When the ThioFab was labeled for the first time under above described conditions, a labeling degree of 0.4% was achieved, thus an optimization of the labeling procedure was necessary. As the availability of the ThioFab was limited, pre-optimization of the labeling procedure was performed with another protein, which also contained a free cysteine for labeling. For this purpose human serum albumin was chosen- its free cysteine is highlighted in red in Figure 8.
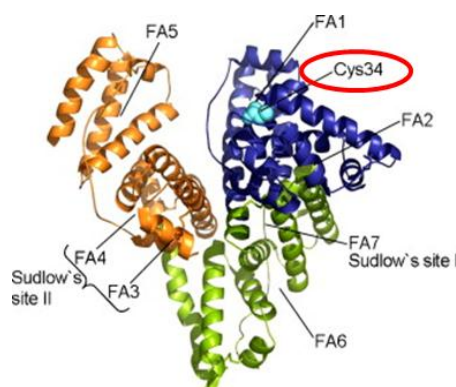

Figure 8: Structure of Human serum albumin modified from (Turell L et al. 2009)

3.1. OPTIMIZATION OF LABELING PROCEDURE

As mentioned previously in section 1.2.2, the pH value plays a fundamental role in chelation as well as in the linking reaction of ThioFab to the elemental label.

For chelation of the lanthanide in the macrocycle DOTA-Mal, the rate of complex formation increases with pH, though lanthanide ions start to form insoluble hydroxo complexes at > pH 6 (De León-Rodríguez LM and Kovacs Z 2008). Therefore, the optimal pH range for the labeling is between 5 and 6. In order to maintain the pH, typically sodium acetate or ammonium acetate buffer (50 mM to 0.5 M) is employed (De León-Rodríguez LM and Kovacs Z 2008).

In the linking reaction, the thiolate anion of the ThioFab is added to the maleimid group of the elemental label in a 1,4-Michael addition (Sinz A 2003) and forms a stable thioether linkage. This reaction is specific for sulfhydryl groups if the pH value ranges from 6.5 – 7.5 (Sinz A 2003, Partis MD et al. 1983). At a pH value of 7 the maleimid reaction proceeds 1000 times faster with thiol groups than with amines. However, in more alkaline conditions, at pH values above 8.5, the maleimid reaction favors primary amines (Brewer CF and Riehm JP 1967) and also hydrolysis of the maleimid group to a non-reactive open maleamic form may occur (Sinz A 2003). Therefore a pH range of 7.0- 7.5 was intended for the linking reaction.

For the following experiments all employed solutions were degassed and as far as possible all steps were performed under oxygen exclusion. Additionally the ionic strength of the labeling buffer (ammonium acetate) was increased from 20 mM to 200 mM as described by Ahlgren *et al.* (Ahlgren S et al. 2008). Reaction conditions for the coordination of the lanthanide to the complexing moiety remained constant- equimolar incubation for 1 h at 37 °C. The reaction time for the attachment of the label to the protein was extended from 1 h to 12 h. The optimization experiments with albumin, as "dummy" protein, were prepared threefold.

In order to monitor also the protein concentration *via* sulfur oxide signal, a final concentration of around 350 µg mL^{-1}, corresponding to approximately 5 µM, HSA was intended. However, it had to be stressed, that these 5 µM HSA are, compared to the available concentration of the ThioFab (1.5- 1.7 µM) considerably high. Hence,

the labeling procedure optimized with albumin, might lead to a different labeling output for the ThioFab and had to be assessed specifically in further detail.

For the optimization in terms of pH and label excess, an inert titanium HPLC gradient system (Transcend Liquid Chromatography System, Thermo Fisher Scientific, Munich, Germany) combined with a metal-free autosampler (HTC-PAL Autosampler, Thermo Fisher Inc., Waltham, USA), coupled to an ICP-QMS detector (iCAP-QMS, Thermo Fisher Scientific Inc.) with a PFA-ST microconcentric nebulizer and a cyclonic spray chamber was used. After assessing these parameters, analysis was conducted as described in section 2.2.5.

3.1.1. Pre-optimization of labeling procedure *via* human serum albumin

The first step was the optimization of the reactions in terms of the pH value. As already mentioned, this was done with albumin. For every experiment, HSA was weighed in freshly and dissolved in PBS, since the recombinantly produced ThioFab was dissolved in same buffer.

In order to evaluate the optimal pH value, the labeling buffer was set to different pH values: 5.5, 5.8 and 6.0. Additionally it was assessed if due to its reductive potential, the addition of 20 mM ascorbic acid to the labeling buffer would improve the labeling degree. Furthermore it was evaluated, if the linking reaction prior to complexation would have a positive impact on the labeling degree

The experiments showed that a pH of 5.8 for the labeling buffer led to a higher labeling degree, 8.8% ± 0.2%, than applying a pH value of 5.5, where 1.4% ± 0.04% was achieved. Since a higher pH value was also favorable for the following maleimid reaction, the pH was raised to 6.0 after optimizing different label excesses.

The addition of ascorbic acid to the ammonium acetate buffer was discarded as it decreased the labeling degree by a mean factor of 1.5.

The labeling procedure, where the attachment of the macrocycle is followed by the complexation of the rare earth element, had to be abandoned as no lanthanide signal was obtained at the respective retention time of the protein. However, it was not

possible to verify if the linking or the coordination reaction did not work, as the mass difference between labeled and unlabeled protein was only approximately 1 kDa and therefore no separation of was achieved *via* the SEC. Though, out of theoretical assumptions, it is presumed to attribute it to the complexation reaction.

