Boosting mass spectrometry-based analytics for biopharma

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Chapter 2

Fast and automated characterization of antibody variants with 4D-HPLC/MS

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2.1 Abstract

Characterization of unknown monoclonal antibody (mAb) variants is important in order to identify their potential impact on safety, potency and stability. Ion exchange chromatography (IEC) coupled with UV detection is frequently used to separate and quantify mAb variants in routine quality control (QC). However, characterization of the chromatographic peaks resulting from an IEC separation is an extremely time consuming process, involving many cumbersome steps. Presented here is an online four-dimensional high performance liquid chromatography-mass spectrometry (4D-HPLC/MS) approach, developed to circumvent these limitations. To achieve this a 2D-HPLC system was extended through the introduction of additional modules, hence enabling fully automated bioseparation of mAbs, fractionation of peaks, reduction, tryptic digestion and reversed-phase (RP) separation of resulting peptides followed by MS detection. The entire separation and analytical process for an unknown peak is performed in less than 1.5 hours, leading to a significant time saving, with comparable sequence coverage. To show the comparability with the traditional offline process, a proof of concept study with a previously characterized mAb1 is presented in this paper.

2.2 Introduction

The chemical amino acid modifications of therapeutic antibodies have been extensively investigated and well reviewed.[2] The most common degradations are oxidation of methionine and tryptophan, deamidation of asparagine, isomerization of aspartate and glycation of lysine residues[3-10] Monitoring of these modifications is important, because they show altered stability as well as an impact on biological function, especially deamidation of asparagine residues located in the complementarity-determining regions (CDRs).[11-15] In addition to deamidation, methionine oxidation also requires monitoring, due to the fact that oxidized mAbs are known to influence in vivo product stability, receptor binding, structure and exhibit accelerated plasma clearance.[16-18]

Normally characterization of mAb modifications is a time-consuming and multi-step offline process, where the samples are fractionated, reduced and alkylated followed by tryptic digestion for several hours. More recently Native MS is receiving increased application,[19] however the structural information obtained is only protein specific and pre-fractionation or post-digestion is still required to localize the detected modifications.

Tryptic digestion in solution presents some disadvantages including trypsin autodigestion, long incubation times and low efficiency.[20] Several methodologies have been reported to improve or automate the digestion step of proteins,[21,22] however in many cases their applicability to routine analysis is limited due to the complexity of the system. López-Ferrer et al. described a system with a pressurized loop, which enabled online digestion of protein samples.[23] In addition, immobilization of trypsin on a monolithic column to generate a trypsin bioreactor, so called immobilized enzyme reactor (IMER) was reported.[24] Hedström et al. discussed a miniaturized system where trypsin was immobilized in a fused silica capillary directly followed by a reversed phase separation.[25] These systems all involve direct digestion of the introduced protein sample, without the possibility to separate variants up-front. The main disadvantage of such methodologies is that protein samples, in reality, often consist of a mixture of different protein variants, and subsequent results are complicated by this heterogeneity.

In 2011, Alvarez et al. presented a 2D LC-MS technique for online reduction, separation and detection of mAb variants. The method enabled detection and localization of larger modifications to the respective reduced protein chains, however the system configuration was rather complex.[26] In a recent publication we presented a fully automated online 2D system also enabling online reduction and separation.[27] These 'middle-down' approaches, whilst informative, share the limitation that the modifications measured are not localizable to the amino acid level (or at least the peptide) and nor are the methods sensitive enough to enable detection of smaller modifications such as deamidation (*m/z* delta 1 Da).

More recently, a 1D method applying ion exchange chromatography directly coupled with

a mass spectrometer for native ESI-MS analysis was reported. The system proved effective for determination of larger mass-shift modifications, however smaller modifications such deamidation, required additional sample preparation steps.[28] Implementing digestion with IdeS prior to the IEC analysis enabled location of the modification to the Fc or Fab fragment, but for the exact position additional peptide mapping would be required.

