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Characterisation of Cetuximab by Different Mass Spectrometric Techniques

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The structural complexity of therapeutic monoclonal antibodies such as cetuximab requires comprehensive analytical strategies to verify identity, integrity and modification patterns throughout production and storage. In this work, cetuximab was characterised using a combined mass-spectrometric and electrophoretic approach aimed at evaluating both intact protein features and fragment-level information. Chemical stressing and enzymatic digestion with trypsin, papain and PNGase F were performed to generate targeted peptide and domain fragments, enabling the assessment of cleavage behaviour, glycan release and sequence-dependent digestion patterns. SDS-PAGE provided an initial separation and visualisation of heavy/light chains and digestion products, while MALDI-ToF mass spectrometry enabled mass profiling of intact subunits and peptide mixtures. Although only single-stage MS analysis was available, theoretical digestion and manual matching allowed preliminary sequence confirmation and evaluation of post-translational modification effects. Together, these complementary techniques demonstrated their utility in the structural characterisation of cetuximab.

Keywords: Cetuximab, Monoclonal antibodies, mAb Characterisation, Trypsin and Papain digestion, PNGase F.

INTRODUCTION

Monoclonal antibodies (mAbs) are often used in the biopharmaceutical industries as approved drugs. More than 40 mAbs and related products are used to treat inflammatory or oncology diseases. mAbs are theimmunoglobulins produced in a single cell line with a tetrameric structure composed of two light chains and two heavy chains. They undergo many posttranslational modifications such as phosphorylation, lipidation or glycosylation and therefore must be extensively characterised prior to use in patients. Heavy chain N-glycosylation of cetuximab can play a critical role in immune cytotoxic functions or plasmatic half-life. Other modifications during production or storage of mAbs can occur and could have a negative impact on patients [1,2]. Regarding that, must be considered the primary and tertiary structure and the correction of posttranslational modifications. The tertiary structure analysis is often done by X-ray crystallography or nuclear magnetic resonance spectroscopy, while mass spectrometry is used to consider the primary structure and possible modifications of proteins [3-5]. In past, protein sequencing was often done by Edman sequencing [6] but also modern technology like nanopore sequencing takes place [7]. Polyacrylamide gel electrophoresis is used to compare protein bands to reference standards [8]. The aim of this method is to confirm identity, molecular weight, quaternary structures of the proteins or to separate a complex protein mixture [6]. In this experiment a well-known mAb cetuximab is used for identification and characterisation. Cetuximab belongs to the immunoglobulins and is composed of two light and heavy chains. To identify and characterize cetuximab have been used, sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionisation (MALDI) coupled to time-of-flight mass spectrometry (ToF) [5].

Due to characterisation purposes provided cetuximab was first stressed or digested with different chemicals and enzymes followed by SDS-PAGE. After that, MALDI-ToF was used for identifications and characterisation purposes. Only the first stage mass spectrometry was possible because post decay source could not be used with all functions, meaning only the 1st stage mass spectrometry was possible [9]. The primary sequence alignment was planned to take place with Mascot Server, a search engine for identifying proteins and peptides using mass spectrometry data. Due to server errors, this method was substituted by theoretical sequence digestion and manual alignment [3].

Antibodies, known as immunoglobulins, are proteins produced in organisms as a reaction to certain substances that are known as antigens. Immunoglobulins are found in blood serum and extracellular fluid. The specific binding of antibodies to the antigens called paratope, represents a significant role in immune defense. Each antibody consists of two identical heavy chains and two identical light chains which are connected by four covalent disulfide bridges, to form a Y-shaped structure. The light chains each have of a variable (VL) and a constant (CL) domain. Each of the heavy chains have one variable domain (VH) and three constant domains (CH1, CH2, CH3). The VL and VH domain form the antigen binding site forms the paratope. Glycosylation sites are located on the CH2 domain, where post-translational modification with oligosaccharides occur. During papain digestion, the immunoglobulin is cleaved to a so called crystallizable fragment (F_c) due to its property to crystallize under specific conditions and the antigen binding fragment (F_{ab}) Fig. 1 [6]. Immunoglobulins are divided into subclasses with five different types of heavy chains, (α, δ, ε, γ, μ). The types of heavy chains differ in size and composition. Antibodies with the same subclass have the same constant region composition but differ in the variable region. Also, light chains can be divided into two classes (λ, κ), which have minor differences in primary sequence. Antibodies of the same class of light chain have the same composition in the constant region of the light chain, but differ in the variable region [10].

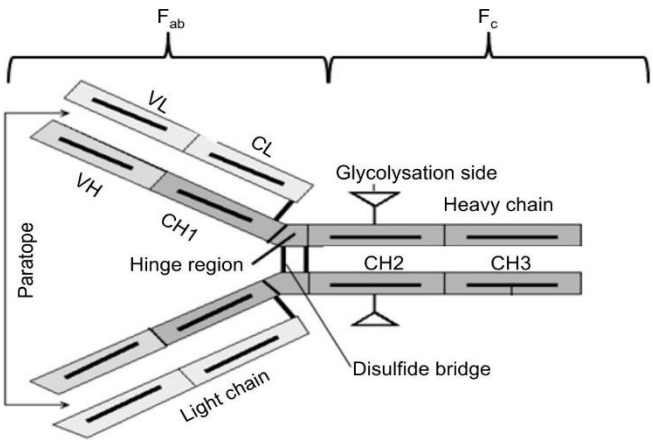

Fig. 1. Schematic structure of immunoglobulins, figure modified from [6]

The mAb used in this experiment was cetuximab, which is a recombinant chimeric human/mouse mAb produced in a single cell line and belongs to the immunoglobulins type G1. This means, cetuximab is composed by γ heavy chain and κ light chain.

