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Published in:
Journal of Chromatography A

DOI:
10.1016/j.chroma.2021.462506

Publication date:
2021

Journal of Chromatography A 1655 (2021) 462506

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

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ARTICLE INFO

Article history:
Received 16 June 2021
Revised 23 August 2021
Accepted 25 August 2021
Available online 28 August 2021

Keywords:
Trastuzumab
Cation-exchange chromatography
Charge variants
pH gradient
Peptide mapping

ABSTRACT

Cation-exchange chromatography is a widely used approach to study charge heterogeneity of monoclonal antibodies. Heterogeneity may arise both *in vitro* and *in vivo* because of the susceptibility of monoclonal antibodies to undergo chemical modifications. Modifications may adversely affect the potency of the drug, induce immunogenicity or affect pharmacokinetics. In this study, we evaluated the application of optimized pH gradient systems for the separation of charge variants of trastuzumab after forced degradation study. pH gradient-based elution resulted in high-resolution separation of some 20 charge variants after 3 weeks at 37°C under physiological conditions. The charge variants were further characterized by LC-MS-based peptide mapping. There was no significant difference in the binding properties to HER2 or a range of Fcγ receptors between non-stressed and stressed trastuzumab.

1. Introduction

Trastuzumab is a humanized monoclonal antibody (mAb) directed against the human epidermal growth factor receptor-2 (HER2) that is used for the treatment of HER2-positive breast cancer patients. It is known that trastuzumab, like other monoclonal antibodies, is heterogeneous [1]. Heterogeneity may be caused by posttranslational modifications due to chemical or biological processes during manufacturing, storage, and *in vivo*, since mAbs are subject to chemical modifications like asparagine deamidation, aspartic acid isomerization, and the oxidation of methionine and tryptophan residues [2–4]. Such modifications lead to changes in their physicochemical properties, which may affect drug potency or induce immunogenicity. Modifications are particularly important when occurring in the complementarity-determining regions (CDRs) affecting antigen binding or in the constant region with possible effects on Fc receptor binding. Numerous studies have been performed to assess whether different modifications have an effect on the potency of the molecule. For example, Vlasak et al. reported that asparagine deamidation in the light chain CDR1 of a humanized IgG1 resulted in reduced antigen binding [5]. Another study reported that deamidation in the heavy chain CDR2 of an unspecified mAb led to a 14-fold reduction of binding affinity to the target antigen [6]. Modification of amino acids in the heavy chain may affect antibody dependent cellular cytotoxicity (ADCC), which can be assessed by Fcγ receptor binding assays, since Fcγ receptor binding correlates with ADCC activity [7].

Ion-exchange chromatography (IEX) and notably cation-exchange chromatography (CEX) is a widely used method for the separation of charge variants of mAbs. Being a non-denaturing technique, IEX allows the isolation of charge variants for further characterization. Modifications such as deamidation, sialylation, and C-terminal lysine truncation can be resolved by CEX [2]. Besides small differences in charge, charge variants may also have minor structural differences. For example, a structural difference due to the isomerization of aspartic acid to iso-aspartic acid has been resolved by CEX [1]. Salt and pH gradient-based separations are the most common approaches for charge variant separation. While salt-based separation is widely used and considered a

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https://doi.org/10.1016/j.chroma.2021.462506

traditional method, pH gradient-based separations provide an alternative approach where charge variants are separated with a change of pH over time. While less frequently used, the application of pH gradient-based separations is emerging and the two approaches have been compared in terms of separation power [8]. While Fekete et al. did not find an advantage of the pH gradient over the salt gradient elution mode [9], Farsang et al. reported that a pH gradient-based separation outperformed a salt gradient-based separation on a strong cation exchange column [10]. Several other studies reported that pH gradient-based separations are simpler in method development and less time-consuming while having comparable or even higher resolution than salt gradient-based separations [11–14].

Clinical grade trastuzumab is heterogeneous. Early work of Harris et al. showed separation of charge variants of trastuzumab on a cation-exchange column using salt gradient elution [1]. Heterogeneity is caused by asparagine deamidation and aspartic acid isomerization in the CDR regions. The level of these modifications likely increases when trastuzumab is administrated to patients because of the slightly basic pH of blood and the temperature of the human body, which are favorable conditions for such modifications. For example, Bults et al. reported up to 24% deamidation of Hc-Asn-55 in plasma samples of breast cancer patients who were treated with trastuzumab [15]. To gain further insight into charge heterogeneity during the course of treatment, we developed a high-resolution CEX method for profiling the charge heterogeneity of trastuzumab after stressing at physiological conditions to mimic *in vivo* conditions. Trastuzumab was stressed for up to three weeks, to simulate a scenario where patients are given subsequent doses of trastuzumab every three weeks based on the half-life of trastuzumab of 28 days [16]. Chemical analysis by LC-MS/MS-based peptide mapping was combined with evaluation of the biological activity in terms of HER2 and Fcγ receptor binding.

2. Materials & methods

2.1. Chemicals and reagents

Trastuzumab (Herceptin®, Lot N3024H10) was purchased from Roche (Grenzach-Wyhlen, Germany). Human HER2 / ErbB2 Protein (His Tag protein, extracellular domain Thr23 - Thr652; cat # HE2-H5225) was obtained from Acrobiosystems (Delaware, USA). Fcγ receptors RIIa, RIIb and RIIIa were purchase from Sino Biological (Beijing, China), and RIIIb receptors were produced as described [17,18].