Recently several different approaches have been presented in the field of mAb analysis using 2D-HPLC, for example the analysis of mAb breakdown products. [29] For a recent overview about different methods for antibody characterization please refer to in Sandra et al.[30]. Different chromatographic support combinations have been tested, such as strong cation exchange (SCX) combined with reversed-phase (RP), RP combined with RP and hydrophilic interaction chromatography (HILIC) combined with RP for the analysis of digested mAbs.[31] Benefits of this technology for mAb analysis have been illustrated by Sandra et al..[32] The combination of cation exchange chromatography (CEX) with RP was also shown to be a powerful tool for separation of mAb fragments or peptides especially combined with MS detection.[33]

Another method, developed by Tran et al., including an IEC separation, on-line reduction and on-line digestion with electrospray time-of-flight (ESI-TOF)-MS characterization has also been reported. The technique was however only applied to smaller proteins and required a customized capillary chromatographic system using a stop-flow technique.[34] Full automation and online digestion of large molecules, which is necessary for pharmaceutical drug product quality control, was not presented.

In this article we describe for the first time a fully automated 4D HPLC-MS system for online separation, reduction, digestion and peptide mapping which addresses the above mentioned disadvantages. The system enables automated fractionation of IEC-separated charged variants, online reduction and tryptic digestion, subsequent chromatographic resolution of the generated peptides and detailed characterization through online coupling to ESI-MS/MS. To achieve full automation of this process, a standard, commercially available, 2D-HPLC was extended by the introduction of further standard modules. A significant advantage of our system is that no adjustments to the IEC separation conditions or buffers is required for introduction to the MS, but rather the methods of commercial QC quality control can be directly used. The system is simple to configure and the resulting methods robust and user-friendly. As such the application of the method in research and development labs is highly feasible and offers tremendous improvements over current workflows. In this paper a proof of concept study of our system with a mAb, previously characterized by standard offline analytical methods[1] is presented.

2.3 Experimental Section

2.3.1 Production of stressed material

To generate stressed material for characterization of mAb variants, a recombinant IgG1 antibody expressed in Chinese hamster ovary cells, hereafter described as mAb2, was used (Roche Diagnostics GmbH, Penzberg, Germany). The hydrophibicity is comparable to a standard IgG1 and the isoelectric point is 9.1. The concentration of the formulation was 25 mg/mL in a 20 mM His-HCl (Sigma-Aldrich, Missouri, USA) buffer system at pH 5.5. The generation of antibody variants, including deamidated, oxidized and glycated versions of mAb2, was achieved using various stress conditions, namely high pH, elevated temperature, H_2O_2 (Merck KGaA, Darmstadt, Germany) and raised levels of glucose. Forced deamidation was achieved by diluting 800 µL of mAb2 with 1.2 mL of 200 mM TRIS-HCl (Sigma-Aldrich, Missouri, USA) at pH 9.0 and incubating for 7 days at 37°C. Forced oxidation was achieved by adding 0.2% H_2O_2 to the formulated mAb2 and incubation for 24 h at 25 °C. For generating glycated mAb2, the formulated antibody was diluted with a stock solution of glucose (Merck KGaA, Darmstadt, Germany) to a final concentration of 1 M. This was followed by incubation for 7 days at 25°C.

2.3.2 4D-HPLC hardware configuration for analysis of charged variants

The 4D-HPLC system was completely configured using standard modules from Agilent Technologies (Waldbronn, Germany). The system is based on a standard 2D-HPLC with the following modules: 1260 Bio ALS autosampler (G5667A), 1290 thermostat (G1330B), 1290 binary pump (G4220A), 1260 quaternary pump (G5611A), 1290 column oven (G1316C) configured with a 6 port / 2 position valve (G4231B) and a 1260 UV detector (G1314F). Critical for the 2D application is the interface module consisting of a 1290 8 port / 2 position valve (G4236A) and two selector valve (decks) with 6 x 40 µL loops each for multiple heart cutting (G4242A multiple heart cutting kit). This standard system was further extended with an additional binary pump (G4220A), a quaternary pump (G1311A), a second column oven (G1316A) configured with a 6 port / 2 position valve (G4231B) and a 6 port / 2 position external valve (G1158B). The system was controlled by two installations of the OpenLab software package, one for the two quaternary pumps, the two column ovens and the external valve, the second for the remaining modules. For full automation, an additional self-designed macro “valve event plugin” from ANGI (Gesellschaft für angewandte Informatik, Karlsruhe, Germany) is necessary. This macro generates a start signal for the modules in the second OpenLab software for each fraction. A start signal for the MS system is also simultaneously triggered and thus a single MS TIC for every fraction is produced. The ‘remote’ start signals are delivered by a contact closure board, which is integrated in the autosampler.