Electrophoresis is an analytical technique for separation of proteins, enzymes, nucleic acids, glycoproteins or carbohydrates. It is used to separate anionic and cationic, small biosubstances or large biopolymers, as well as particles and cells with the help of electricity. The principle is based on the migration of charged molecules under the influence of a direct current field in aqueous solution, where the anionic or cationic species migrate to the poles with opposite charges. Electrophoretic separation is carried out in substrate-supported media, typically a polyamide gel or within free solutions. An electrophoresis apparatus consisted of anode and cathode was used to perform the separation [6]. In this experiment, a SDS-PAGE was used. This type of polyacrylamide gel electrophoresis separates denatured proteins based on their molecular weight instead of their charge. Sodium dodecylsulfate as anionic surfactant masks the charge of the protein to a uniform negative charge and therefore, a separation based on molecular weight can take place. Larger molecules migrate slower in direction to the anode than small molecules. After separation, the proteins are sorted by size and can be modified by *e.g.*, coomassie-brilliant blue for visualizing purposes. Also, a standard protein with known molecular size was separated by SDS-PAGE to compare weights of the standard with the proteins found in the sample [6].

Only molecular weight characterisation of the intact protein is not possible since there are too many proteins with similar molecular weights. For this reason, the analysed proteins are usually converted into smaller peptides by enzymatic digestion. This step increases the amount of measurable information [6]. In this experiment, one of the used enzymes for digestion was trypsin with defined cleavage sites. Trypsin cuts carboxyterminal of arginine, lysine, but only if the amino acids are not followed by proline. The number and mass of the resulting peptides are thus directly dependent on the primary structure of the protein [6].

Often, proteins are modified by glycosylation in post-translational modifications. For structure elucidation of the glycolsylated chains or the nonglycolysated protein, enzymatic digestion with PNGase F (35 kDa) is carried out to release the N-glycosidic bound oligosaccharides from an mAb [6]. PNGase F is able to modify asparagine residues to aspartic acid (deamidation) and therefore releasing the bound oligosaccharides [11]. Papain is an enzyme from the papaya plant, which can leave immunoglobulins in the hinge region after activation with L-cysteine. As the digestion result, F_c and $F_{ab}/2$ fragments, were analysed. It was observed that papain also cleaves F_c fragments into smaller fragments of ~ 10-12 kDa in time-course experiments [6,12].

Antibodies are often connected by disulphide bridges. To increase information in the analytical process, disulphide bonds are reduced with reducing agents such as mercaptoethanol, dithioreithol or in this case with *tris*(2-carboxyethyl) phosphine (TCEP). Reducing disulphide bonds is an equilibrium reaction that causes denaturation of the protein and to prevent the protein from join in the reverse reaction, the resulting thiol group is alkylated with iodacet amide (IAA) [6]. The matrices used for MALDI are carboxylic acids such as α-cyano-4-hydroxycinnamylic acid (HCCA), 2,5-dihydroxybenzoic acid or sinapine acid (SA). Usually, the matrix is mixed in excess with the analyte and applied to a target plate. During the subsequent drying process, the matrix and analyte cocrystallize. The right matrix choice of for MALDI depends on the molecule to be analysed. In several cases, different matrices yield comparable analytical results; however, certain molecules occasionally necessitate the use of a highly specific matrix within a defined concentration range to achieve optimal performance. The suitable matrix for solving a specific analytical problem can be determined empirically. The analyte-matrix ratio is usually 1:1,000 to 1:10,000. The prepared carrier

plate is inserted into the system and the resulting crystals are processed with a pulsed laser. The applied energy destroys the matrix crystals and evaporate the analyte molecules with the matrix molecules into the gas phase. MALDI ionisation technique results in positively or negatively charged ions with little to no fragmentation, this is why MALDI is considered as soft ionisation technique. Typical analytes are, for example, biomolecules such as proteins, peptides or polymers like polyethylenglycol [13]. Due to different ionisation models, the typical charging states for molecules are lower than in other soft ionisation techniques such as electrospray ionisation. In electrospray ionisation, the ionisation process is explain-able with three models, such as the charge residue model, the ion evaporation model, or the chain ejection model. All three models could explain molecules with multiple charging states. In MALDI, typically the matrix is ionised and the charge is transferred to analyte molecules causing for small peptides single or double charged ions and increasing for larger proteins the charging state up to z = 5.

In ToF, the ions formed in the ion source re accelerated with the help of a voltage U into the mass analyzer. The transferred kinetic energy E_{kin} to an ion depends on the charge state z of the ion. The separation according to differences in the mass-to-charge ratio (*m/z*) is made by the flight time t. Heavy molecules need longer to flight through flightpath *s* than lighter molecules and therefore need higher E_{kin} for the same acceleration, which results in the lower velocity v. After rearranging the equation and applying the rate-time-distance equation *m/z* depends on the acceleration voltage U (eqns. 1a and 1b).

$$E_{kin} = U \times z = \frac{1}{2} m \times v^2 \quad (1a)$$

$$\frac{m}{z} = \frac{2 \times U}{s^2} \times t^2 \quad (1b)$$

The ToF operates in two different modes, the so-called linear mode and the reflector mode. In linear mode, the accelerated ions hit the linear detection device opposite the ion source. In reflector mode, the accelerated ions are deflected over a grit and hit the reflection detector. When deflecting, the flight time is roughly doubled, which leads directly to a higher mass resolution. Another effect in reflector mode is the ions focus by the reflection grit, which also leads to a higher mass resolution than in linear mode [13].

EXPERIMENTAL

A series of solutions and reagents were prepared to enable the enzymatic digestion, reduction/alkylation, electrophoretic separation and mass-spectrometric characterisation of cetuximab. Two digestion buffers were prepared *viz*. digestion buffer No. 1 (50 mM) consisted of 50 mM NH_4HCO_3, 20 mM EDTA and 20 mM cysteine dissolved in water, while digestion buffer No. 2 (100 mM) contained the same components with NH_4HCO_3 adjusted to 100 mM. A reducing agent solution of 30 mg/mL tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was obtained by dissolving 47 mg TCEP in 1.56 mL water and an alkylation agent solution of 18 mg/mL iodoacetamide (IAA) was prepared by dissolving 31 mg IAA in 1.72 mL water. Both solutions were stored cold and protected from light. For gel electrophoresis, NuPAGE MES SDS running buffer (20×) was diluted by mixing 40 mL stock buffer with 760 mL water. Trypsin stock solution was provided by the supervisor and diluted 1:10 in water. For MALDI-ToF analysis, two matrix solutions were prepared by dissolving 3 mg α-cyano-4-hydroxycinnamic acid (HCCA) and 4 mg sinapinic acid (SA), respectively, in 200 µL of TA30 solution.