Optimizing the labeling procedure in regard to elemental label excess:

In the following the labeling condition was optimized in terms of elemental label excess. Four different molar excesses, 3-, 5-, 7.5- and 10-fold were evaluated.

Increasing the excess from 3-fold to a 10 fold molar excess of the lanthanide-DOTA-maleimid, the labeling degree improved by a factor of 1.4 leading to a labeling degree of 22.7% ± 0.3%. Comparing the 5- fold (20.7 ± 0.7%) to the 10-fold molar excess, the labeling degree improved marginally, therefore a higher molar excess was not evaluated.

The chromatogram of the Ho-DOTA-Mal labeled HSA separated on a SEC column (Waters Biosuite 125, 4 µm UHR SEC, 4.6 x 300 mm) using 100 mM ammonium acetate buffer, pH 6.5 is depicted in Figure 9. As it can be seen in the chromatogram in Figure 9, the holmium background is high. This is due to the fact that rare earth elements tend to interact easily and therefore "stick" on the SEC column as well as in the whole system. The problem was solved, when hydrochloric acid instead of nitric acid was used as blank injection after every lanthanide containing sample, since lanthanides are prone to form chloro-complexes.
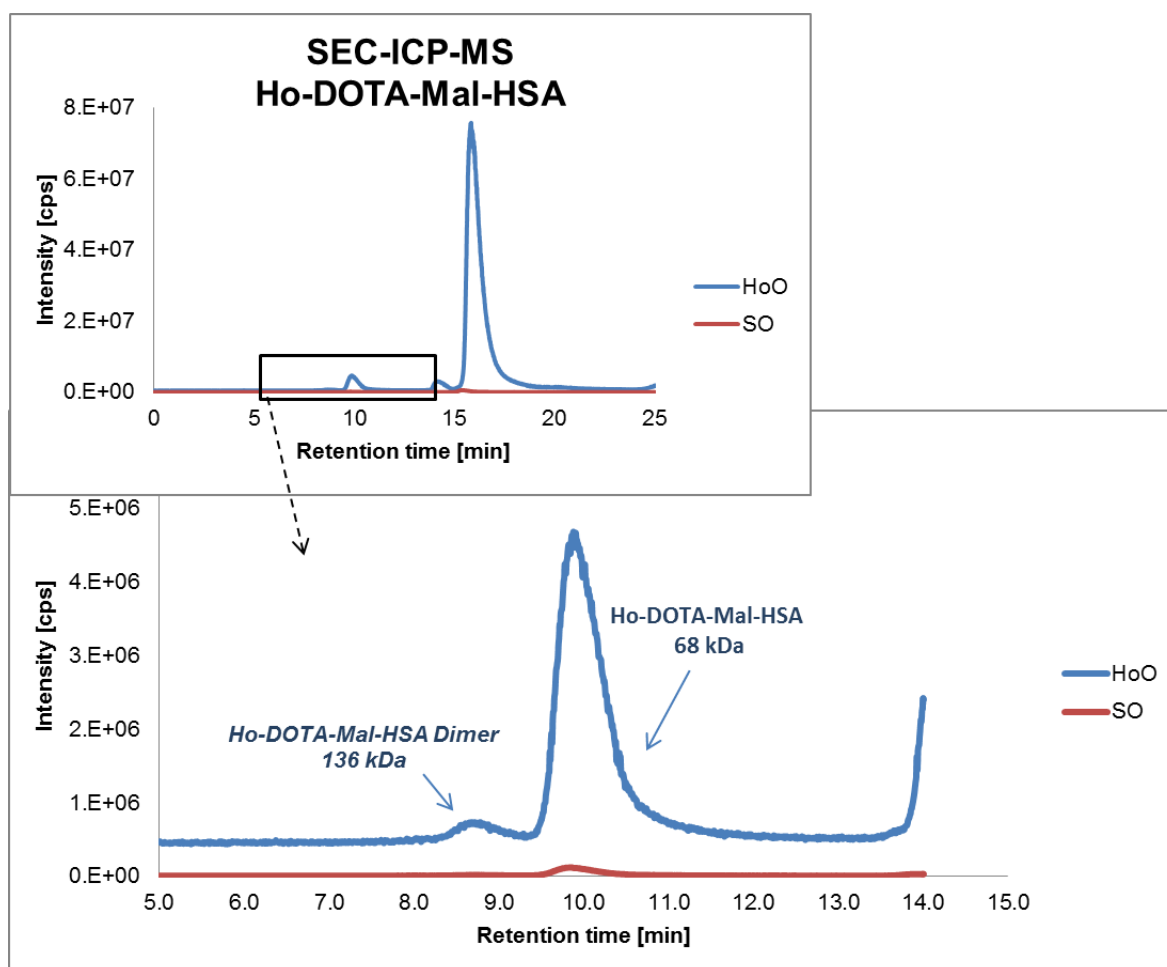
Figure 9: Chromatogram of Ho-DOTA-Mal-HSA [1 μM]

Optimizing the linking reaction:

In order to evaluate if a different labeling setup would lead to a higher labeling output, the described labeling conditions were compared in terms of labeling degree with a modified labeling protocol published by Waentig *et al.* (Waentig L et al. 2012).

The labeling protocol by Waentig *et al.* was modified with respect to the employed elemental label, as instead of MeCAT® label, the in-house produced complex Ho-DOTA-Mal was used. In addition no reduction step was performed.

Furthermore, two different ways of incubation for the attachment step were evaluated. It was assessed if incubation overnight on a shaker would lead to a higher labeling output than incubation at 37 °C.

