Intact Mass Analysis of Monoclonal Antibody (MAb) Charge Variants Separated Using Linear pH Gradient

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Overview

Purpose: Intact mass analysis of monoclonal antibody (MAb) charge variants separated using linear pH gradient.

Methods: The separation of MAb charge variants is achieved using linear pH gradient method on a cation-exchange column. The intact mass information is acquired on the Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer.

Results: This linear pH gradient enables the high resolution separation of MAb charge variants. The intact mass analysis characterizes the structural difference of the MAb variants.

Introduction

Monoclonal antibodies can be highly heterogeneous due to modifications such as sialylation, deamidation, and C-terminal lysine truncation. Salt gradient cation-exchange chromatography has been used with some success in characterizing MAb charge variants. However, additional effort is often required to tailor the salt gradient method for an individual MAb. In the fast-paced drug development environment, a platform method is desired to accommodate the majority of the MAb analyses.

In 2009, Dell and Moreno reported a method to separate MAb charge variants using pH gradient ion-exchange chromatography. The buffer employed to generate the pH gradient consisted of piperazine, imidazole, and tris, covering a pH range of 6 to 9.5. While good separation was observed, the slope of the pH increase was shallow at the beginning and steep towards the end.[1] In this study, we present a novel pH gradient method for cation-exchange chromatography that is more linear. This method features a multi-component buffer system in which the linear gradient was run from 100% eluent A (low pH buffer) to 100% eluent B (high pH buffer). Using an online pH meter, it was confirmed that a linear pH gradient was achieved. Furthermore, a plot of measured pH values at the retention time of model proteins versus their pI values exhibited a high correlation. Once the approximate pH elution range of the target MAb has been established in the initial run, further optimization of separation can simply be achieved by running a shallower pH gradient in a narrower pH range.

Methods

Sample Preparation:

All standard proteins were purchased from Sigma. Harvest cell culture and monoclonal antibodies were a gift from a local biotech company. Proteins and MAb were dissolved in deionized water.

Column and Buffer

Thermo Scientific™ MAbPac™ SCX-10, 10 µm, 4 × 250 mm (P/N 074625)

CX-1 pH Gradient Buffer Kit (P/N 083274)

Liquid Chromatography

HPLC experiments were carried out using a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS System equipped with:

- SRD-3600 Membrane Degasser
- DGP-3600RS Biocompatible Dual Gradient Rapid Separation Pump
- TCC-3000SD Thermostatted Column Compartment with two biocompatible 10-port valves
- WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler
- VWD-3400RS UV Detector equipped with a Micro Flow Cell
- PCM-3000 pH and Conductivity Monitor

Column and Buffer

The CX-1 pH buffer kit consists of one bottle of 10X buffer A (pH 5.6) and one bottle of 10X buffer B (pH 10.2). Eluent A and B each was prepared by simply diluting the corresponding 10 X buffer 10 fold using deionized water.

Linear pH Gradient Chromatography
The linear pH gradient was generated by running linear gradient from 100% eluent A (pH 5.6) to 100% eluent B (pH 10.2). For pH gradient analysis carried out on the MAbPac SCX-10, 10 µm, 4 × 250 mm, cation-exchange columns, the gradient method in Table 1 was used unless further stated.

Table 1. 30 min linear gradient method for the MAbPac SCX-10, 10 µm, 4 × 250 mm, cation exchange columns. Total run time is 40 min. The linear pH range covers from pH 5.6 to pH 10.2. UV wavelength was set at 280 nm.

Time (minutes)	Flow rate (mL/min)	%A	%B
0-1	1	100	0
1-31	1	100-0	0-100
31-34	1	0	100
34-40	1	100	0

LC-MS
First dimension HPLC: in a scale up purification, 1 mL of IgG was purified from 3.8 mL of HCC using Thermo Scientific™ Pierce Protein A beads. The protein concentration was determined at ~ 0.5 mg/mL. About 33 µL of the purified IgG was injected onto a MAbPac SCX-10, 10 µm, 4 × 250 mm column and separated via linear pH gradient from pH 6.52 to pH 9.28. The column was equilibrated at 40% B. Three minutes after sample injection, a linear gradient was run from 40% to 100% B in 30 minutes. Fractions were collected onto a 96-well plate at a rate of 0.2 min per fraction from 10 to 26 min.

Second dimension LC-MS: Thermo Scientific™ ProSwift™ RP-10R monolithic column (1 × 50 mm) was used for desalting. LC solvents were 0.1% formic acid in H_2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Column was heated to 50 ºC during analysis. Flow rate was 100 µL/min. After injection of MAbs, a 5 min gradient from 10% B to 95% B was used to elute the mAbs from the column.