Several sample types were generated to characterize cetuximab under native, stressed, digested or chemically modified conditions. For oxidative stress treatment, a 0.05 % (v/v) H_2O_2 solution was prepared by diluting 30 % H_2O_2 and 45 µL of this solution was added to 5 µL cetuximab (5 µg/µL), followed by incubation at room temperature for at least 1 h. The PNGase F-treated samples were prepared by adding 40 µL of 50 mM ammonium bicarbonate to 5 µL cetuximab, followed by the addition of 2 µL recombinant PNGase F (500,000 U/mL) and incubating the mixture at 37 ºC for 3 h. Papain digestion was carried out by preparing papain at 0.1 mg/mL in Digestion buffer No. 1, activating it for 15 min at 37 ºC, then adding 5 µL activated papain and 40 µL Digestion buffer No. 1 to 5 µL cetuximab, followed by incubation for 3 h at 37 ºC.

Native antibody samples were prepared in two dilutions. For native mAb 1:10, 45 µL water was mixed with 5 µL cetuximab (5 µg/µL). For native mAb 1:5, 40 µL water was combined with 10 µL cetuximab. Reduced and alkylated samples were produced from three sample types *viz*., native mAb 1:10, H_2O_2-stressed mAb and PNGase F-treated mAb. To 20 µL of each sample, 25 µL digestion buffer No. 2 and 5 µL TCEP solution were added, followed by heating at 95 ºC for 5 min. After cooling, 8 µL IAA solution was added and the samples were incubated for 20 min at room temperature in the dark. A 10 µL aliquot of each reduced and alkylated sample was transferred to a new tube, mixed with 1 µL trypsin stock (1:10) and digested overnight at 37 ºC.

For SDS-PAGE analysis, reduced samples were mixed with 4× sample buffer and water by combining 6 µL sample, 5 µL buffer and 9 µL water. Unreduced samples were prepared by mixing 2 µL sample with 5 µL buffer and 13 µL water. The following samples were analysed: reduced/alkylated native mAb 1:10, reduced/alkylated H_2O_2-stressed mAb, reduced/alkylated PNGase F-treated mAb, papain-digested mAb, native mAb 1:10, H_2O_2-stressed mAb and PNGase F-treated mAb. All samples were loaded onto an SDS-PAGE gel and electrophoresed in running buffer for 25 min. For MALDI-ToF mass spectrometry, each sample was mixed in a 1:9 ratio with matrix solution. The analysed samples included native mAb 1:5 and 1:10, H_2O_2-stressed mAb, papain-treated mAb, reduced and alkylated samples and all trypsin-digested variants. Tryptic digests were combined with the HCCA matrix, while all other samples were mixed with the SA matrix. One microliter of each sample-matrix mixture was spotted onto a MALDI target plate and allowed to dry at room temperature for 30 min before analysis.

RESULTS AND DISCUSSION

Cetuximab belong to the immunoglobulin family and is well characterised. It consisted of two light and heavy chains

with a molecular weight of 145781.6 Da composed with chemical formula $C_{6484}H_{10042}N_{1732}O_{2023}S_{36}$ [14]. The F_c fragment weight approximately 50 kDa and the F_{ab} fragment approx. 100 kDa resulting in an average weight of 150 kDa [14]. Ayoub *et al.* [15] stated the weight of the glycosylated cetuximab with 152235 Da, a light chain with 23369 Da (214 amino acids) and a heavy chain with 49372 Da (449 amino acids). Based on that, average weight of N-glycans should be around 6 kDa, which indicates four glycosylation sides with an average weight of 1.5 kDa, two glycosylation sides at asparagine 299 and 88 in each heavy chain [16].

To improve clarity, a summary table comparing theoretical and experimentally observed molecular weights for intact cetuximab, its chains and major digestion fragments has been shown in Table-1, which also consolidates the theoretical literature values with experimental SDS-PAGE and MALDI-ToF data obtained in this study, providing improved clarity.

The sequence of cetuximab in heavy chain and light chain is given in Fig. 2. Another sequence for cetuximab was shown in Fig. 3, introduced as the correct sequence for cetuximab. The orange marked amino acids differ from source [14] to source [15].

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>Cetuximab heavy chain
QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSGGNTDYN
TPFTSRLSINKDNSKSQVFFKMNSLQSNDTAIYYCARALTYYDYEFAYWGQGTLVTVSAA
STKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG
LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGP
SVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS
TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ
QGNVFSCSVMHEALHNHYTQKSLSLSPGK