2.3.3 First dimension - IEC separation and fractionation

IEC separation was performed to separate charged variants from the main mAb species. For separation, a 4.0 x 250 mm ProPac WCX-10 analytical cation exchange column (Dionex, Thermo Scientific, Massachusetts, USA) was used. A step gradient from 5 to 100% B in 56 min with a flowrate of 1 mL/min of 20 mM MES pH 6.2 as solvent A and 20 mM MES (Merck KGaA, Darmstadt, Germany), 0.75 M NaCl (Sigma – Aldrich, Missouri, USA), pH 6.2 as solvent B was applied. The column temperature was set to 40°C and UV detection at 280 nm. For each injection 175 µg mAb2 was used.

2.3.4 Second dimension – RP trapping, reduction and elution

A 2.1 x 12.5 mm AdvancedBio RP cartridge column (Agilent Technologies, Waldbronn, Germany) was used for subsequent trapping and reduction. As mobile phase A and B, 0.1% FA (Fluka, Missouri, USA) in H_2O and in ACN (Merck KGaA, Darmstadt, Germany), respectively were used. Reduction was achieved using 20 mM DTT (Dithiothreitol) (mobile phase C) for 10 minutes, followed by isocratic elution of the cartridge with 60% B to the 3rd dimension column for 25 min. Longer reduction times were evaluated, however even after 10min incubation full reduction of the antibody was obtained. After reduction no intact material could be detected by MS. To prevent carryover, the cartridge was subsequently flushed with 100% mobile phase B for 5 mins at the end of each run. For gradient details see **Table 1**.

Table 1. Gradient and valve position information for the second dimension trapping, reduction and elution.

Time (min)	A (%)	B (%)	C (%)	Flow (mL/min)	Valve 3 switching (Position)
0.00	99.0	1.0	0.0	0.50	1-2
5.00	99.0	1.0	0.0	0.50	
5.01	0.0	0.0	100.0	0.10	
15.00	0.0	0.0	100.0	0.10	
15.01	99.0	1.0	0.0	0.50	
20.00	99.0	1.0	0.0	0.50	
20.01	40.0	60.0	0.0	0.06	1-6
45.00	40.0	60.0	0.0	0.06	1-2
45.01	0.0	100.0	0.0	0.10	
47.00	0.0	100.0	0.0	0.10	
47.00	0.0	100.0	0.0	0.50	
50.00	0.0	100.0	0.0	0.50	
51.00	99.0	1.0	0.0	0.50	

2.3.5 Third dimension - tryptic digestion

The reduced mAb chains eluted from the second dimension RP column (60% B, 60 µL/min) were directed to a 2.1 x 100 mm StyrosZyme TPCK-Trypsin column, (OraChrom, Massachusetts, USA) in the third dimension. The column temperature was set to 40°C. Digestion buffer comprising of 50 mM TRIS, 10mM $CaCl_2$ (Merck KGaA, Darmstadt, Germany) at pH 8.0 was permanently introduced via a T-junction with a flow rate of 250 µL/min. This leads to a final solvent composition of 11.6% ACN diluted in digestion buffer for elution of the peptides to the fourth dimension RP column. The effective digestion time or contact time on the trypsin column is approximately one minute, given the flow rate of the second dimension column and added digestion buffer. Subsequent cleaning of the column after digestion was achieved with 50 % ACN and 50 % digestion buffer at a flowrate of 0.5 mL/min for 5 min.