Peroxidase-conjugated F(ab)$_2$ Fragment Rabbit Anti-Human IgG (cat # 309-035-006), specific for the Fc part of human IgG, was obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, US). Trypsin/Lys-C Mix, Mass Spec Grade, (cat # V5073) was obtained from Promega (Madison, WI, USA). Difluoroacetic acid (DFA, cat # 162120025) was acquired from Acros Organics (Fair Lawn, NJ, USA).

2-(N-Morpholino)ethanesulfonic acid, 4-Morpholineethanesulfonic acid monohydrate (MES monohydrate, cat # 69892), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, cat # H4034)), N,N-bis(2-hydroxyethyl)glycine (bicine, cat # B3876), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO, cat # C2278), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, cat # C6070), sodium chloride (cat # 746398), DL-Dithiothreitol (DTT, cat # D0632), iodoacetamide (cat # 16125), Bovine Serum Albumin (BSA, cat # 03117332001), Tween-20 (cat # P9416), sodium deoxycholate (cat # 30970), and sodium bicarbonate (cat # 31437) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Sodium hydroxide (cat # 6346) was obtained from Merck (Darmstadt, Germany). Phosphate-buffered saline 10X (PBS, cat # 14200-067), 3,3′,5,5′- tetramethylbenzidine (TMB, 1-Step® Ultra, cat # 34028) solution, and stop solution (cat # N600) for the enzymatic reaction were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Forced degradation study

30 mg/mL stock solution of trastuzumab was diluted to 10 mg/mL with PBS, pH 7.4. The diluted samples were stressed at 37°C for 3 weeks with a 1-week sample collection interval. To prevent deamidation of the stressed samples during storage, MES buffer was added to a final concentration of 20 mM to adjust the pH to 6.

2.3. Cation-exchange chromatography

An Agilent 1200 HPLC system was used with a MabPac SCX-10 (4 × 250 mm, 5 µm, Thermo Fisher Scientific, cat # 078655) column for the separation of trastuzumab charge variants with pH gradient buffers. pH gradient buffers were prepared according to Lingg et al. [11]. Buffer A (HEPES, Bicine, CAPSO, CAPS) had a pH of 8.0, and buffer B had a pH of 10.5 (Bicine, CAPSO, CAPS), respectively. Charge variants were eluted with a linear gradient of B changing from 0 to 60% over 10 column volumes (CV) at 0.5 mL/min (62.8 min at a flow rate of 0.5 mL/min) and room temperature. The autosampler temperature was set to 10 °C. UV absorbance was measured at 280 nm. A pH/C-900 unit (Amersham Biosciences) with pH electrode and flow cell was coupled after the UV detector to follow the pH change online over the gradient.

Fractions were collected into Protein LoBind 96 well plates (cat # 0030504208; Eppendorf, Hamburg, Germany). The time window for fraction collection was based on the retention time and chromatographic peak width (valley to valley). To prevent further deamidation during fraction collection, plates were filled with 150 µL of 300 mM MES buffer (pH 6) for pH neutralization. Collected fractions were first concentrated and afterwards buffer exchanged to 10 mM MES pH 6 with Amicon Ultra-2 Centrifugal Filter Units, 50 kDa (UFC205024, Merck Millipore, Darmstadt, Germany) to a final volume of 20-40 µL.

2.4. Peptide mapping

Samples from the forced degradation study and fractions from cation-exchange chromatography at the concentration of 0.5 mg/mL were denatured and reduced in the presence of 0.5% sodium deoxycholate (SDC) and 5 mM DTT by heating at 60 °C for 30 min. Alkylation was performed by adding iodoacetamide (IAA) to a final concentration of 15 mM at room temperature for 20 min in the dark. IAA was quenched with an excess of DTT. Subsequently, a Trypsin/Lys-C mix was added to the samples at a ratio of 25:1 (protein: enzyme), and proteins were digested for 6 hours at 37°C. SDC was removed by precipitation prior to LC-MS analysis by adding 0.1% final concentration of DFA and centrifugation at 14000 rpm for 10 min.

Tryptic peptides were separated on a PepMap C18 column (0.3 × 150 mm, 2 µm, 100 Å, Thermo Fisher Scientific). Mobile phase A consisted of 0.1% formic acid in water, mobile phase B was 0.1% formic acid in acetonitrile (AcN). The gradient changed from 2 to 35% B in 65 min at the flow rate of 5 µL/min and a column temperature of 40 °C. Samples were stored at 8 °C during the analysis.

LC-MS analysis of the digest was performed on an Eksigent NanoLC 425 system with a microflow pump (1-10 µL) coupled to a TT6600 quadrupole-time-of-flight (QTOF) mass spectrometer with an OptiFlow® source (SCIEX, Toronto, Canada). The source settings were as follows: Ion Source Gas 1 (GS1) 10 psi, Ion Source Gas 2 (GS2) 20 psi, Curtain Gas (CUR) 25 psi, Temperature (TEM) 100 °C, IonSpray Voltage Floating (ISVF) 4500 V, and Declustering Potential

(DP) 90 V. MS/MS analyses were performed in the data-dependent mode in which one cycle consisted of an MS scan from 350 to 2000 m/z, followed by MS/MS of the top five most intense precursor ions detected at a minimum threshold of 500 counts per second. Precursor ions with charge state 2 to 5 were selected for MS/MS fragmentation with an exclusion window of 4 seconds after two occurrences. The Rolling Collision Energy option was activated where the collision energy is calculated based on the m/z and charge state of the candidate precursor ion.

Data analysis was performed with the BPV Flex 2.1 software (SCIEX, Toronto, Canada) with a precursor mass error tolerance of 15 ppm and a fragment mass error tolerance of 0.03 Da. Carbamidomethylation was set as a fixed modification, while methionine oxidation and asparagine deamidation were set as variable modifications.