>Cetuximab light chain
DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESISGIPS
RFSGSGSGTDFTLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELKRTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT
LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
```

Fig. 2. Letter code for heavy and light chain of cetuximab [14]

SDS-PAGE: For the samples prepared, the SDS-PAGE was performed in Fig. 4. Sample K shows the spectra multicolour broad range protein ladder with 10 bands at 235, 120, 80, 70, 50, 40, 30, 25, 15 and 10 kDa. Based on the bands of the reference standard, the bands for the different prepared samples were assigned. For sample A, the reduced and alkylated native mAb 1:10, the gel displayed two bands at 65 kDa and 31 kDa.

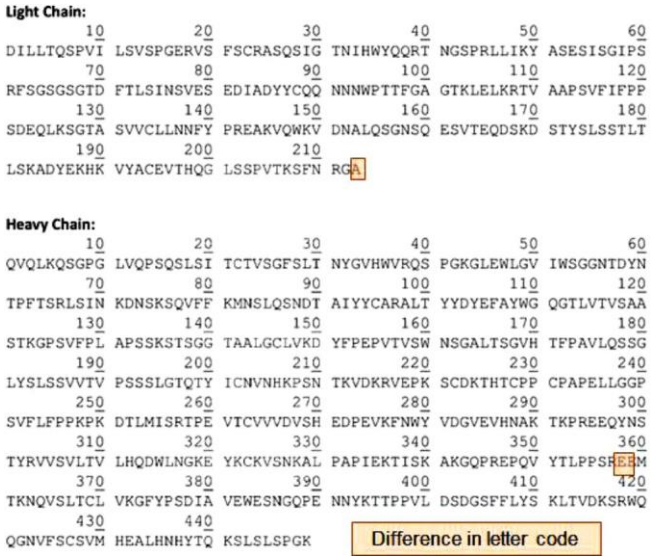

Fig. 3. Letter code for heavy and light chain of cetuximab modified from [15] with differences in the letter code found in [14]

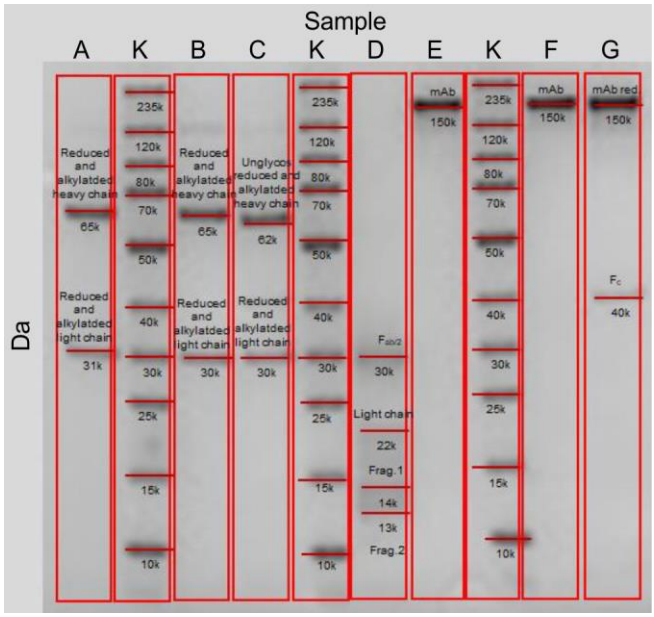

Fig. 4. SDS-PAGE analysis of cetuximab under reducing and non-reducing conditions. The gel shows separation of intact mAb, reduced heavy and light chains, deglycosylated fragments and papain digestion products. Expected molecular weight ranges for each band are indicated to clarify the identification of major structural domains

TABLE-1
COMPARISON OF THEORETICAL AND OBSERVED MOLECULAR WEIGHTS OF CETUXIMAB, ITS CHAINS AND MAJOR FRAGMENTS

Component	Theoretical MW (Da)	Observed MW (SDS-PAGE)	Observed MW (MALDI-ToF)
Intact cetuximab (glycosylated)	152,235	~150 kDa	152,220
Heavy chain	49,372	~50 kDa	~49–50 kDa (if detected)
Light chain	23,369	~25–30 kDa	23,637
Papain fragment (large)	–	30 kDa	9,791–8,353 (digested peptides)
Papain fragment (medium)	–	22 kDa	Not detected (converted to peptides)
Papain fragments (small)	–	14–13 kDa	7,500–16,000 Da (peptide range)
PNGase F major fragments	–	~40 kDa (deglycosylated Fc)	24,300/24,051/23,938/23,891
PNGase F peptides	–	–	12,139/11,823/8,588/7,881

Sample B, the reduced and alkylated stressed mAb, displayed two bands at 65 kDa and 30 kDa. For sample C, the reduced and alkylated PNGase F treated mAB, displayed two bands at 62 kDa and 30 kDa. For sample D, the papain digested sample, displayed four fainted bands at 30, 22, 14 and 13 kDa. Sample E, the native mAb 1:10 and sample F, the H_2O_2 stressed mAb, displayed one single band at 150 kDa. Sample G, the PNGase treatet mAb, displayed two bands at 150 kDa and 40 kDa.

The bands in sample E and F represents the hole mAb cetuximab. The H_2O_2 stressed sample F showed no changes due to oxidation compared to the diluted mAb in sample E. Resolution in SDS-PAGE was too low, to observe the change for adding ^{16}O at a methionine residue. Based on the Knowledges, sample G should theoretically display a band at approximately 146 kDa because of the released N-glycosylation moieties located on VH and CH2 of the heavy chain [16], but the mass resolution in SDS-PAGE was not proper to show the difference between 152 kDa and 146 kDa. The second, fainted band at 40 kDa for sample G was suggested as F_c fragment, which lost all N-glycans with a theoretical weight of approximately 46 kDa. A way to find out the primary sequence of the band at 40 kDa, was that the band could be cut out of the gel and prepared for MALDI-ToF with post source decay for analysis. After sequencing, the hypothesis of suggested F_c fragment could be stated or not. Papain could cleave in the hinge-region of a mAb, resulting in F_c and $F_{ab}/2$. Theoretical weight of F_c fragment was 50 kDa and for $F_{ab}/2$ fragments 50 kDa as well. No band was observed for 50 kDa in-sample D, but smaller bands at 30, 22, 14 and 13 kDa. These bands are explainable based on the knowledge, that papain cleaves immunoglobulins into $F_{ab}/2$ and F_c fragments over time [12] and also further conversion of fragments into smaller peptides could not be ruled out. The band at 30 kDa can be assigned to residues from glycosylated $F_{ab}/2$ domain (approx. 23 kDa + 2 kDa) and 22 kDa for a light chain or papain [17]. The smaller fragments of 13 and 14 kDa could belong to the $F_{ab}/2$ and F_c domain formed by further digestion with papain. Further investigations by mass spectrometry could clear up uncertainties. For reduced and alkylated samples, different reduced fragments with 25+X, 50+X, 75+X, 100+X, 125+X kDa could be suggested. X represents the added mass due to alkylation reaction for amino acid cysteine. Based on the letter code [14], 11 possible acetyl amine (11*58 Da) groups could be added to the heavy chain and five to the light chain by reducing and alkylation. The reduced samples A, B and C showed he same pattern with bands at approx. 65 kDa representing the reduced and alkylated heavy chain and the band at approximately 30 kDa was assigned to the reduced and alkylated light chain. The difference of sample C in band 62 kDa resulted to a possible loose in N-glycans cleaved of from cetuximab heavy chain by PNGase F treatment. For the lower mass bands, the resolution was higher than for higher mass bands. But also, no significant differences in sample A and B could be observed. Mass spectrometry is more powerful to resolve mass shifts of *m/z* 16 or 32 for adding ^{16}O and therefore oxidation sensitivity should be evaluated with MALDI-ToF.