The modified labeling protocol was conducted as following: 100 μL of a 1.0 mg mL^{-1} HSA solution was put into a centrifugal filter device (with a 30 kDa molecular weight

limit membrane) and diluted to 400 µL with Tris buffer (20 mM Tris; 150 mM NaCl, pH 7.4). After reaching the holdup volume by centrifuging at 7500 x g, one washing step followed by adding Tris buffer. The next step was the labeling of albumin with a 10-fold molar excess of Ho-DOTA-Mal. The reagent mixture was shaken for 60 minutes. Afterwards a clean-up procedure of the labeled albumin was performed by two washing steps as described above (Waentig L et al. 2012).

It is important to notice that for the label protocol from Waentig *et al.* 100 µg or a minimum amount of 50 µg should be employed for labeling. Therefore the concentration was set at approximately 1.0 mg mL^{-1} (corresponding to 15 µM HSA), whereas for the in-house optimized labeling procedure a concentration of approximately 350 µg mL^{-1} was employed. As previously mentioned in section 1.2.2, as well as in the beginning of this chapter, the concentration may also play a certain role in the labeling output.

In Figure 10 the results of this comparison are shown. In general the labeling degree does not vary between the different approaches to a large extent. Though with the modified labeling protocol of Waentig *et al.* (Waentig L et al. 2012), the highest labeling degree was achieved. In addition, the incubation on a shaker had an advantageous impact on the obtained standard deviations.

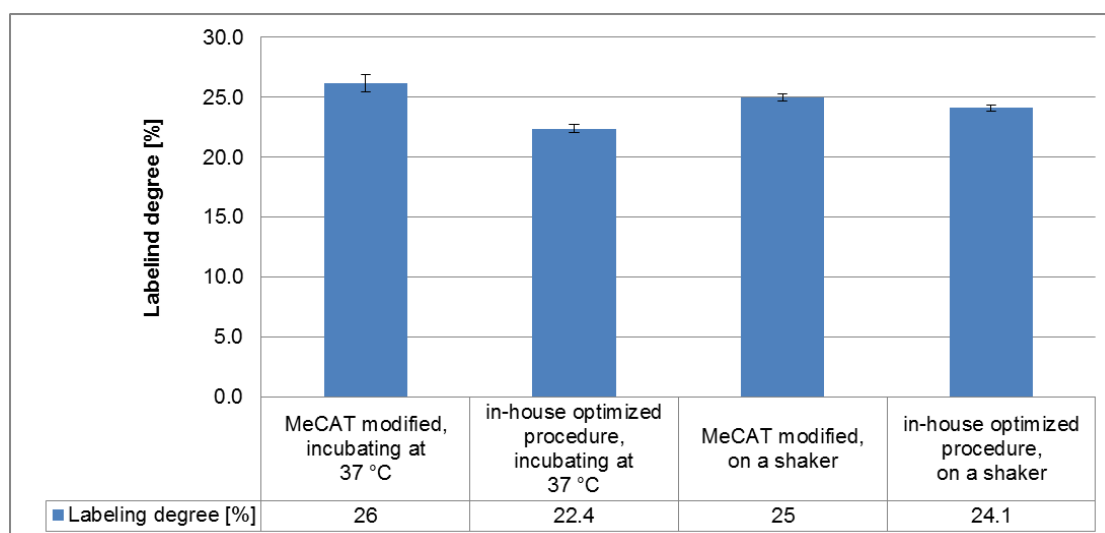|	MeCAT modified, incubating at 37 °C	in-house optimized procedure, incubating at 37 °C	MeCAT modified, on a shaker	in-house optimized procedure, on a shaker
■ Labeling degree [%]	26	22.4	25	24.1

Figure 10: Comparison of labeling procedures *via* albumin

3.1.2. Adaption of labeling procedure to the ThioFab

When the labeling procedure was optimized as far as possible with albumin, it was applied to the Anti-HER2-ThioFab. By employing the established labeling procedure to label the ThioFab, the labeling degree was still in the low range of 0.8%. This difference in labeling degree corresponds with the findings of Lewis *et al.*, as he describes that applying the same labeling procedure to different proteins yielded to different labeling degrees (Lewis MR, Raubitschek A and Shively JE 1994). Consequently, two different approaches were assessed in order to increase the labeling output of the ThioFab.

Since the labeling also depends on the concentration of the protein, the ThioFab was pre-concentrated *via* 10 kDa molecular weight cut-off filter (Viva Spin 2 sample concentrator, 10.000 molecular weight cutoff value, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). A pre-concentration by a factor of 10, to 6.9 µM was obtained and was assessed photometrically. However, when labeling the pre-concentrated ThioFab, only a minor improvement of the labeling degree to 1.3% was achieved.

Thus there was reason to presume that the additional sulfhydryl group of the ThioFab might not be free anymore. Hence, a mild reduction step had to be performed. For this propose TCEP was employed, since it does not contain any thiol groups and therefore no reaction with the maleimid group of the elemental label is expected to occur.

In order to assess the appropriate molar excess of TCEP, seven different concentrations, starting form 2.5-fold, to 5-, 10-, 50-, 100-, 150- and 300 fold molar excess, were added. The reduction step was performed prior to the linking reaction and TCEP, which was dissolved in 100 mM Tris acetate, pH 7.0, was incubated with the ThioFab for 45 min on a shaker. As the pH value plays a crucial role in the linking reaction, it was ensured that due to the addition no severe change was obtained.