MS: The Q Exactive Orbitrap mass spectrometer was used for this study. Intact MAb was analyzed by ESI-MS for intact molecular mass. The spray voltage was 4kV. Sheath gas flow rate was set at 10. Auxiliary gas flow rate was set at 5. Capillary temperature was 275 ºC. S-lens level was set at 55. In-source CID was set at 45 eV. Resolution was 17,500. The AGC target was set at 3E6 for full scan. Maximum IT was set at 200 ms.

Data Processing: Full MS spectra of intact MAbs were analyzed using Thermo Scientific™ Protein Deconvolution 1.0 software that utilizes the ReSpect algorithm for molecular mass determination. Mass spectra for deconvolution were produced by averaging spectra across the most abundant portion of the elution profile for the MAb. The averaged spectra were subsequently deconvoluted using an input m/z range of 2000 to 4000 m/z, an output mass range of 140000 to 160000 Da, a target mass of 150000 Da, and a minimum of at least eight consecutive charge states from the input m/z spectrum to produce a deconvoluted peak.

Results

Linear pH gradient
The linear pH gradient was achieved by employing a multi-component buffer system containing multiple zwitterionic buffer species with pI values ranging from 6 to 10. Eluent A was titrated to pH 5.6 and eluent B was titrated to pH 10.2. In this pH range, each buffer species was either neutral or negatively charged. Therefore they were not retained by cation-exchange column stationary phase and served as good buffers for the mobile phase and the stationary phase.

Using the gradient method shown in Table 1, six proteins with a range of pI values from 6 to 10 were effectively separated on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. These proteins were lectin (including three isoforms, lectin-1, lectin-2, and lectin-3), trypsinogen, ribonuclease A, and cytomchrome C. The chromatogram was shown in Figure 1. The pH value measured in this experiment as a function of time was plotted in Figure 2. The pH gradient was essentially linear from pH 5.6 to pH 10.2 over a 30 minute period. The correlation coefficient value R^2 was 0.9996.

An analysis was performed to show that there is a correlation between the elution pH for the peaks and the corresponding pI values of the protein components. Figure 3 is a graph comparing the measured pH values for six protein component peaks in Figure 1 as a function of the corresponding pI values. The measured pH values for the six protein component peaks exhibited a strong linear correlation to the literature based pI values. Thus, after a calibration procedure, this example supports the fact that linear regression coupled with the gradient method described here can be used to estimate the pI of a protein component based on the peak retention time and measured pH.

FIGURE 1. Chromatogram of six proteins separated on a 30-min linear pH gradient on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. Protein name, retention time, and corresponding pH values are labeled for each protein peak.

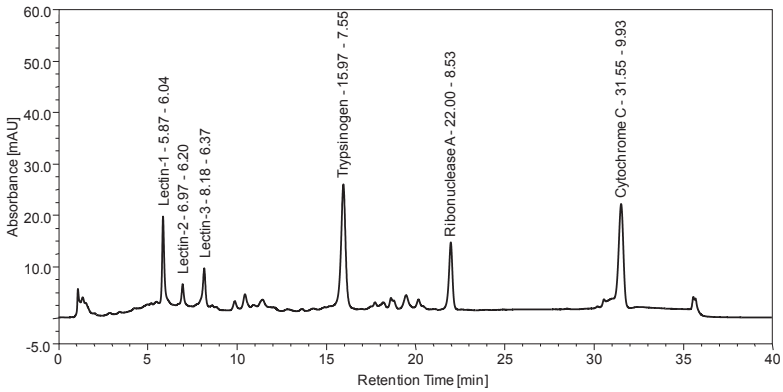


FIGURE 2. A graph showing measured pH values as a function of time. The measured pH values were exported from the same experiment shown in Figure 1. The measured pH values are labeled using blue diamond shape.