To summarize the results observed from SDS-PAGE, could not be observed untypical bands for cetuximab. This leads to the conclusion, that the analysed mAb has suggested characteristics for cetuximab.

The distinction between the experimentally confirmed findings and hypotheses has now been clarified. The results obtained directly from SDS-PAGE and clearly detected MALDI-ToF peaks (*e.g.* intact mAb, reduced heavy and light chains) represent confirmed experimental observations. In contrast, interpretations related to oxidation sensitivity, assignment of small fragments and peptide-level sequence attribution are presented as hypotheses because they are based on single-stage MALDI-ToF data and manual peptide matching. Without MS/MS fragmentation, these interpretations cannot be confirmed with full confidence and should be considered tentative.

Stressed antibody: In this experiment, spectra for H_2O_2 stressed and reduced as well as alkylated cetuximab have been measured (Fig. 5). The used matrix compound for ionisation was SA and the spectra were recorded over *m/z* 5000-170000 in linear mode with positive ionisation. Fig. 5a for the stressed mAb showed peaks at *m/z* 152220, 78000 and 10000, which could be assigned to the hole stressed mAb, the stressed mAb losing a light and heavy chain (-75000 kDa) and different fragments were formed from stressed mAb. Fig. 5b representing the stressed, reduced and alkylated mAb showed peaks at *m/z* 99230, 23637, 11818 and 9000 representing the stressed mAb losing two light chains, light chains and different fragments formed from stressed mAb.

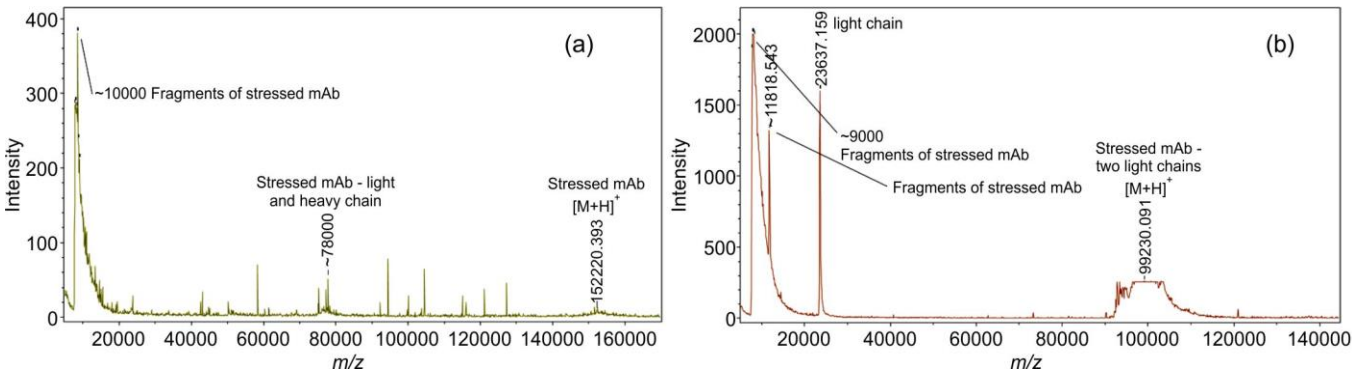

Fig. 5. MALDI-ToF spectra of stressed and reduced/alkylated cetuximab in linear positive mode (*m/z* 5000-170000). Major peaks corresponding to intact stressed mAb, chain losses and oxidation-related fragments are annotated. This figure highlights how chemical stressing and reduction alter the mass distribution of cetuximab

N-Glycosylated cetuximab has a weight of 152235 Da [15] and the obtained *m/z* for protonated stressed cetuximab was 152220 Da. The obtained peak matches the theoretical value. The peak at *m/z* 78000 showed significant gain of weight compared to the theoretical weight of a light and heavy chain of 72741 Da added up with the approximately 3 kDa of the N-glycosylation sides to 75741 Da. Without further information, possible fragments for the peak at 10000 could not be assumed but implies that cetuximab is sensitive to oxidation. The comparing results from MALDI-ToF to SDS-PAGE, MALDI sample was stored one week in the freezer prior to analyzation. In SDS-PAGE no fragments in the lower mass range at 10 kDa were obtained, while the resulting peaks in MALDI-ToF had to be formed during storage phase.

For reduced and alkylated, stressed cetuximab, no peak for the hole mAb could be observed but a peak at *m/z* 99230 (cut off by detector overload) representing the mAb losing two light chains by reduction. The weight matches the approximately weight of 94000 Da for two heavy chains added by approx. 6000 Da for N-glycosidic residues. Also, the peak at *m/z* 23637 is comparable to the theoretical value for a light chain *m/z* 23369 gaining weight of approximately *m/z* 270 showing slight mass gain, perhaps by alkylation and/or oxidation. Nevertheless, also fragments at lower mass range showed sensitivity to oxidation for cetuximab during storage. A temperature and time dependent experiment could clarify right the storage conditions for cetuximab.

The use of single-stage MALDI-ToF in this study introduces inherent limitations in peptide identification and post-translational modification analysis. Without MS/MS fragmentation data, peptide sequence confirmation, discrimination between isobaric residues and reliable localisation of oxidation or glycan-related mass shifts cannot be achieved with high confidence. Since, peptide interpretation relied on theoretical digestion and manual matching, these assignments must be considered tentative. Incorporating MS/MS-capable workflows or automated database searching would significantly increase sequence coverage and analytical certainty.

A short comparison between MALDI-ToF and ESI-MS workflows is useful to contextualize the analytical depth of the present study. MALDI-ToF provides rapid mass profiling, allows detection of intact biomolecules and is well suited for identifying major subunits or fragmentation patterns. In contrast, electrospray-ionisation mass spectrometry (ESI-MS), particularly when coupled with MS/MS, typically offers higher mass accuracy, improved sequence coverage and more reliable localisation of post-translational modifications. Moreover, the present work relied exclusively on single-stage MALDI-ToF, the analytical depth is inherently more limited than that achievable with ESI-based middle-down or bottom-up workflows. This contextual difference is important for interpreting the level of structural detail obtained.