Due to the low labeling degree and the general low sensitivity for sulfur, the results of this experiment, shown in Figure 11, are not very reliable and therefore a standard error of 25% was assumed. Still, it can be presumed that the labeling degree applying a 50-fold molar excess led to the highest labeling degree.
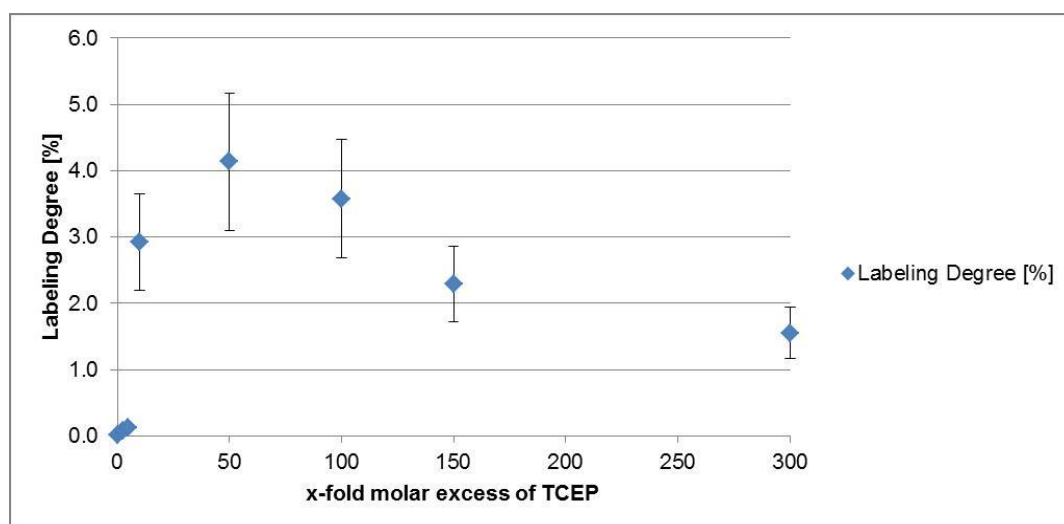
Figure 11: Assessment of TCEP excess for a mild reduction step of the Anti-HER2-ThioFab

Though the labeling degree of the genetically modified ThioFab was increased by approximately a factor of 10, the need for further improvement was still essential.

However, it has to be stressed, that the ThioFab employed for this fine-optimization was frozen at -20 °C for already two years, and might have experienced some losses and changes due to unintentional freeze-thaw cycles. Hence, for the following experiments a new recombinantly produced Anti-HER2-ThioFab in a concentration of 1.7 µM (80 µg mL^{-1}) was employed.

The further optimized labeling procedure was also compared with the above mentioned labeling protocol from Waentig *et al.* (Waentig L et al. 2012). Though, in contrast to the HSA-approach, a reduction step with TCEP was performed and in addition, the MeCAT ® reagent was added as described in (Waentig L et al. 2012). The resulting labeling degree is depicted in Figure 12.
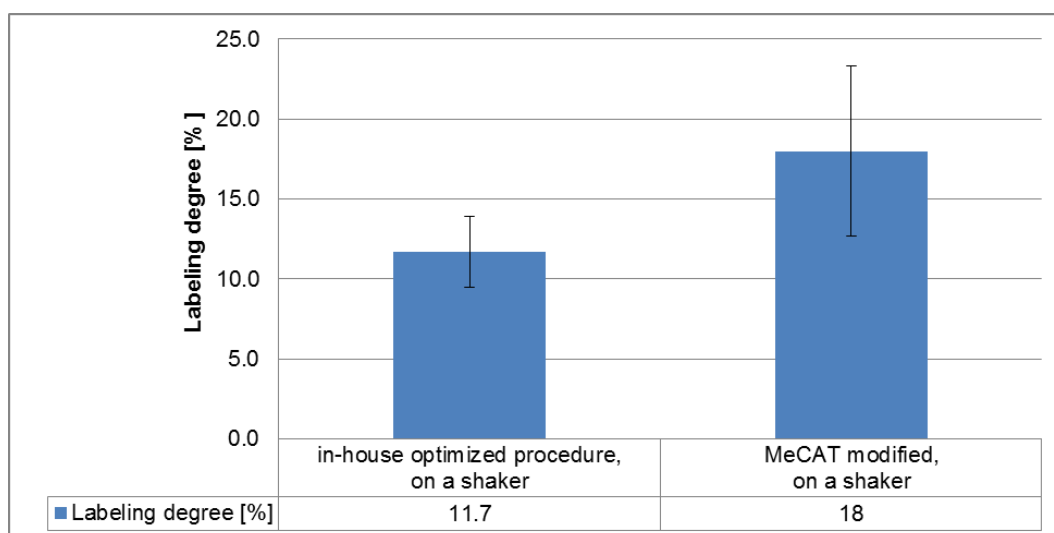
Figure 12: Comparison of labeling procedures for the Anti-HER2-ThioFab

As pointed out before, this labeling protocol requires a minimum concentration of 50 μg, which was not achieved with the way of producing the ThioFab. This may explain the relative standard deviation of 30%. Though, a higher mean value of the labeling degree was achieved, the labeling protocol of Waentig *et al.* (Waentig L et al. 2012) was not applied, due to the lower reproducibility.