2.5. HER2-binding assay

A Maxisorp 96-well plate (Thermo Fisher Scientific, Cat # 439454) was coated overnight at 4 °C with 100 µL of 1 µg/mL HER2 in PBS buffer pH 7.4. The next day, the plate was washed three times with 300 µL of wash buffer (0.05% Tween-20 (v/v) in PBS) followed by a blocking step with 300 µL of 1% BSA in PBS for 2 hours. Stressed samples were diluted in PBS buffer with 0.5% BSA to 50 ng/mL trastuzumab. Next, the plate was washed three times with 300 µL of wash buffer, after which 100 µL of diluted samples were transferred to the plate in triplicate and incubated for 1 hour at room temperature. Then, the plate was washed five times with 300 µL of wash buffer, and 100 µL of 0.016 µg/mL Peroxidase-conjugated $F(ab)_2$ Fragment Rabbit Anti-Human IgG, specific for the Fc part of human IgG, was added to each well and incubated for 45 min at room temperature. Subsequently, the plate was washed three times with wash buffer and three times with PBS. Next, 50 µL of TMB solution was added, and the plate was incubated for 15 min at room temperature. The reaction was stopped by adding 100 µL of stop solution. The absorbance was measured on a FLUOstar optima plate reader (BMG LABTECH, Offenburg, Germany) at 450 nm. The measured concentration of the stressed samples was compared with the expected concentration of 50 ng/mL. The calibration curve (5-100 ng/mL) was made by diluting the non-stressed sample with PBS buffer containing 0.5% BSA. Protein concentration in the stock samples was determined on a NanoPhotometer® N120 (Implen GmbH, Munich, Germany) at 280 nm.

2.6. Fcγ receptor binding assays

2.6.1. FcγRIIIa affinity chromatography

Affinity chromatography was performed on an Agilent 1200 HPLC system equipped with a TSKgel FcγRIIIa-NPR column (4.6 × 75 mm, Tosoh Bioscience, cat # 0023513). 50 mM citric acid at pH 6.5 was used as mobile phase A, and 50 mM citric acid at pH 4.5 as mobile phase B. A gradient of 0 to 100%B in 17 min was applied at a flow rate of 1 mL/min at room temperature. UV absorbance was measured at 280 nm.

2.6.2. Fcγ receptor binding assay

Affinity of all IgG-Fcγ receptors was performed by surface plasmon resonance (SPR) as previously described [19]. In short, c-terminally biotinylated human FcγRIIa (both H- and R131 variants), FcγRIIb and FcγRIIIa (both F- and V158 variants) and FcγRIIIb (both NA1- and NA2-variants) were spotted using a Continuous Flow Microspotter (Wasatch Microfluidics, Salt Lake City, UT) on a SensEye G-streptavidin sensor (Ssens, Enschede, The Netherlands). All receptors were spotted in three-fold dilutions in PBS, 0.075% Tween-80, pH 7.4 (Amresco, Solon, OH), ranging from 10 nM to 1 nM for FcγRIIa H131, FcγRIIIa F158 and the FcγRIIIb's, from 100 nM to 3 nM for the FcγRIIIa V158 and from 10 nM to 0.3 nM for FcγRIIa R131 and FcγRIIb. Samples containing trastuzumab were injected over the SPR sensor at 2 dilutions of 0.49 nM to 1000 nM in PBS in 0.075% Tween-80. Regeneration with acid buffer (10 mM Gly-HCl, pH 2.4) was carried out after each sample. The dissociation constant (KD) was calculated for each ligand concentration by equilibrium fitting as described in [19]. All binding data was analyzed using Scrubber software version 2 (Biologic Software, Campbell, Australia).

2.7. Intact protein analysis of fractionated glycoforms

Intact protein analysis of glycoforms fractionated by FcγRIIIa affinity chromatography was measured on a Maxis Plus QTOF mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled to a Waters Acquity UPLC (Waters, Milford, USA). LC-MS analysis was performed using a HALO® Diphenyl column (2.7 µm, 2.1 × 100 mm, 1000 Å) where mobile phase A was 0.05% DFA in water and mobile phase B was 0.05% DFA in AcN. The gradient changed from 10 to 50% B in 8 min at a flow rate of 0.4 mL/min. The column temperature was set to 80 °C. Samples were stored in the autosampler at 10 °C. MS spectra were obtained in positive ion mode using the following instrumental parameters: capillary voltage 4500 V; nebulizer 3 bar; dry gas rate 12 L/min; dry gas temperature 250 °C; funnel RF 400 Vpp; isCID 120 eV; multipole RF 400 Vpp; ion energy 4.0 eV; collision energy, 8.0 eV; collision RF 3000 Vpp.

3. Results & discussion

3.1. Cation-exchange chromatography

In their previous study, Lingg et al. reported optimized buffer systems for linear pH gradient separations of charge variants of monoclonal antibodies (mAbs) by cation-exchange chromatography in the alkaline pH range [11]. Linearity was achieved by maintaining a constant buffering capacity over the entire pH range of the gradient. One of the important parameters to consider when designing a pH gradient system is that the linearity of the pH gradient should not be affected due to the interaction of buffer components with functional groups on the stationary phase. Baek et al. showed that the interaction of positively charged components of the buffer with a negatively charged stationary phase may affect the linearity of the pH gradient [20]. In our study, no deviation in linearity of the pH gradient was observed with and without a column installed showing that the buffers are suitable for separating charge variants of mAbs on a cation-exchange column in a reproducible manner (**Fig. S-1**).