Papain digested antibody: Papain digested, reduced and alkylated sample was measured *via* MALDI-ToF with SA as matrix compound in linear mode and positive ionisation (Fig. 6) over the mass range *m/z* 5000-17000. No peaks in the higher mass range could be found and a increase of *m/z* 5000-27000 region was displayed with assigned peaks at *m/z* 9791, 9350, 8909, 8353 and 7991.

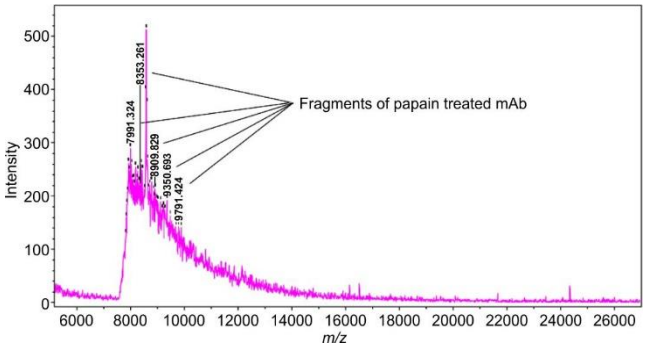

Fig. 6. MALDI-ToF spectrum of papain-digested, reduced and alkylated cetuximab. The broad peak distribution (*m/z* 7500-16000) reflects peptide mixtures formed during enzymatic digestion and storage. This figure illustrates the transition from defined SDS-PAGE fragments to complex peptide mixtures in mass spectrometry

The peaks in the papain digested cetuximab spectra was randomly assigned by software algorithm and did not represent the peak itself. Mass spectra from small compounds show a clear peak shape with normal distribution and a peak top representing the *m/z* of the measured compound. In this case, the peak for papain digested sample shows distribution over a mass range of *m/z* 7500-16000 representing different peptides formed from cetuximab which cannot be separated in linear mode. Compared to SDS-PAGE results, the fragments with 30 kDa and 22 kDa disappeared. During sample storage these fragments were digested to smaller peptides in between 7500-16000 Da. The band of 22 kDa in SDS-PAGE was with mass spectrometry clearly assigned to a fragment of cetuximab. In MALDI-ToF spectra, no peak for approximately 22 kDa was found, so this fragment formed in sample preparation must be digested during storage and therefore could not be papain.

PNGase treated antibody: PNGase F treated, reduced and alkylated sample was measured with MALDI-ToF in linear mode and positive ionisation over a mass range of *m/z* 5000-170000 and a rise of region *m/z* 5000-30000 were displayed (Fig. 7). Fragments with *m/z* 24300, 24051, 23938, 23891, 23865, 12139, 11823, 8588 and 7881 could be found.

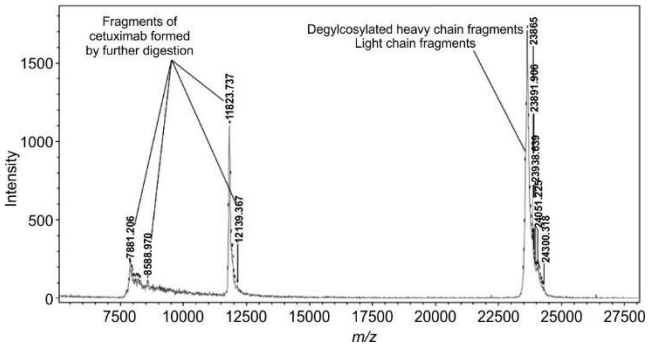

Fig. 7. MALDI-ToF spectra of PNGase F-treated cetuximab showing deglycosylated fragments and secondary digestion products. Peaks around *m/z* 24300-23800 correspond to major deglycosylated fragments, while peaks near *m/z* 12000-8000 represent further cleavage. This figure supports the assessment of glycan removal and fragmentation behaviour under PNGase F treatment

As seen in SDS-PAGE, cetuximab was first deglycolsated followed by reducing and alkylation of disulfide bridge to form deglycolysated heavy chains and light chains. In the

obtained mass spectra different fragments with *m/z* 24000 were recorded, representing different fragments of light chain and possible heavy chain fragments. No peaks in the higher mass range were recorded, so it was possible that also deglycosylated heavy chain and light chain were digested into smaller fragments over the displayed mass range. This hypothesis matches with the obtained fragments at *m/z* 12139 and 11823. Both fragments give in sum a possible weight of approx. 24 kDa. Also, the fragments at *m/z* 8588 and 7881 could be formed by further digestion during storage of sample.

Tryptic digested antibody: For MALDI experiment, native mAb, stressed mAb and PNGase F treated mAb were tryptic digested, reduced and alkylated. For the Spectrum Analysis Report an alignment with Mascot was not possible due to server errors, so a manual alignment takes place. The used letter code differs from literature [15] in position 358 of the heavy chain (D=E) and 213-214 of the light chain (EC=A) and matches another found letter code for cetuximab [14]. A theoretical tryptic digestion on the lettercode was performed by the software with and without possible carboxymethyl modification from alkylation.

To support interpretation of the peptide-mapping results, it is important to note that the large extraction tables compile many peptides with overlapping theoretical masses and small *m/z* differences. Since, the single-stage MALDI-ToF does not provide MS/MS fragmentation, some peptides cannot be distinguished solely by mass. For this reason, the tables should be understood as summarised peptide-coverage information rather than definitive sequence confirmation, which improves clarity and accessibility for the reader.

The covered sequence found in the tryptic digested mAb are shown in Tables 2-4 with the measured *m/z*, theoretical *m/z*, found sequence in letter code, the deviation between theoretical and measured *m/z* in Da, the domain and position of the found sequence. The allowed tolerance for deviation was set to 5 Da.