2.3.6 Fourth dimension peptide trapping and peptide mapping

Peptides were trapped on an UPLC ACQUITY BEH C18 column 2.1 x 50 mm, 1.7 µm (Waters Corporation, Massachusetts, USA). Mobile phases of 0.1% FA in H_2O and 0.1% FA in ACN were used as A and B, respectively. A gradient of 1% to 100% B in 87 minutes was applied, however the starting conditions are, in reality, closer to 11.6% B, due to the relatively high composition of ACN in the elution solvent from the second dimension (60%), despite dilution with digestion buffer in the 3rd dimension. The trapped peptides were first washed 5 min with 1% ACN, and thereafter eluted to the MS system with the gradient shown in **Table 2**. The solvent composition is maintained at 1% mobile phase B for the first 50 min until introduction of the digested peptides from the third dimension column, following that the elution gradient begins. The MS analysis was performed with an Impact II ESI Q-TOF (Bruker, Bremen, Germany). The connection between mass spectrometer and 4D HPLC were fused silica capillaries. For the MS data acquisition, the HyStar™ software was used and for the evaluation of results the DataAnalysis software. The spectra were generated in positive ion mode over the *m/z* range 300 – 2500 and a frequency of 1 Hz. The endplate offset was set to 500 V and the capillary to 4500 V. The theoretical Peptide masses and modifications were obtained by in silico trypsin digestion with GPMAW (General Protein/Mass Analysis for Windows).

Table 2. Fourth dimension RP column elution gradient conditions (for peptide mapping).

Time (min)	A (%)	B (%)	Valve 2 switching (Position)	Valve 4 switching (Position)
0.00	99.0	1.0	1-2	1-2
20.00	99.0	1.0	1-6	
50.00	99.0	1.0		1-6
51.00	99.0	1.0		
58.00	85.0	15.0		
65.00	81.0	19.0		
82.00	65.0	35.0		
86.00	30.0	70.0		
87.00	0.0	100.0		
90.00	0.0	100.0	1-2	1-2
92.00	99.0	1.0		
96.00	99.0	1.0		

2.3.7 Process workflow

In the first dimension charged variants are separated by IEC separation. Fractions of interest can be stored in one of the 12 loops of deck A and B. After the first selected fraction is stored in one of the loops, valve one is switched and the fraction is flushed, by the second dimension quaternary pump, to the 2D RP trapping cartridge (AdvancedBio RP 2.1 x 12.5 mm). Reducing agent is subsequently delivered to the trapping cartridge for 10 min by the same pump enabling protein reduction. To prevent degradation of the enzymatic column by the reducing agent, the trapping cartridge is first washed with 1% mobile phase B prior to elution of the reduced mAb chains to the 3D trypsin cartridge. The fourth pump delivers digestion buffer via a T-junction. The mAb is digested by elution through the trypsin cartridge and the peptides are trapped on a second RP column (UPLC ACQUITY BEH C18 column; 2.1 x 50 mm, 1.7 µm). The trapped peptides are washed to prevent contamination of the MS system by the high-salt containing ($CaCl_2$ and TRIS) digestion buffer. Subsequently, the peptides are separated using the third pump and analyzed by ESI-QTOF MS/MS. The entire process is depicted schematically in the flowchart shown in **Figure 1**.