To find optimal conditions for the separation of charge variants of trastuzumab, chromatographic parameters such as gradient slope and length were investigated. Firstly, to check the dependence of elution on pH, we evaluated gradients from 0 to 40, 50, and 60% B corresponding to a measured final pH of 8.75, 9, and 9.25, respectively. The pH at 40% B was not high enough for the elution of all charge variants from the column, since the main peak eluted after the gradient had reached 40% B (**Fig. S-2A**). In gradients up to 50 and 60% B, retention times at which the main peak eluted from the column corresponded to a measured pH of 8.9 (**Fig. S-2B and S-2C**) independent of gradient slope. We decided to continue with the gradient up to 60% B where the final pH was high enough for complete elution of all variants.

In our study, trastuzumab charge variants eluted from the column between pH 8.2 and 9.15. Baek et al. reported the separation of charge variants of trastuzumab between pH 7.2 and 7.7 with commercial Thermo Scientific CX-1 pH gradient buffers on

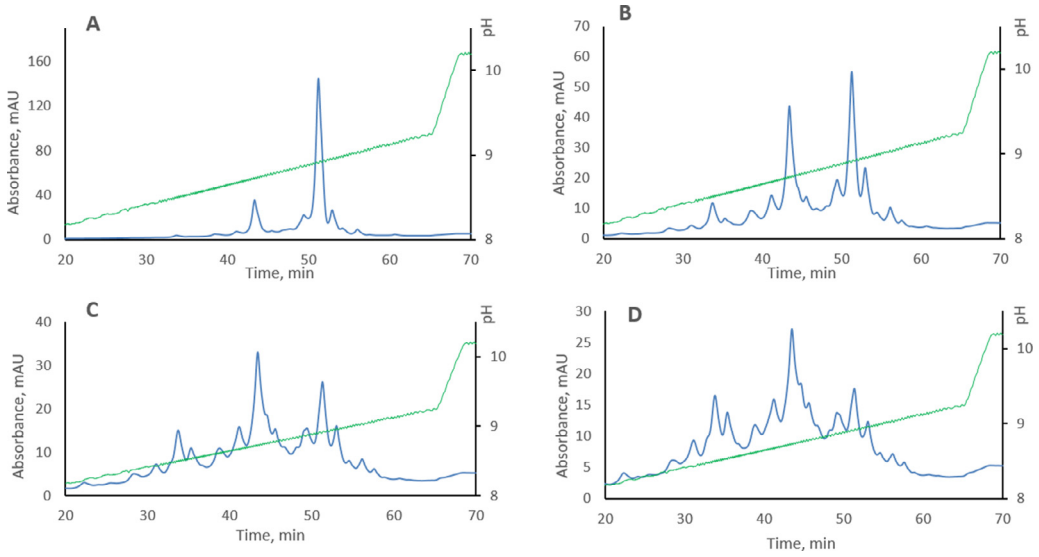

Fig. 1. Charge state profile of trastuzumab upon stressing for 3 weeks at pH 7.4; 37°C in PBS. (A) Starting material (clinical grade trastuzumab); (B) 1 week stressed trastuzumab; (C) 2 weeks stressed trastuzumab; (D) 3 weeks stressed trastuzumab. The green line indicates the measured pH gradient. Absorbance was measured at 280 nm.

Table 1
Relative composition of acidic, main, and basic forms after stressing under physiological conditions (PBS, pH 7.4, 37°C) for up to 3 weeks. Numbers are an average of 3 runs.

Sample	Acidic forms, %	Main form, %	Basic forms, %
Non-stressed	34.5 ± 0.6	53.4 ± 0.5	12.1 ± 1.2
1 week stressed	64.5 ± 0.4	22.5 ± 0.8	12.9 ± 1.1
2 weeks stressed	77.9 ± 0.5	11.9 ± 0.5	10.3 ± 3.1
3 weeks stressed	83.8 ± 0.1	7.8 ± 2.6	8.4 ± 1.8

a MabPac SCX-10 RS (5 µm, 2.1 × 50 mm) column [20]. Commercial buffers have a higher ionic strength compared to the optimized buffer systems used here, which may explain why the charge variants of trastuzumab eluted at lower pH values (**Table S-1**). To assess the influence of ionic strength on the retention of charge variants, we doubled the concentration of our buffer components thereby increasing the average buffering capacity from 5 to 10 mM, which resulted in shorter retention times and elution at lower pH values (**Table S-2**). With the 5 mM buffer the main peak eluted at pH 8.9 whereas it eluted at pH 8.2 with the 10 mM buffer (**Fig. S-3**). This shows that pH gradient and ionic strength are the main parameters affecting the retention of charge variants on ion-exchange columns [21].

Gradients of 5, 10, and 15 CV were evaluated to find a compromise between run time, number of resolved variants, and resolution between peaks. An increase in peak resolution was observed with an increase in gradient length as expected (**Fig. S-4**). Since 10 and 15 CV gradients showed quite similar chromatographic profiles while a 5 CV gradient was clearly inferior, we opted to continue with a gradient of 10 CV because of the shorter run time.

Clinical grade trastuzumab is already heterogeneous in terms of charge, as described previously [1]. When analyzed in our pH gradient system, around 53% of the original material corresponded to the main form, while the remainder consisted of a mixture of acidic and basic forms. After stressing under physiological conditions (PBS, pH 7.4, 37°C) for up to 3 weeks the relative area of acidic peaks increased to more than 80%, while the relative area of the main peak decreased to about 8% (**Table 1**). The relative peak area of basic forms, eluting later than the main peak, remained almost unchanged at 8-13%. The pH gradient system resolved about 20 different peaks of trastuzumab in the 3 weeks stressed sample (**Fig. 1**).