For the peak in MALDI-ToF at *m/z* 1266 were found four possible fragments within the native mAb, stressed mAb and PNGase F treated mAb. All fragments from position 136-149, 329-340 and 325-336 from the heavy chain and position 50-61 from the light chain have an acceptable deviation from less than ± 2 Da. Information obtained in this MALDI-ToF experiment is not sufficient to select a favourite sequence for the alignment. Also, for fragments *m/z* 755 and *m/z* 1875 two different fragments could be possible. Further information was necessary to determine the correct sequence for the peak at *m/z* 755, 1266 and 1875. A MALDI-ToF experiment with post decay source could help for this task but, was not available at the time of experiment. Most found sequences lay between *m/z* 1200-2600. Only two fragments with *m/z* < 1000 were found. It can be mentioned that fragments formed by tryptic digestion with a sequence length of 12-24 amino acids could be analysed with this method. Smaller or bigger fragments are less likely to be found. From theoretical digestion also fragments up to *m/z* 7000 was possible to found but to receive such big fragments only a few possible cleavage sides

TABLE-2
EXTRACTED SPECTRUM ANALYSIS REPORT FROM TRYPTIC DIGESTED NATIVE mAb; ALLOWED TOLERANCE 5 Da, *m/z* AND THEORETICAL *m/z* FROM THE ALIGNED SEQUENCE WITH DEVIATION IN Da, DERIVED FROM DOMAIN HEAVY OR LIGHT CHAIN AND POSITION IN LETTER CODE, GREEN = SPECIFIC CETUXIMAB FRAGMENT[a]

m/z	*m/z* theor.	Found sequence	Deviation (Da)	Domain	Position
1266.696	1266.742	VSNKALPAPAIEK	-0.045	Heavy chain	329-340
1266.696	1267.762	ALPAPIEKTISK	-1.066	Heavy chain	325-336
1266.696	1266.632	YASESISGIPSR	0.064	Light chain	50-61
1788.500	1788.878	ASQSIGTNIHWYQQR	-0.378	Light chain	25-39
1797.481	1797.895	SGTASVVCLLNNFYPR 8: Carbamidomethyl (C)	-0.414	Light chain	127-142
1854.444	1849.821	MNSLQSNDTAIYYCAR	4.623	Heavy chain	82-97
1875.433	1873.922	TTPPVLDSDGSFFLYSK	1.511	Heavy chain	395-411
1875.433	1875.927	VYACEVTHQGLSSPVTK 4: Carbamidomethyl (C)	-0.494	Light chain	197-207
1923.557	1924.075	DILLTQSPVILSVSPGER	-0.518	Light chain	1-18
1945.478	1946.027	TVAAPSVFIFPPSDEQLK	-0.549	Light chain	109-126
2101.379	2102.128	RTVAAPSVFIFPPSDEQLK	-0.749	Light chain	108-126

[a]Blast against κ light chain or γ heavy chain

TABLE-3
EXTRACTED SPECTRUM ANALYSIS REPORT FROM TRYPTIC DIGESTED STRESSED mAb; ALLOWED TOLERANCE 5 Da, *m/z* AND THEORETICAL *m/z* FROM THE ALIGNED SEQUENCE WITH DEVIATION IN Da, DERIVED FROM DOMAIN HEAVY OR LIGHT CHAIN AND POSITION IN LETTER CODE, GREEN = SPECIFIC CETUXIMAB FRAGMENT[a]

m/z	*m/z* theor.	Found sequence	Deviation (Da)	Domain	Position
1266.696	1267.762	ALPAPIEKTISK	-1.066	Heavy chain	329-340
1266.696	1266.742	YASESISGIPSR	0.064	Light chain	50-61
1734.478	1733.980	LLIKYASESISGIPSR	0.499	Light chain	46-61
1788.497	1788.878	ASQSIGTNIHWYQQR	-0.381	Light chain	25-39
1797.461	1797.895	SGTASVVCLLNNFYPR 8: Carbamidomethyl	-0.434	Light chain	127-142
1849.400	1849.821	MNSLQSNDTAIYYCAR	-0.421	Heavy chain	82-97
1923.527	1924.075	DILLTQSPVILSVSPGER	-0.548	Light chain	1-18

[a]Blast against κ light chain or γ heavy chain

TABLE-4
EXTRACTED SPECTRUM ANALYSIS REPORT FROM TRYPTIC DIGESTED PNGase F TREATED mAb; ALLOWED TOLERANCE 5 Da, m/z AND THEORETICAL m/z FROM THE ALIGNED SEQUENCE WITH DEVIATION IN Da, DERIVED FROM DOMAIN HEAVY OR LIGHT CHAIN AND POSITION IN LETTER CODE, GREEN = SPECIFIC CETUXIMAB FRAGMENT[a]

m/z	*m/z* theor.	Found sequence	Deviation (Da)	Domain	Position
755.344	755.409	SQVFFK	-0.065	Heavy chain	76-81
755.344	755.350	VSFSCR 5: Carbamidomethyl (C)	-0.007	Light chain	19-24
869.403	869.357	SFNRGEC 7: Carbamidomethyl (C)	0.046	Light chain	208-214
1266.575	1267.762	ALPAPIEKTISK	-1.187	Heavy chain	329-340
1266.575	1266.742	VSNKALPAPIEK	-0.167	Heavy chain	325-336
1266.575	1266.632	YASESISGIPSR	-0.058	Light chain	50-61
1266.575	1264.657	STSGGTAALGCLVK	1.918	Heavy chain	136-149
1286.602	1286.674	EPQVYTLPPSR	-0.072	Heavy chain	347-357
1672.363	1671.809	TKPREEQYNSTYR	0.554	Heavy chain	291-303
1788.789	1788.878	ASQSIGTNIHWYQQR	-0.088	Light chain	25-39
1797.351	1797.895	SGTASVVCLLNNFYPR 8: Carbamidomethyl (C)	-0.544	Light chain	127-142
1874.261	1873.922	TTPPVLDSDGSFFLYSK	0.339	Heavy chain	395-411
1877.246	1875.927	VYACEVTHQGLSSPVTK 4: Carbamidomethyl (C)	1.319	Light chain	191-207
1904.244	1904.942	EPQVYTLPPSREEMTK	-0.699	Heavy chain	347-362
1923.871	1924.075	DILLTQSPVILSVSPGER	-0.204	Light chain	1-18
1945.294	1946.027	TVAAPSVFIFPPSDEQLK	-0.732	Light chain	109-126
2101.208	2102.128	RTVAAPSVFIFPPSDEQLK	-0.92	Light chain	108-126
2542.670	2544.131	GFYPSDIAVEWESNGQPENNYK	-1.461	Heavy chain	373-394

[a]Blast against κ light chain or γ heavy chain

must be digested by trypsin. For this experiment, the reaction for tryptic digestion was performed overnight, so it might be more likely to have smaller fragments. The smaller fragments than *m/z* 750 could also not be measured in this experiment because of the cut-off from calibration range.