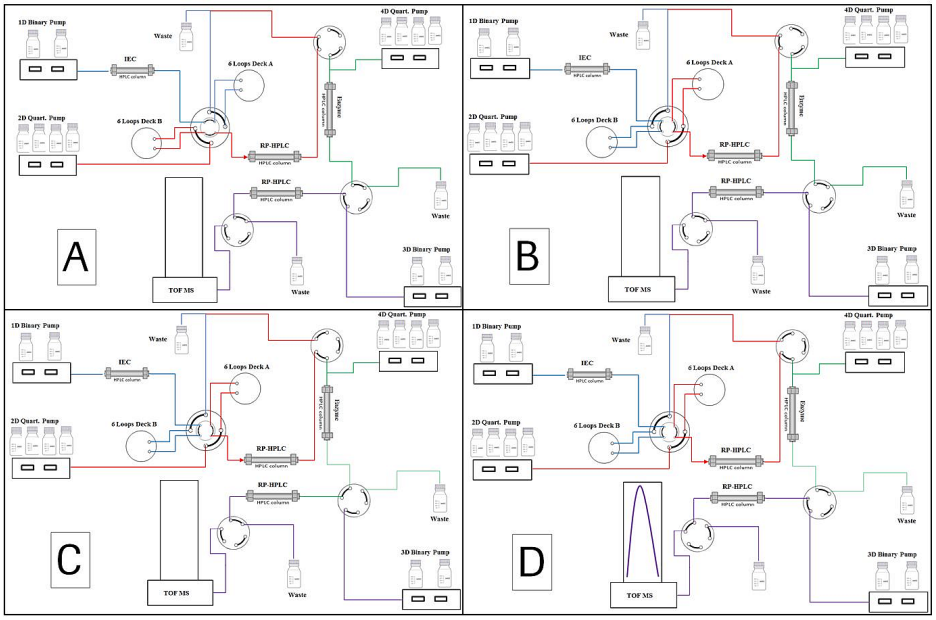

Figure 1. Schematic representation of the automated process workflow, enabling protein isolation, online reduction, digestion and detection. A) The IEC fractions of interest from the 1D separation are stored in Deck A or Deck B. B) Valve 1 is switched, enabling transfer of the isolated 1D fraction(s) to the 2D RP column. The sample is subsequently reduced and washed. C) Valves 2 and 3 are switched, hence eluting the reduced mAb chains from the 2D RP column to the 3D digestion column. The digested peptides are subsequently trapped on the online 4D RP column. D) Valves 2 and 4 are switched, enabling chromatographic elution and LC/MS detection of the trapped peptides from the 4D RP column.

2.4 Results and Discussion

2.4.1 Proof of concept study

In order to demonstrate the productive efficiency of the developed 4D-HPLC-MS system a proof of concept study was performed. A previously characterized therapeutic antibody (mAb2) was used as the benchmark. Results of this previous characterization work, which was performed using the traditional offline process, have been published by Haberger et al..[1] The goal was to detect and localize all reported modifications including deamidation, oxidation and glycation in stressed and non-stressed samples.

Samples exposed to pH stress conditions and reference material were analyzed. High pH stress was chosen, as compared to temperature stress, in order to impart higher levels of deamidation. Online fractionation was carried out for the acidic peak of both samples. Measurement of the reference material was also performed to determine sensitivity to low

levels of deamidation (see **Table 3** for expected levels). Additionally, the main IEC peak was also fractionated in order to enable determination of the efficiency of the IEC separation. Ideally no deamidation should be detected in the main peak fraction, as the loss of positive charge resulting from deamidation leads to a shift to the acidic region of the IEC separation (**Figure 2**).

Table 3. Quantitation results of deamidated peptides in reference material and pH stressed material. Values sourced from Ref[1].

	Relative amount of deamidated peptide (%)*	
Peptide	**Reference material**	**pH stressed sample**
LC-deamid-30,31	**1.8**	**11.0**
LC-deamid-50,52,53	**0.5**	**0.9**
LC-deamid-92	**7.5**	**27.1**
HC-deamid-384,389,390	**1.6**	**26.0**

* Values calculated relative to wild-type peptide (LC/MS peptide mapping)