3.2. Peptide mapping

It has been reported previously that charge heterogeneity of trastuzumab is mainly due to Asn deamidation and Asp isomerization in the CDR regions of the antibody [1]. To assign the major modification sites, we first performed peptide mapping analyses of stressed trastuzumab samples from the forced degradation study. Modifications were assessed by LC separation of modified and unmodified peptides followed by MS/MS confirmation of the modification sites (**Fig. S-5** and **Fig. S-6**). In the case of Asp isomerization, differentiation was based on the difference in retention times, since both peptides had identical MS and MS/MS spectra. The modification percentage was quantified by calculating the ratio of the area of the modified peptide to the sum of the areas of the modified and unmodified peptide. As this does not take the difference in MS ionization efficiency of modified versus unmodified peptides into account, the results must be considered good estimates rather than accurate numbers (**Table 2**). Deamidation of Lc-Asn-30, which is located in CDR1, was most pronounced increasing from 7.1 to 52% after 3 weeks. Deamidation of Hc-Asn-55, which is located in CDR2, was rather moderate compared to Lc-Asn-30 increasing from 0.6 to 6.7% after 3 weeks, while isomerization of Hc-Asp-102 in CDR3 increased from 7.1 to 27.6%. In addition to these modifications in the CDRs, pyroGlu formation was observed at the N-terminus of the heavy chain increasing from 1.6 to 13.2% after 3 weeks at pH 7.4 and 37°C. Deamidation at Hc-Asn-392 in the crystallizable fragment region (Fc region) of trastuzumab increased from 2.1 to 14% after 3 weeks of stress.

To study the modifications of stressed trastuzumab in greater detail and to relate them to the cation exchange profile (see **Fig. 1B**), fractions were collected as shown in **Fig. 2** and characterized by peptide mapping (**Table 3**). Fraction M, which corresponds to the main form, contains already some modifications as shown in Table 3. Part of these modifications may be due to sample handling after fractionation (fraction collection, concentrating fractions, buffer exchange) and sample preparation for peptide mapping analysis. This is a potential drawback of manual fraction

Table 2
Assessment of modifications in trastuzumab upon forced degradation at 37°C, pH 7.4. Numbers are based on the average of two runs.

Sample	Lc-Asn-30 deamidation, %	Hc-Asn-55 deamidation, %	Hc-Asp-102 isomerization, %	Hc-Asn-392 deamidation, %	Hc-N-terminus pyroGlu formation, %
Non-stressed	7.1 ± 0.2	0.6 ± 0.1	7.1 ± 0.3	2.1 ± 0.3	1.6 ± 0.1
1 week stressed	23 ± 0.1	2.5 ± 0.1	15.3 ± 0.1	6 ± 0.6	4.9 ± 0.4
2 weeks stressed	47.9 ± 0.2	5.7 ± 0.1	25.1 ± 0.6	13.8 ± 0.3	12.4 ± 0.4
3 weeks stressed	52 ± 0.2	6.7 ± 0.1	27.6 ± 0.4	14 ± 0.2	13.2 ± 0.1

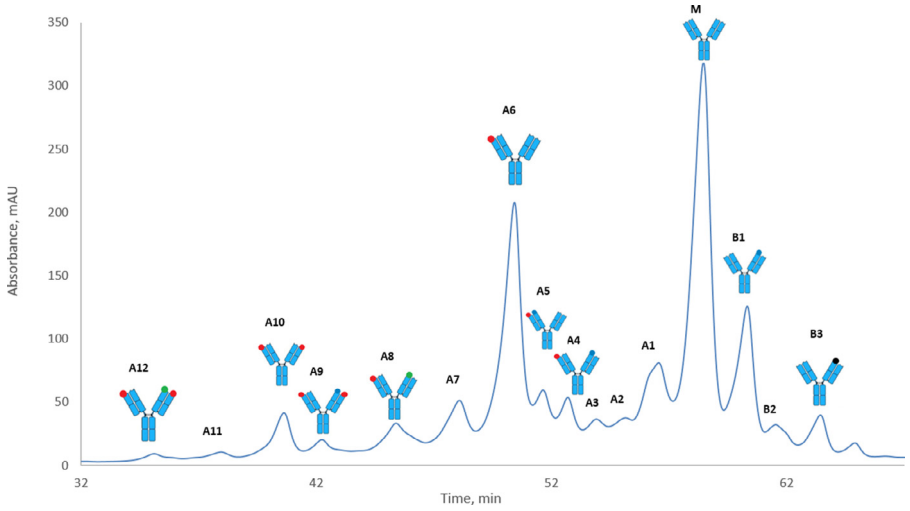

Fig. 2. Cation-exchange chromatography of trastuzumab after 1 week at 37°C, pH 7.4 in PBS. Fraction M contains the major form of clinical grade trastuzumab while fractions A1 to A12 comprise charge variants eluting across a more acidic pH region than the major form and fractions B1 to B3 comprise variants eluting at more basic pH than the major form. Deamidation at Lc-Asn-30 (red dot); deamidation at Hc-Asn-55 (green dot); isomerization at Hc-Asp-102 (blue dot); N-terminal pyroglutamate (black dot). Absorbance was measured at 280 nm.

Table 3
Characterization of charge variants of trastuzumab separated by cation-exchange chromatography by peptide mapping (**Figure 2**). Numbers are based on the average of two runs.