Still, for the final labeling procedure an additional clean-up step, employing 30 kDa molecular weight cut-off filter, was applied in order to remove the unreacted label. The established labeling method is described in section 2.2.4. By optimizing the labeling procedure, the labeling degree of the genetically modified ThioFab was increased from initial 0.4% to 40%. The chromatogram of the Tm-DOTA-Mal labeled Anti-HER2-ThioFab is shown in Figure 13.
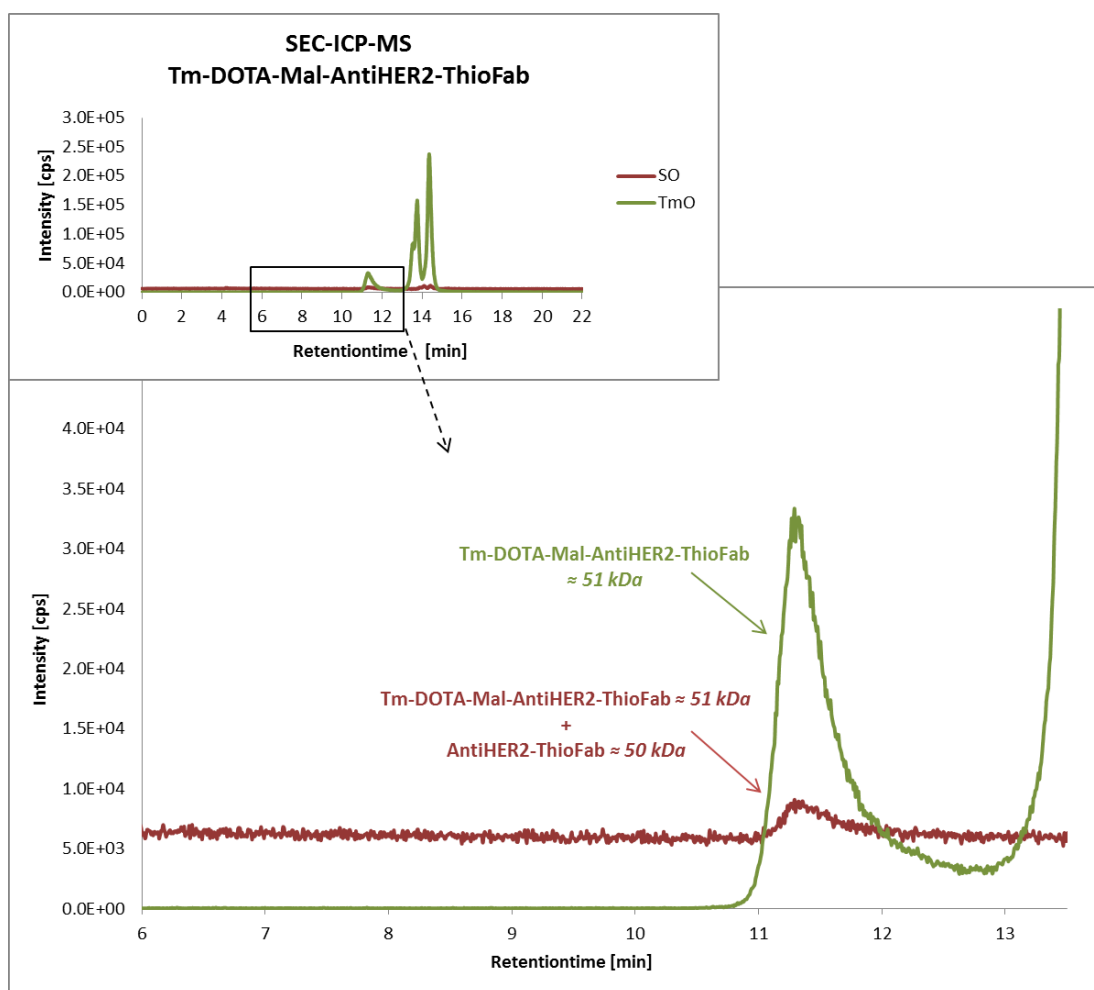
Figure 13: Chromatogram of Tm-DOTA-Mal Anti-HER2-ThioFab [1.55 µM]

For calculating the sensitivity factor, the calibration curves obtained *via* flow injection were employed. The calibration of the rare earth element thulium is depicted in Figure 14, whereas the calibration curve for sulfur is shown in Figure 15.
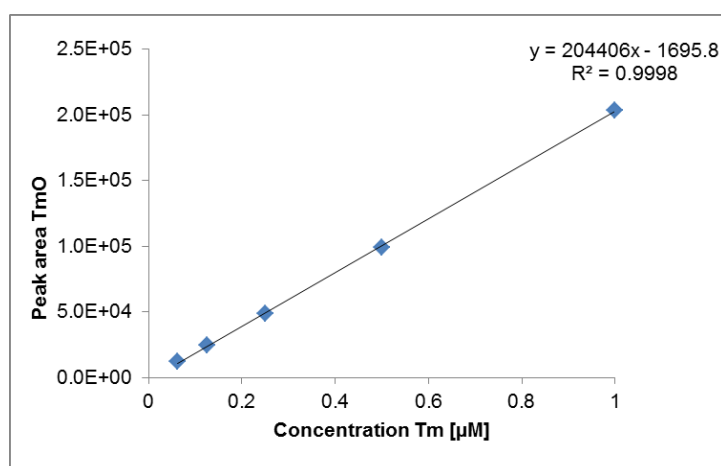

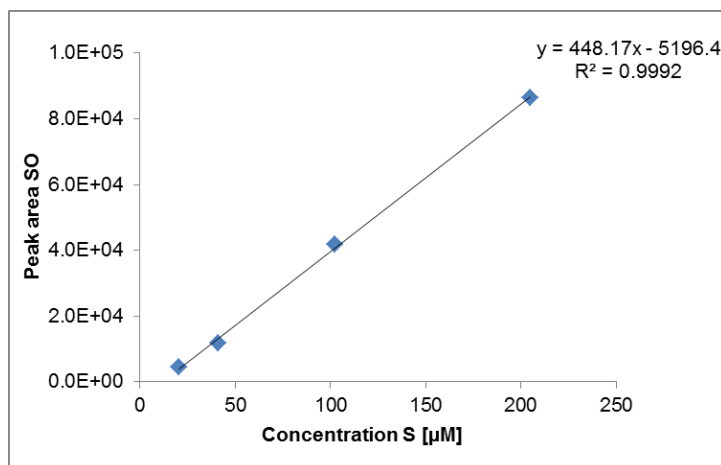**Figure 14:** Calibration curve of thulium