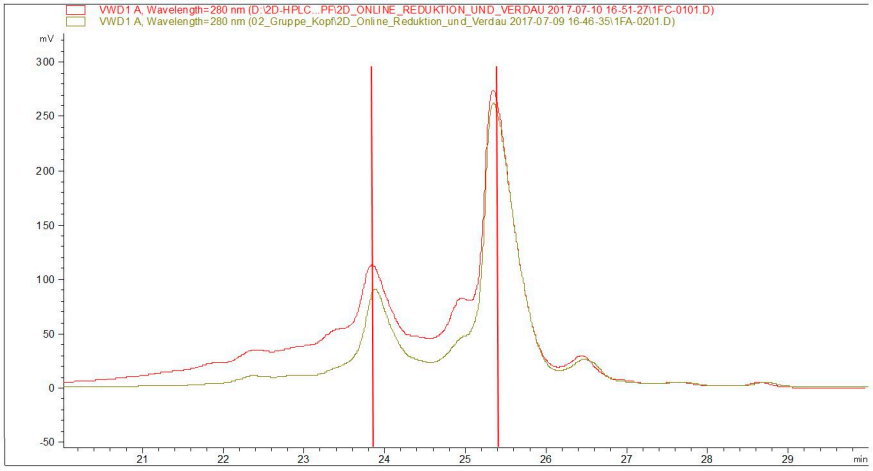

Figure 2. IEC-UV chromatogram of pH stressed material (red trace) and reference material (brown trace). Fractions (approximately 3.4 µg of AP2) are depicted by red bars.

All four deamidation sites could be detected in both the acidic peak fraction (AP2) of the pH stressed and the non-stressed reference material, as shown in **Figure 3**. The mass difference was the expected 1 Da. The high sensitivity of our system is demonstrated by our ability to detect the deamidation of the light chain at position 50,52,53, which is only abundant with an amount of 0.5% in the non-

stressed reference material. As can be seen in **Figure 3B**, this peptide could also be detected in the main peak fraction, while the other three deamidated peptides are only detectable in the acidic region. One possible explanation for this is that the asparagine residues on this peptide are buried in the hydrophobic core of the mAb and hence interaction with the IEC matrix is inhibited. This is further corroborated by the fact, that also during pH stress nearly no deamidation occurs at these three asparagine residues. The second signal also detected (**Figure 3B**) is attributed to a co-eluting peptide.

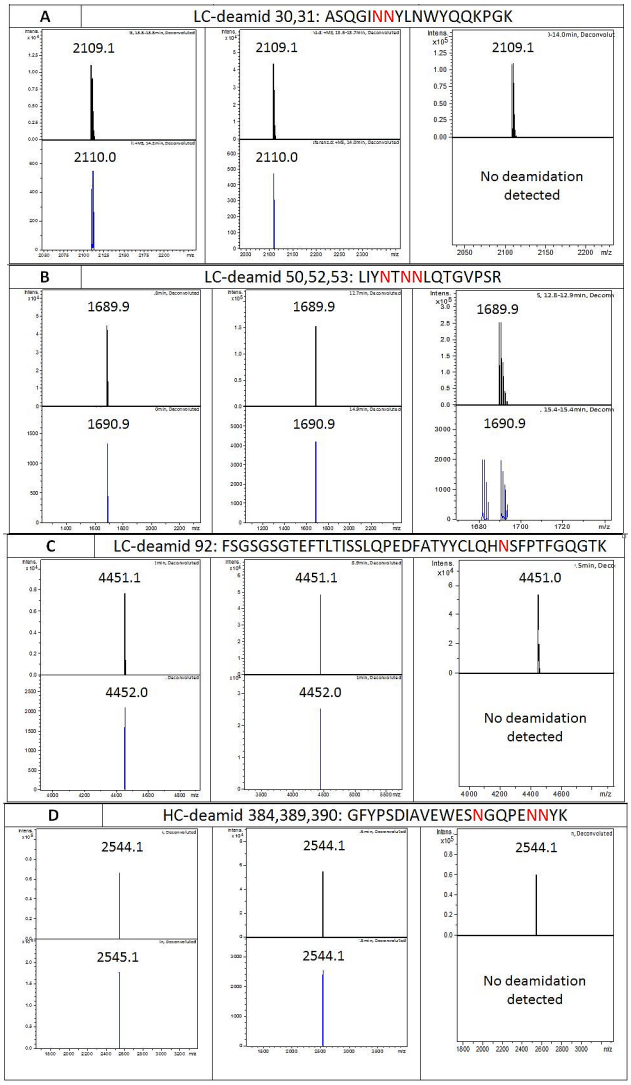

Figure 3. (A-D) Spectral results showing the deamidated peptides detected in the pH-stressed, reference material and fractionated main peak samples.