Fraction	Lc-Asn-30 deamidation, %	Hc-Asn-55 deamidation, %	Hc-Asp-102 isomerization, %	Hc-Asn-392 deamidation, %	Hc-N-terminal pyroGlu formation, %
M	5	n.d.	9	3	2
A1	16	n.d.	9	14	8
A2	31	n.d.	11	11	15
A3	33	n.d.	18	9	9
A4	46	1	40	4	5
A5	48	1	40	3	3
A6	43	1	12	3	2
A7	28	2	12	12	2
A8	40	14	11	7	5
A9	73	9	39	4	3
A10	83	7	10	3	2
A11	81	10	11	6	2
A12	72	20	14	3	2
B1	4	n.d.	49	n.d.	3
B2	10	n.d.	42	4	17
B3	11	n.d.	14	n.d.	32

collection and sample handling which may be reduced by using integrated online systems [22].

Trastuzumab has two identical light and heavy chains and modification can happen in either one of them or in both chains. Peptide mapping analysis of a pure variant after isolation by cation-exchange chromatography with a single light chain deamidation should thus result in a deamidation level of 50%, and a pure variant in which both chains are deamidated should result in a deamidation level of 100%. This is the ideal situation, which can, however, not be reached with present-day separation systems due to the fact that the variants are not baseline separated from each other. Even though our pH gradient-based separation results in a higher number of peaks than what has been described previously [1], charge variants were not baseline separated on the cation-exchange column and there is overlap between neighboring fractions. This makes it difficult to link quantitative assignments of certain modifications to a particular variant. In spite of this, it is possible to link some of the major variants to individual peaks. For example, deamidation of Lc-Asn-30 did not reach 50% in fractions A1-A8, so these fractions contain variants where one of two light chains is deamidated together with other variants. This is particularly noticeable in fractions A1, A2, A3, and A7. Of note is that these fractions had higher levels of deamidation of Hc-Asn-392 compared to the other fractions. Besides, fractions A1-A3 showed an increased level of pyroglutamate formation.

Fractions A4, A5, A6, and A8 had similar deamidation levels at Lc-Asn-30 that were close to 50%. However, the fact that they are still separated on the cation-exchange column indicates that there must be other modifications present. Fraction A6 was assigned as a fraction where one of two light chains is deamidated since the deamidation at Lc-Asn-30 was the major modification in this fraction. Fraction A8 had an increased level of Hc-Asn-55 deamidation compared to other fractions and we labeled this fraction as a variant with deamidation in one light chain and one heavy chain. The amount of deamidation at Lc-Asn-30 and isomerization at Hc-Asp-102 was very similar in fractions A4 and A5. They may correspond to variants where one light chain is deamidated and one heavy chain is isomerized as reported by Harris et al. who confirmed the existence of such a variant by analysis at the Fab level by hydrophobic interaction chromatography [1]. Since fractions A4 and A5 showed quite similar levels of modifications while being separated in the pH gradient, they may correspond to variants where deamidation and isomerization occur either in the same Fab region or in separate Fab regions. Further analyses at the $F(ab)_2$ and Fab level are needed to verify this hypothesis.

Fractions A9-A12 had deamidation levels of Lc-Asn-30 of up to 83%, indicating a high level of deamidation in early eluting acidic peaks. Fraction A9 had also a considerable amount of Hc-Asp-102 isomerization, so we assigned it as a variant where Lc-Asn-30 is deamidated in both chains and Hc-Asp-102 is isomerized in one chain as shown in **Fig. 2**. Fraction A10 was assigned as a variant, where Lc-Asn-30 is deamidated in both chains (83%). The presence of some deamidated Hc-Asn-55 in fraction A12 allowed us to assign peak A12 to a variant with two deamidated light chains at Asn-30 and Hc-Asn-55 deamidated in one chain. It was not possible to make clear assignments for the unlabeled peaks in **Fig. 2** based on the results of peptide mapping indicating that the heterogeneity of charge variants exceeded the separation capacity of the CEX, pH gradient system.

Fractions B1 and B2 showed a similar level of Hc-isoAsp-102 formation (49% versus 42%, respectively). Previously Harris et al. reported two basic forms eluting after the main peak in cation-exchange chromatography using a salt gradient [1]. The first peak contained Hc-isoAsp102 and the second peak was due to an intermediate succinimide form at the same position. As succinimide intermediates are known to be unstable, it is possible that such a form hydrolyzed to the corresponding isoAsp during fraction handling and sample preparation. That may explain the detection of two forms with similar amounts of modifications. Peptide mapping of fractions B2 and B3 showed the presence of increased levels of Hc-N-terminal pyroglutamate albeit in different amounts. Since fraction B3 had two times more pyroglutamate compared to fraction B2, we assigned this fraction to a variant where one heavy chain N-terminus converted to the pyroglutamate form. The presence of some pyroglutamate in fraction B2 can be due to the overlap of fraction B2 with fraction B3. After localization of modification sites in the different fractions, we investigated whether HER2 binding and Fcγ receptor binding are affected by stressing in PBS, pH 7.4 at 37°C.

3.3. HER2-binding assay

SPR measurements of binding of trastuzumab charge variants to HER2 have been reported in the literature with contradictory results. While some studies showed no significant changes in HER2 binding between acidic, main, and basic forms [7,23], others reported that acidic variants of trastuzumab showed reduced affinity to HER2 [24,25]. In our study, we were interested in whether the occurrence of charge variants would affect the quantification of the concentration of trastuzumab in a HER2 binding assay, which is relevant in view of pharmacokinetic and pharmacodynamic studies. Stressed samples were diluted to 50 ng/mL and HER2 binding was measured relative to the non-stressed sample by comparing the expected and measured concentrations. Overall, there was no notable decrease in receptor binding after stressing for up to 3 weeks (**Table 4**). Expected and measured concentrations were similar within 10% deviation, which is likely due to analytical variability.