Figure 15: Calibration curve of sulfur

For the determination of the limit of detection (LOD) the threefold standard deviation of the blank signal was employed whereas for the limit of quantification (LOQ) the tenfold standard deviation was used. A LOD of 1.4 nM and a LOQ of 4.7 nM for the thulium labeled Anti-HER2-ThioFab was achieved by using the measurement setup described in 2.2.5. Apart of the obtained LOD and LOQ, in Table 4 the precision of the SEC-ICP-MS analysis is shown and was assessed by injecting the sample 5 times consecutively. The recovery of the ThioFab, also shown in Table 4, is 47.1%. This may seem low, though for size exclusion chromatography quite common. In comparison the recovery for human serum albumin was 61%.

Table 4: Analytical characteristics

Compound	Retention time [min]	Recovery S [%]	LOD [nM]	LOQ [nM]	RSD (n=5)
Tm-DOTA-Mal Anti-HER2-ThioFab	11.3	47.1	1.41	4.70	5.6%

In order to evaluate the freeze-thaw stability of the elemental labeled ThioFab, the protein was frozen for 1 month at -80 °C and subsequently analyzed by injecting the sample three times. As the mean labeling degree resulted in 37.2% with a precision of 5.6%, the label seemed to be stable.

3.2. PROOF OF CONCEPT- IMMUNOASSAY

As a proof of principle the formation of the final ThioFab/ antigen conjugate employing Anti-HER2/ HER2 as model compound, was demonstrated.

Therefore 148 nM antigen HER2 (~135 kDa) was incubated in liquid with the Tm-DOTA-maleimid labeled ThioFab (~51 kDa) in a 5-fold molar excess for 2 h on a shaker and subsequently measured by SEC-ICP-MS analysis.

The identification of the thulium labeled ThioFab/ antigen conjugate (~ 186 kDa) was performed *via* mass shift in the size exclusion chromatogram. A chromatogram of the ThioFab/ antigen conjugate separated on a SEC column using 100 mM ammonium acetate buffer, pH 6.5 is depicted in Figure 16. When the concentration of the ThioFab/ antigen conjugate was calculated *via* the thulium oxide peak area, a concentration of 10.3 nM was obtained. This indicates that the antigen HER2 might not be as pure as expected, since the conjugate should correspond to approximately 75 nM.

In Table 5 the obtained LOD and LOQ for the elemental labeled ThioFab/ antigen conjugate are depicted. As, based on the mean value of healthy individual ± 2 standard deviations, the normal HER2 concentration in serum is under 15 ng mL^{-1} (corresponding to 0.11 nM) (Asgeirsson KS et al. 2007) the presented setup for liquid based incubation is not yet sufficiently optimized.

Table 5: LOD and LOQ of ThioFab*/ antigen conjugate

Compound	Retention time [min]	LOD [nM]	LOQ [nM]
Tm-DOTA-Mal Anti-HER2-ThioFab/ HER2 conjugate	7.6	0.35	1.17
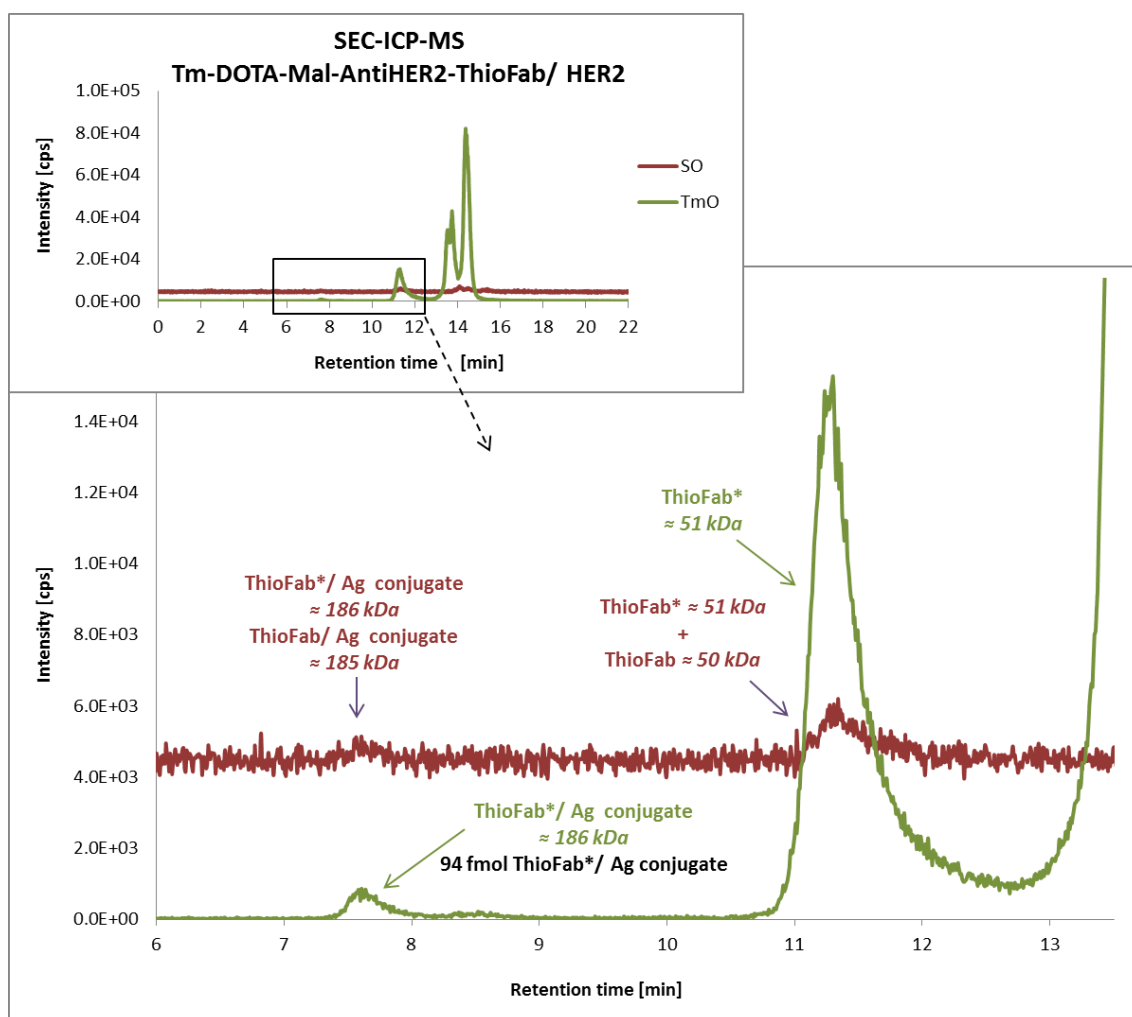
Figure 16: Chromatogram of Tm-DOTA-Mal Anti-HER2-ThioFab/ HER2 conjugate [9.4 nM]