In addition to deamidation, glycation was also one of the modifications found in the molecule.[1] In order to determine if glycation at high and low levels can also be detected with our system, artificially glycated material and the reference material were analyzed. The main peak, in which no glycation should be detected, was also fractionated and analyzed to confirm the efficiency of the IEC separation. The IEC chromatogram, including the fractionation, is depicted in **Figure 4**.

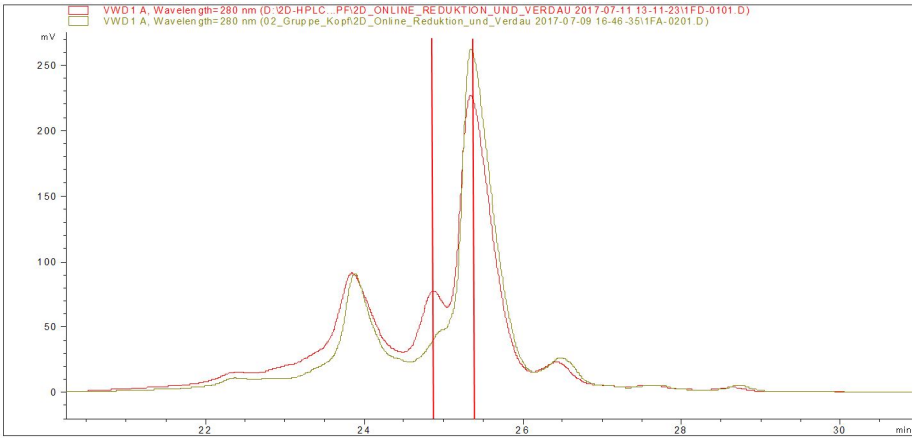

Figure 4. IEC chromatogram of material stressed with high amount of glucose (red trace) and reference material (brown trace). Fractions (approximately 1.68µg of AP1) are depicted by red bars.

The expected mass difference of 162 Da for a glucose molecule could be detected in both the stressed material and in the reference material. The glycation was confirmed at the same position (HC-Lys-33) as reported previously.[1] In the main peak fraction no glycation could be detected, see **Figure 5**.

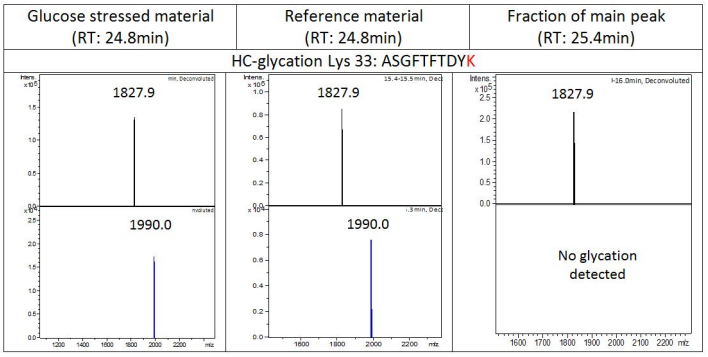

Figure 5. Spectral results showing the glycated peptide, as detected in the glucose-stressed, reference material and fractionated main peak samples.

Detection of the six oxidation sites, also reported for mAb2,[1] was also investigated with the 4D HPLC-MS system. For this purpose, the main IEC peak, as well as the basic fraction were

analyzed. The relative amounts of oxidized peptides detected in the H_2O_2-stressed sample and the reference material are provided in **Table 4**.[1]

Table 4. Quantitation results of oxidized peptides in reference material and oxidation stressed material, as sourced from Ref[1].