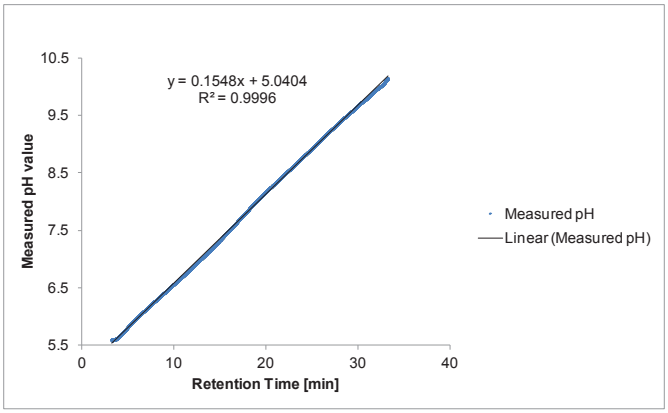
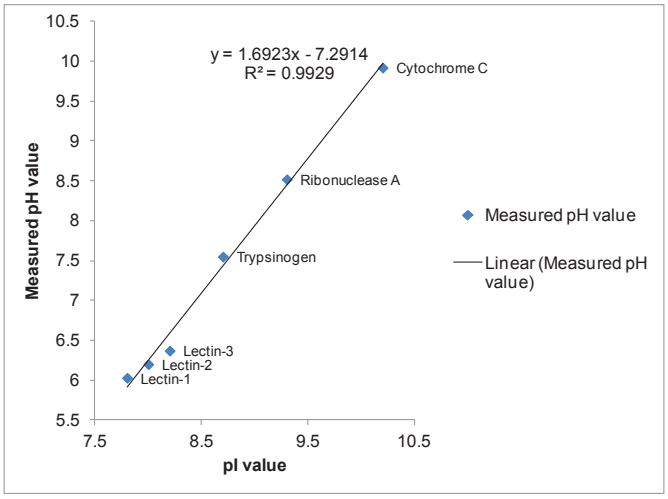


FIGURE 3. A graph plotting the measured pH values for six protein component peaks as a function of the corresponding pI value. The measured pH values of all six components were exported from the same experiment shown in Figure 1.

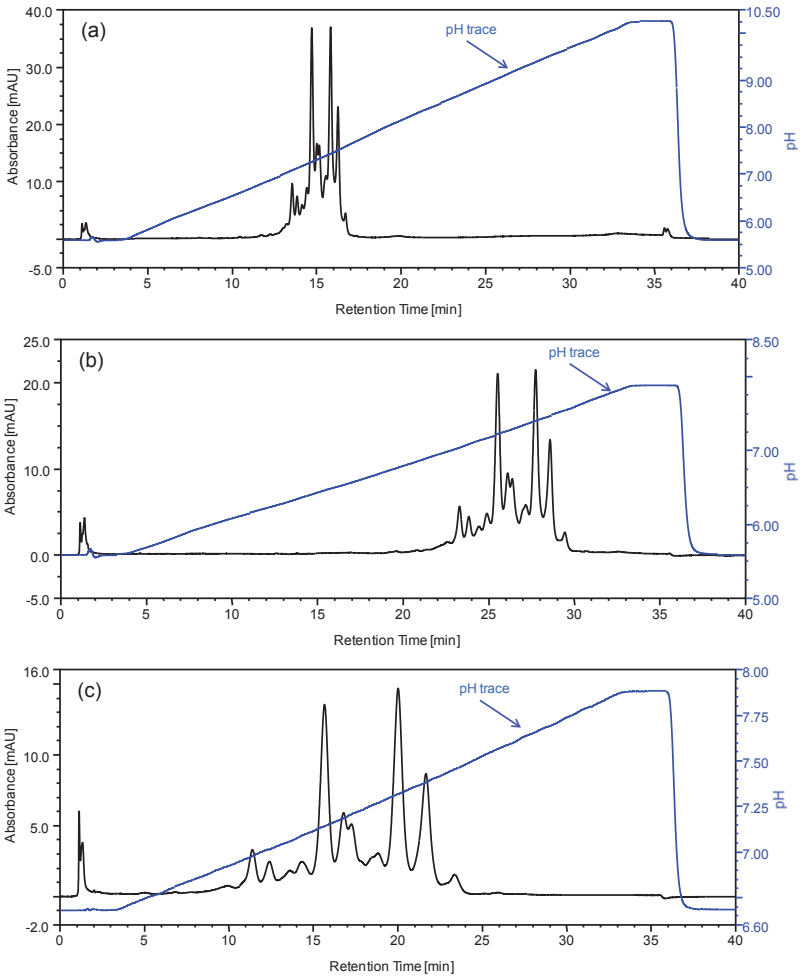


pH Gradient Separation Platform for MAb Variants

Most MAbs have pI values in the range of 6 to 10. Our pH gradient separation method can serve as a platform for charge variant separation. Using a full range of pH gradient from pH 5.6 to pH 10.2, we established the pH elution range in the initial run (Figure 4a) with a pH gradient slope of 0.153 pH unit/min. Further optimization of separation can simply be achieved by running a shallower pH gradient in a narrower pH range. Figure 4b showed the separation profile from pH 5.6 to pH 7.9 with pH gradient slope at 0.076 pH unit/min. Figure 4c showed the separation profile from pH 6.75 to pH 7.9 with pH gradient slope at 0.038 pH unit/min. The pH traces in Figure 4a, 4b, and 4c demonstrated that the pH gradient maintain linear when the slope was reduced to ½ or ¼ of the initial run.