Table 4
Quantitative HER2-binding of trastuzumab after stressing for up to 3 weeks in PBS, pH 7.4 at 37°C. The measured concentrations are an average of two experiments.

Sample	Expected concentration, ng/mL	Measured concentration, ng/mL
1 week stressed	50	50.2
2 weeks stressed	50	49.6
3 weeks stressed	50	54.2
Fraction M	50	50.2
Fraction A4	50	55.3
Fraction A5	50	54.9
Fraction A6	50	46.5
Fraction A8	50	53.8
Fraction B1	50	52.5

In order to check whether particular modifications in the CDRs could affect HER2 binding of trastuzumab, several fractions with different combinations of modifications were tested in the same way as described above. None of the fractions showed a significant change in receptor binding (**Table 4**). Previously Harris et al. reported that the CEX fraction where Hc-Asp-102 isomerized in one arm dramatically reduced the biological activity of trastuzumab in a cell-based assay [1]. Another study reported almost no change in HER2 binding upon SPR analysis for the fraction where Hc-Asp-102 was isomerized in one arm [26]. These seemingly contradictory results may be reconciled when taking the results from an early trastuzumab humanization study into account, which showed that HER2 binding and activity in a cell proliferation assay are not necessarily correlated [27]. The authors showed that trastuzumab variants with less affinity to HER2 showed higher anti-proliferative activity and vice-versa. This indicates that further investigations are required to study the effect of modifications on HER2 binding and anti-proliferative activity.

3.4. Fcγ receptor binding assays

3.4.1. FcγRIIIa affinity chromatography

Antibody-dependent cellular cytotoxicity (ADCC) is one of the main anti-tumor mechanisms of action of trastuzumab. The FcγRIIIa receptor is known to mediate ADCC. In order to investigate whether FcγRIIIa receptor binding is affected by stressing, we performed FcγRIIIa affinity chromatography analysis of stressed samples. The structure of N-glycans plays an important role in FcγRIIIa receptor binding. It is well-established that the absence of core fucose enhances affinity to FcγRIIIa and trastuzumab is a highly fucosylated antibody [28]. Galactosylation is another important feature and according to a recent publication, galactosylation increases FcγRIIIa binding of therapeutic antibodies [29]. FcγRIIIa affinity chromatography of trastuzumab showed 3 main peaks, which were classified as low-, medium-, and high-affinity forms according to the elution order **(Fig. 3)**. Intact protein analysis by mass spectrometry of these peaks after fractionation showed an increased level of galactosylation with later elution times (**Table S-3** and **Fig. S-7**). These results are consistent with previously reported data showing that the TSKgel FcR-IIIA-NRP column mainly separates based on differences in the level of galactosylation [30]. Stressed samples showed no major changes in chromatographic profiles compared to non-stressed samples indicating that FcγRIIIa binding was not affected by stressing.

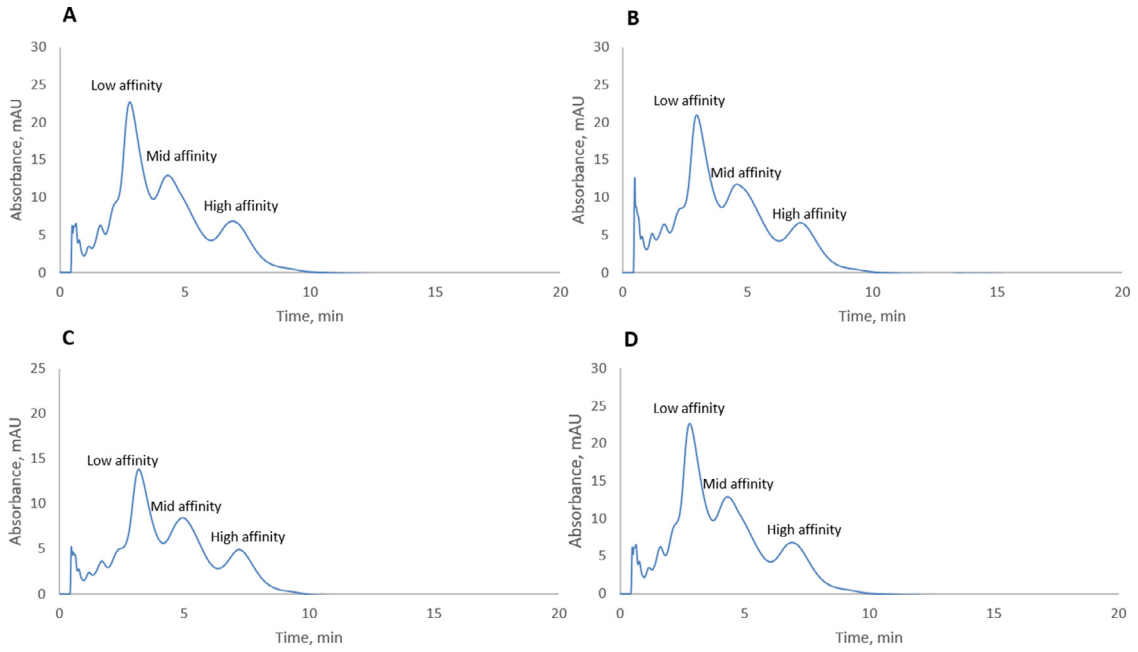

Fig. 3. FcγRIIIa affinity chromatography profile of non-stressed and stressed trastuzumab. (A) Non-stressed sample; (B) 1 week stressed sample; (C) 2 weeks stressed sample; (D) 3 weeks stressed sample. Absorbance was measured at 280 nm.