The fragment with theoretical *m/z* 1849 could be found in native and stressed mAb, which was formed by tryptic digestion to form a fragment with position 82-97 from the heavy chain. This fragment contains a methionine at position 82, which can be oxidised with H_2O_2 and it can be mentioned that position 82 was not oxidised in the stressed mAb. Other possible oxidation sides at position 254 or 429 of the heavy chain could not be evaluated. No fragment with this oxidation side could be found in native and stressed mAb. The sensitivity to oxidation of cetuximab in the MALDI experiment remains unclear and cannot be conclusively assessed. From spectra of stressed mAb further investigations for oxidation sensitivity of cetuximab was needed.

The fragment with theoretical *m/z* 1849 was also interesting for not being found in the PNGase F treated mAb. At this fragment, at position 87 of the heavy chain was a N-glycolysation side reported [16] and was not released by PNGase F. N-Glycolysation is a post-translational modification with sugar moieties with different structure and molecular weight. From this fragment, it can be mentioned that modification with oligosaccharides at the asparagine residue cannot be described as complete. Otherwise, this fragment could not be found in native and stressed mAb. The fragment with the theoretical *m/z* 1671 with position 291-303 of the heavy chain contains at position 299 the other possible N-Glycolysation side of cetuximab. This fragment could only be found in the PNGase F treated sample. Since the treatment with PNGase F release the bound sugar chains from the mAb, this fragment was not likely found in native or stressed mAb.

Another information was gained by fragment *m/z* 1904 with position 347-362 of the heavy chain. The used letter code for sequence alignment differs in position 358 of the heavy chain (D=E). From this experiment it can be stated that the correct amino acid in this fragment was glutamic acid (E) and not aspartic acid (D). The used sequence for manual alignment was not representing the correct primary sequence of cetuximab. Also, the used code differs in position 213-214 of the light chain from the "correct" letter code for cetuximab [15]. This seemed to be not the correct sequence, due to the fact that fragment *m/z* 869 confirms the sequence SFNRGEC found in [14].

In the manual sequence, alignment only modification with carboxymethyl from alkylation reagent IAA was selected. This reveals four confirmed alkylated cysteine in the sequence of the light chain at position 23, 134, 194 and 214. For oxidised sample, there was no possible modification with oxygen performed, maybe this could reveal oxidation sides in stressed sample. Reviewing again the raw data with the possible modification, could be revealed the lack of information about oxidation stage of cetuximab.

Conclusion

In this work, different mass spectrometric techniques were performed to identify and characterise cetuximab. No untypical bands in SDS-PAGE was identified whereas MALDI-ToF has shown typical fragments, which can be assigned to cetuximab but also to other immunoglobulins. The covered sequence found in spectrum analysis report from tryptic digested native, has shown stressed and PNGase F treated mAb with possible N-glycolysation sides, confirmed alkylation sides, confirmed unoxidised oxidation sides, trypsin cleavage sides and deviation from the used sequence. Five specific fragments for cetuximab could be found in the variable region of light

and heavy chain, which identifies the analysed mAb as cetuximab. Furthermore, some unspecific fragments from constant region of the light and heavy chain could be found to confirm the subclass of the analysed mAb. Due to the fact, that the post decay source did not work for this experiment, sequence coverage of 33% for 1st stage mass spectrometry was a good result. Most of the found sequence were located in the light chain of the mAb, while only few sequences were found in the heavy chain. This could be caused by post translational modification with N-glycans in the heavy chain. This hypothesis is proved by the fact, that in the PNGase F treated sample more sequence fragments could be found. One possible oxidation site appeared unoxidised in this study; however, without MS/MS confirmation and considering possible storage effects, this observation remains preliminary and cannot be interpreted conclusively. Most of the found fragments were 10-20 amino acids long. The theoretical digestin performed with trypsin as digestion agent could theoretically form fragments smaller or longer than 10-20 amino acids. Smaller fragments could not be found due to mass cut-off from calibration. Larger fragments may not be found due to the allowed deviation of 5 Da from theoretical mass.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

DECLARATION OF AI-ASSISTED TECHNOLOGIES

The authors declare that no AI tools were used in the preparation or writing of this research/review article.

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