Because the chromatographic profile of the variants were predictable when running a shallower pH gradient. Pump methods for chromatogram shown in Figure 4b and 4c can be automatically generated by writing a post-acquisition script using the MAb variant pH elution range information collected in the initial run (Figure 4a). This example illustrates the advantages of using pH gradient separation platform, which is to simplify and automate the method development for MAb charge variant separation.

FIGURE 4. An example of MAb charge variant separation by linear pH gradient. The separation was carried out on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. (a) Separation by pH gradient, 0% B (pH 5.6) to 100% B (pH 10.2), gradient method was shown in Table 1; (b) Separation by pH gradient, 0% B (pH 5.6) to 50% B (pH 7.9); (c) Separation by pH gradient, 25% B (pH 6.75) to 50% B (pH 7.9).



Intact Mass of MAb variants

An IgG sample was purified from harvest cell culture using the Protein A bead. This sample was analyzed via linear pH gradient and the fractions were collected via a time-based method (Figure 5). Major fractions collected off the pH gradient were analyzed on a Q Exactive mass spectrometer. On-line desalting using a reversed-phase monolithic column was carried out prior to MS detection. Figure 6 showed the deconvoluted mass spectra of peak 1, 2, 3, 4, and 5. The deconvoluted spectra showed that the major component in Peak 1 has a *m/z* at 147993. Adjacent peaks at *m/z* 148155 and 148317 correspond to different glycoforms with 1 and 2 additional hexoses. The major component in peak 2 has a *m/z* at 148121. The delta mass between Peak 1 and Peak 2 is 128 amu, corresponding to one lysine. The deconvoluted spectra of Peak 3 and peak 4 have the same MS profile as Peak 1 and Peak 2, suggesting they are structural isomers. The major component in Peak5 has a *m/z* at 148250. The delta mass between Peak 4 and Peak 5 is 129 amu. These data suggest that Peak 3 and Peak 4 correspond to lysine truncation variants of Peak 5.

FIGURE 5. pH gradient separation of purified IgG on a ion-exchange column. The separation was carried out on a MAbPac SCX-10, 10 µm, 4 × 250 mm column via a 30 min linear pH gradient from 40% B (pH 6.52) to 80% B (pH 9.28

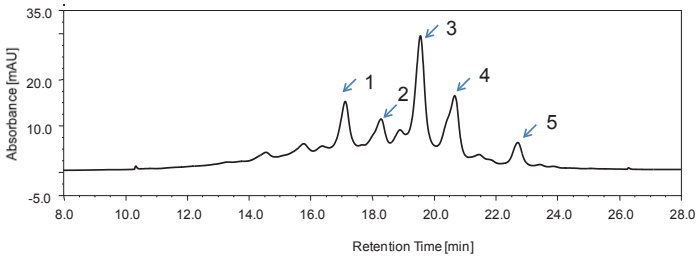
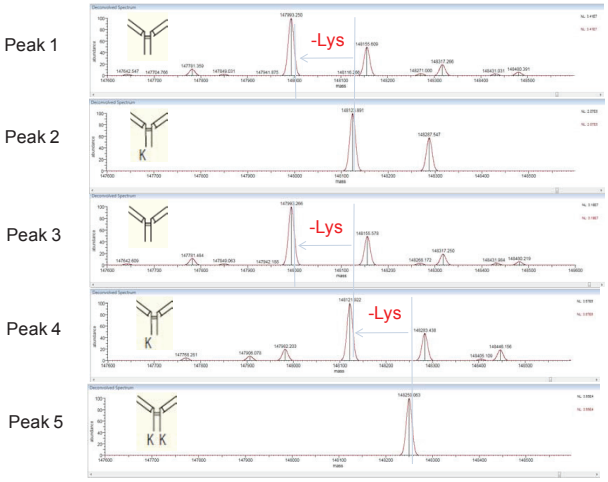


FIGURE 6. Deconvoluted MS Spectra.



Conclusions

- A linear pH gradient from pH 5.6 to pH 10.2 was generated using a multi-component zwitterionic buffer system on a cation-exchange column.
- A linear pH gradient separation platform enables high resolution, fast and rugged MAb charge variant analysis and automation of method optimization.
- The combination of off-line IEC separation and on-line LC mass spectrometry detection provides an efficient way to obtain structural information of MAb variants.

References

1. Farnan, D and Moreno, T. Multiproduct high-resolution monoclonal antibody charge variant separations by pH gradient ion-exchange chromatography. *Anal. Chem.,* 2009, 81, 8846–57.

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