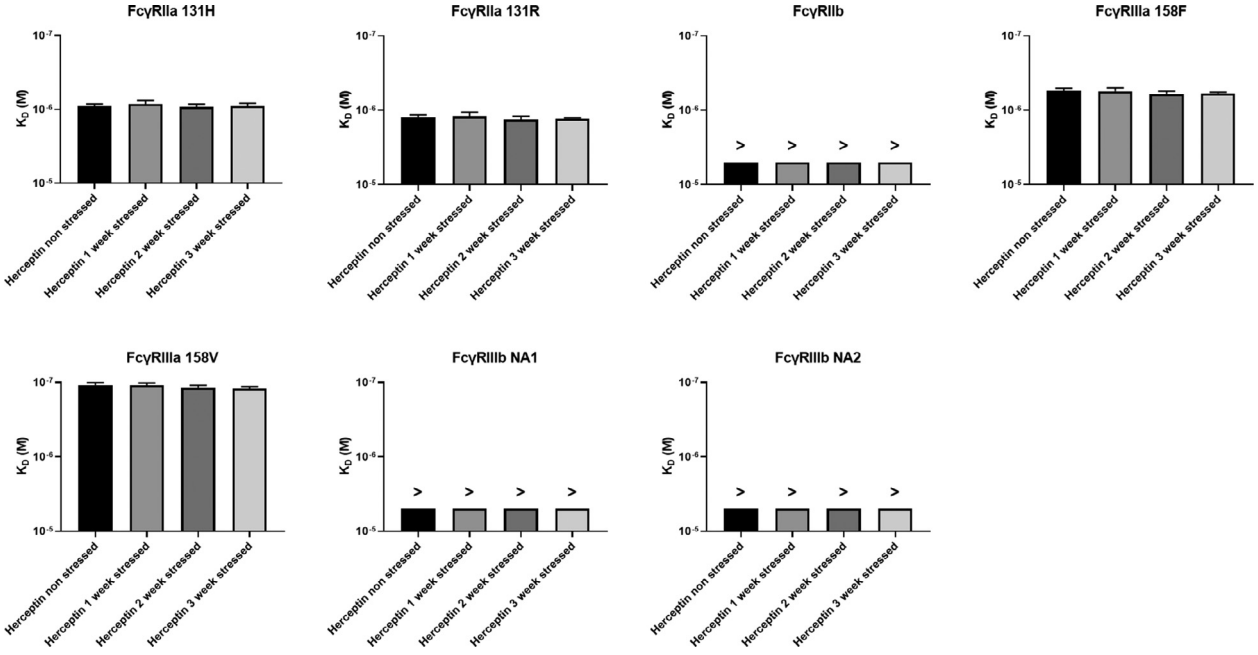

Fig. 4. Binding affinity of trastuzumab to various Fcγ receptors as measured by SPR for non-stressed sample; 1 week, 2 weeks and 3 weeks stressed samples, respectively.

3.4.2. Fcγ receptor binding assay

Although all human FcγR recognize human IgG in a structurally similar fashion, subtle differences are known depending on the allotypic variant of each of these receptors, expression of which differs between individuals. We therefore tested binding of stressed trastuzumab to all polymorphic variants of the human FcγR by SPR [31]. As expected, the lowest affinities were found for the FcγRIIb and the FcγRIIIb variants, which were below 1000 nM. As expected the affinity was similar to the two allotypic forms of FcγRIIa, but elevated affinity was observed for the V158V variant of FcγRIIIa [32]. Importantly, no difference in binding to any FcγR was observed between the stress conditions (**Fig. 4** and **Fig. S-8**).

4. Conclusions

In this study, we demonstrated that pH gradient elution on a cation-exchange column is highly efficient for the separation of charge variants of the therapeutic antibody trastuzumab. The pH gradient was shown to be linear and not affected by the cation-exchange column using a fully characterized, customized buffer system. While this separation system resulted in a high-resolution separation of trastuzumab charge variants, our results show also that a complete separation of all possible charge variants is currently not possible.

While peptide mapping is a powerful approach for localizing modifications sites, by itself it is not sufficient for the full char-

acterization of charge variants, since the context of the entire protein is lost. Other orthogonal approaches like middle-down or top-down MS are thus needed to place the observed modifications in the context of the intact mAb.

While our HER2-binding assay was not designed to reveal subtle changes in binding affinity, it did show that there were no major changes in binding upon stressing that would affect the quantitation of trastuzumab using this assay. Binding of trastuzumab to a range of Fcγ receptors was not affected by stressing. This is an important observation, since triggering ADCC through FcγRIIIa receptor activation is one of the main mechanisms of anti-tumor action.

Credit author statement

B.S. contributed to the concept of the work, performed experimental work and wrote the manuscript

O.O. performed experimental work and contributed to the writing of the manuscript

N.L. critically reviewed the manuscript and contributed to the writing of the manuscript

N.E.H.B. performed experimental work and contributed to the writing of the manuscript

N.G. critically reviewed the manuscript and contributed to the writing of the manuscript

J.H. performed experimental work and contributed to the writing of the manuscript

N.v.d.M. critically reviewed the manuscript and contributed to the writing of the manuscript

G.V. critically reviewed the manuscript and contributed to the writing of the manuscript

A.J. critically reviewed the manuscript and contributed to the writing of the manuscript

R.B. contributed to the concept of the work, critically reviewed the manuscript and contributed to the writing of the manuscript

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

B.S. and O.O. are funded by a grant of the European Commission (H2020 MSCA-ITN 2017 “Analytics for Biologics”, grant agreement ID 765502)

We would like to thank Barry Boyes from Advanced Materials Technology for providing us with HALO® Diphenyl columns

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.462506.

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