	Relative amount of oxidized peptide (%)	
Peptide	Reference material	H2O2 stressed sample
LC-Met 4	3.3	47.9
HC-Met 48	1.8	1.8
HC-Met 80	1.9	1.9
HC-Met 100c	3.3	99.4
HC-Met 252	3.0	99.0
HC-Met 428	1.6	80.4

Five out of the six modifications were determined in the two fractions on our system. The peptide corresponding to the sixth modification (HC-Met 252) however could not be detected. This is a result of the fact that the acetonitrile content needed to elute the reduced chains from the 2D RP cartridge leads to a composition of approximately 12% ACN over the subsequent RP trapping column, despite maximal dilution with digestion buffer. Subsequently small, polar peptides (< 1.3kDa), including the HC-Met 252 peptide, are not retained in the trapping step and therefore not detected during peptide mapping.

The online reduction and digestion steps of the developed system have also been successfully applied to different antibody formats confirming high repeatability of this set-up (data not shown). After online reduction and digestion no intact material or intact chains were detected. Reported here is the proof of concept of the 4D online method carried out with the previously fully characterized mAb.[1] The goal to detect all previously published modifications was successful in terms of all four deamidation sites and the glycation at HC Lysine 33. These modifications could also be detected at the low levels present in the reference material, including the deamidation of the LC-Asn-50,52,53 peptide (total amount of 0.5% in the reference material), showing the sensitivity of the assay. In addition, no deamidation-modified peptides were detected in the fractionated IEC main peak demonstrating that the IEC separation performs efficiently.

In addition to deamidation and glycation, known oxidation modifications[1] were also investigated. Since oxidation does not result in a different charge pattern, separation of the oxidized from the native species by IEC is not expected. Therefore, the main peak and basic peaks from the 1D IEC were fractionated and analyzed by the 4D system. With our assay

we could determine five out of the six expected oxidation sites, however the HC-Met-252 oxidized peptide could not be detected. This is due to inability to trap the peptide resulting from the slightly higher amount of ACN compared to the common process. This inability to bind small, hydrophilic peptides is largely related to the dimensions of the peptide mapping column. This column that is used behind the trypsin column is rather short to achieve a backpressure below the limit of the trypsin column. Other alternatives potentially enhancing trapping of small hydrophilic peptides as well as providing additional resolution to separate aspartate isomerization, such as C30 or monolith columns, will be further investigated.

2.5 Conclusion

With the 4D HPLC setup presented here it is possible to characterize an unknown peak within a mAb formulation in a minimum timeframe. The characterization of five peaks of an IEC chromatogram, which is a typical number for IEC characterization, takes only approximately 9 h, as compared to 52 h using the common process. This time saving is achieved through full automation of the entire process from fractionation to peptide mapping followed by MS detection. Only the fractions of interest from the first dimension separation are chosen, as in normal 2D HPLC. Compared to our previous system [27] (in press) which was limited to online reduction of mAb variants and subsequent separation of the reduced chains, this 4D HPLC system enables detection of low molecular weight modifications, including i.e. deamidation. Due to the coupling with online MS/MS, another benefit of this system is that the modifications can be localized to amino acid level. Sequence coverage of mAb2 (light chain 94%, heavy chain 86%) is comparable to the offline procedure (light chain 94%, heavy chain 94%). Especially advantageous is that, the presented 4D HPLC system also enables pre-fractionation by IEC so that separated, selected charged variants, rather than the whole sample, are reduced and digested, as in other systems.[35, 36] Through this preselection of a charged variant of interest, higher overall signal intensity and sensitivity is achieved. With a single IEC separation run it is possible to fractionate up to nine peaks, and as such, only a minimum amount of material is required for complete IEC separation and isolation. The proposed system hence also offers enhanced possibilities for characterization of unknown peaks in early phase mAb development, where often only limited material is available. The system is configured such that, in addition to IEC separation, SEC could also be used as the separation mechanism of the first dimension. With this configuration size variants could hence also be characterized. The 4D system presented here was developed such that commercial quality control separation methods could directly be applied in the first dimension without any adaptation necessary. This offers significant benefits and time-saving in method transfer. Further experiments are ongoing and include optimization of peptide retention and development of methods using alternative digestion enzymes such as pepsin and LysC.

2.6 Supporting information

Supplementary information is available free of charge on the ACS website: DOI: 10.1021/acs.analchem.7b04372

2.7 Acknowledgments

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