Beside of the proof of concept also the employed freshly labeled ThioFab was analyzed and a labeling degree of 40% was evaluated. Interestingly, the labeling degree of the unbound Anti-HER2-ThioFab changed when incubated with the antigen HER2. When calculating the labeling degree of the unreacted ThioFab in the immunoassay (at a retention time of 11.3 min in the chromatogram depicted in Figure 16) it turned out to be around 30%. As the labeled and unlabeled ThioFab could not be separated from each other regarding the sulfur signal, it led to the assumption that the binding kinetics of derivatized and underivatized protein might vary.

4. CONCLUSION

The major part of the work dealt with the optimization of a method to label the engineered antigen binding fragment, carrying a free thiol group, with a lanthanide label *via* maleimid chemistry. Establishing a basic labeling procedure was feasible in form of pre-optimization by applying human serum albumin as a dummy protein. Final adaption steps of the labeling procedure had to be performed on the genetically modified ThioFab.

As quality control is a crucial step for reliable application, the labeling degree of the elemental labeled antibody was assessed considering the sensitivity factors *via* size-exclusion chromatography coupled to ICP-MS. By optimizing the labeling procedure, the labeling degree of the recombinantly produced ThioFab was increased from 0.4% to 40%. The application of a ThioFab instead of an antibody circumvent the problems coming along with a harsh reduction step, additionally a stoichiometric labeling of only one cysteine residue can be assumed.

As a proof of principle for a further application as immunoassay, the formation of the final ThioFab/ antigen conjugate, employing Anti HER2/ HER2 as model system, was demonstrated. Identification and quantification of the labeled conjugate was possible due to the mass shift of bound and free lanthanide labeled ThioFab.

The immunoassay shows great potential for multiplexed and highly automated quantitative analysis of biological samples e.g. employment as a diagnostic tool for biomarker quantification. However, further optimization is needed regarding the liquid incubation of lanthanide labeled ThioFab with antigen. An improvement of sensitivity is necessary and may be achieved *via* pre-concentration steps.

Nevertheless, the integration of the presented concept of liquid based immunoassay may simplify the approach of elemental labeling combined with automated ICP-MS detection for absolute quantification of bio-molecules in routine diagnostic.

5. SUMMARY

The derivatisation of antibodies with elemental labels and their ICP-MS based detection offers the possibility for indirect quantification of various targets (e.g. cancer markers or cellular metabolites). This straight-forward technique is applicable for e.g. immunoassays *via* ICP-MS based absolute quantification of the targets in biological samples. As the assessed samples are characterized by a complex matrix, limited sample volume and low concentrations of the analyte (e.g. a biomarker), quantification requires highly sensitive, selective and robust strategies.

The aim of this master thesis was the development of a labeling procedure for a genetically modified antigen binding fragment (Fab), so-called ThioFab (Junutula JR et al. 2008), with rare earth element label macrocycles, and its analysis *via* liquid chromatography inductively coupled plasma mass spectrometry (LC-ICP-MS).

For the first time a labeling procedure for a ThioFab with lanthanide label macrocycles was developed. The employment of this ThioFab, which carries an additional recombinantly introduced cysteine, allows a straight-forward labeling. Due to this introduced cysteine residue, only a mild reduction step, prior to the attachment of the elemental label lanthanide-DOTA-maleimid, was necessary and therefore no fragmentation was obtained.

The optimization of the labeling procedure was performed in terms of coordination of selected lanthanides by the complexing moiety and the linking reaction of the complex to the ThioFab. Pre-optimization steps were performed with human serum albumin as model protein. By applying the optimized labeling procedure the labeling degree of the genetically modified ThioFab increased from 0.4% to 40%.

As a proof of concept the formation of the final ThioFab/ antigen conjugate employing Anti-Her2/ Her2 as model system was successfully demonstrated.

6. ZUSAMMENFASSUNG

Die Derivatisierung von Antikörpern mit elementmarkierten Liganden und deren Detektion mittels hochempfindlicher induktiv gekoppeltes Plasma Massenspektrometrie bietet die Möglichkeit zur indirekte Quantifizierung unterschiedlicher Analyten in biologischen und biomedizinischen Fragestellungen (z.B.: Krebs-Biomarker oder zelluläre Metabolite). Diese Art der Detektion findet beispielsweise Anwendung bei Immunoassays mit absoluter Quantifizierung in biologischen Proben. Die zu analysierenden Proben zeichnen sich durch eine komplexe Matrix, ein begrenztes Probenvolumen und niedrige Konzentrationen des Analyten (z.B. ein Biomarker) aus. Daher ist die Entwicklung von hochempfindlichen, selektiven und robusten Quantifizierungsstrategien erforderlich.

Ziel dieser Masterarbeit war die Entwicklung einer Markierungsmethode für ein gentechnisch verändertes Antigen bindendes Fragment (Fab), ein sogenannter ThioFab (Junutula JR et al. 2008), mit einem Elementlabel, bestehend aus dem Makrozyklus DOTA und einem Lanthanid als Chelation. Die Messung erfolgte über Flüssigkeitschromatographie kombiniert mit induktiv gekoppeltes Plasma-Massenspektrometrie (LC-ICP-MS).

Zum ersten Mal wurde eine Markierungsmethode für einen ThioFab mit einem Elementlabel entwickelt. Der Einsatz dieses ThioFabs, welcher ein rekombinant eingeführtes Cystein trägt, ermöglicht ein einfaches Labelingprozedere. Durch diesen eingeführten Cysteinrest ist lediglich ein milder Reduktionsschritt vor der Umsetzung mit dem Elementlabel Lanthanoid-DOTA-Maleimid, notwendig. Dadurch kommt es zu keiner Fragmentierung des ThioFabs und daher zur Beibehaltung der hohen Selektivität des Fab.

Die Optimierung der Markierungsmethode wurde einerseits im Hinblick auf die Koordinierung des ausgewählten Lanthanids durch den Komplexbildner DOTA und andererseits bezüglich der Reaktion des Komplexes mit dem ThioFab, durchgeführt. Vorversuche wurden mit humanem Serum Albumin als Modellprotein durchgeführt. Durch die Anwendung der optimierten Markierungsmethode wurde der Markierungsgrad des gentechnisch veränderten ThioFab von 0.4% auf 40% erhöht.

Als *Proof of Concept* wurde die Bildung des ThioFab/ Antigen Konjugates mit Anti-Her2/ Her2 als Modellsystem erfolgreich gezeigt.

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8. CURRICULUM VITAE

Teresa Mairinger, Bakk.rer.nat. MSc

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Personal Information

- o date of birth: 09-26-1988
- o place of birth: Lucerne, Switzerland
- o nationality: Austrian

Education

11/2012 – date **Master thesis**

"Elemental labeling of antibody fragments for LC ICP MS based immunoassay"

- supervised by ao. Prof.Dr.Regina Krachler, Institute of Inorganic Chemistry, University of Vienna and Prof. Dr. Stephan Hann, Department for Chemistry, University of Natural Resources and Life Science, Vienna

2009 - date **Master degree program Chemistry**

- with focus on analytical chemistry and food chemistry

University of Vienna, Austria

2009 - 2012 **Master degree program Nutritional Sciences**

- with focus on Molecular Nutrition
- master thesis (Feb 2011-Jan 2012): "Development of a multimethod for the analysis of photoinitiators migrating into foodstuffs" supervised by Prof.Dr.Karl-Heinz Wagner, Department of Nutritional Sciences, University of Vienna and Dr. Christoph Czerwenka, Competence Centre for Residue Analysis, Austrian Agency for Health and Food Safety
- March 2012: MSc; passed with distinction

University of Vienna, Austria

2006 - 2009 **Bachelor degree program Nutritional Sciences**

- bachelor thesis: "Flavonoide in der Ernährung- Sekundäre Pflanzeninhaltsstoffe in der Ernährung, am Beispiel der Flavonoide" supervised by Prof. Dr. Robert Ebermann, Department of Nutritional Sciences, University of Vienna
- July 2009: Bakk. rer. nat.

University of Vienna, Austria

Awards

2009 performance scholarship granted by University of Vienna

Publication

07/2013   Poster Presentation
"Elemental labeled antibody fragments for LC-ICP-MS based immunoassays" at the 4th international Symposium on Metallomics in Oviedo, Spain

11/2011   Poster Presentation
"GC-MS multimethod for the analysis of photoinitiators migrating from packaging material into foodstuff" at the 5th International Symposium on Recent Advances in Food Analysis in Prague, Czech Republic

Work Experience

07-10/2012   **Collaboration**
-   GC-MS method development for the analysis of phytosterols
work group of Prof. Dr. Karl-Heinz Wagner, Department of Nutritional Sciences, University of Vienna

11/2010   **Internship**
-   Cell culture, Western Blot and Immunodetection
work group of Prof. Dr. Hildegard Laggner, Department of Medical Chemistry, Medical University of Vienna

07/2010   **Internship**
-   Determination of food contaminants (GC-MS, HPLC-MS, LC-MS/MS)
Competence Centre for Residue Analysis, Austrian Agency for Health and Food Safety

02/2010   **Internship**
-   Immunoassays of human blood samples (ELISA)
Department of Nutritional Sciences, University of Vienna

03-06/2009   **Voluntary collaboration**
-   Taxidermy of guinea pig & intracellular microelectrode technique
work group of Prof. Dr. Christian Studenik, Department of Pharmacology and Toxicology, University of Vienna, Austria

07/2008   **Internship**
-   Microbiological quality control in food production:
Mautner Markhof Feinkost GmbH, Austria

Languages

German   mother tongue
English   fluent
French   fair