A Fast and Robust Linear pH Gradient Separation Platform for Monoclonal Antibody (MAb) Charge Variant Analysis

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Overview

Purpose: Evaluate the loading capacity, elution pH, and the throughput using linear pH gradient separation method for MAb variant analysis.

Methods: A linear pH gradient from pH 5.6 to pH 10.2 is generated over time by running a linear pump gradient from 100% Thermo Scientific™ CX-1 pH Gradient Buffer A (pH 5.6) to 100% CX-1 pH Gradient Buffer B (pH 10.2) . Online monitoring of the mobile phase pH value confirmed that a linear pH gradient was achieved.

Results: 1. Linear UV response with up to 300 μg of protein loading; 2. The elution pH values of MAbs are in linear relationship with their corresponding pI values; 3. a fast separation of MAb charge variants is achieved in a 10-min cycle time.

Introduction

Recombinant monoclonal antibodies (MAbs) 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-pace drug development environment, a fast and robust platform method is desired to accommodate the majority of the MAb analyses.

We recently introduced a cation-exchange pH gradient buffer system which meets the fast and robust platform method requirement (ref. 1) This buffer system consists of a low-pH buffer A at pH 5.6 and a high-pH buffer B at pH 10.2. A linear pH gradient from pH 5.6 to pH 10.2 is generated over time by running a linear pump gradient from 100% buffer A to 100% buffer B. Using various MAbs with pI values ranging from 7 to 10, we demonstrated that the linear pH gradient method separates charge variants consistently. The loading capacity on a Thermo Scientific™ MAbPac™ SCX-10 column when running pH gradient is investigated by injecting increasing amount of protein standards. The study shows that there is a linear signal response up to 300 μg of protein loading. Furthermore, a fast separation of charge variants is achieved in a 10-min cycle time using 2 mL/min flow rate on a MAbPac SCX-10, 5 μm, 4 × 50 mm column.

Methods

Sample Preparation:

All standard proteins were purchased from Sigma-Aldrich®. Monoclonal antibodies was a gift from a local biotech company. Proteins and MAb were dissolved in deionized water.

Columns and Buffers

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

MAbPac SCX-10, 5 μm, 4 × 50 mm (P/N 078656)

CX-1 pH Gradient Buffer A (pH 5.6), 125 mL (P/N 083273)

CX-1 pH Gradient Buffer B (pH 10.2), 125 mL (P/N 083275)

Liquid Chromatography

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

SRD-3600 Membrane Degasser

DGP-3600RS Biocompatible Rapid Separation Pump

TCC-3000SD Thermostated Column Compartment with two biocompatible 10-port valves

WPS-3000TBRS Biocompatible Rapid Separation Thermostated Autosampler

VWD-3400RS UV Detector equipped with a Micro Flow Cell

PCM-3000 pH and Conductivity Monitor

Preparation of Eluents

Eluent A and B each was prepared by simply diluting the corresponding 10 X CX-1 pH Gradient Buffer 10 fold using deionized water.

Gradients

The linear pH gradient was generated by running a 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 columns, the gradient method in Table 1 was used. For pH gradient analysis carried out on the MAbPac SCX-10, 5 µm, 4 × 50 mm columns, the gradient method in Table 2 or Table 3 was used. All methods cover the pH range from pH 5.6 to pH 10.2.

Table 1. A 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.

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

Table 2. A 15 min linear gradient method for MAbPac SCX-10, 5 µm, 4 × 50 mm, cation exchange columns. Total run time is 20 min. The linear pH range covers from pH 5.6 to pH 10.2.

Time (minutes)	Flow rate (mL/min)	%A	%B
0–1	1	100	0
1–16	1	100–0	0 –100
16–17	1	0	100
17–20	1	100	0

Table 3. A 7.5 min linear gradient method for MAbPac SCX-10, 5 µm, 4 × 50 mm, cation exchange columns. Total run time is 10 min. The linear pH range covers from pH 5.6 to pH 10.2.

Time (minutes)	Flow rate (mL/min)	%A	%B
0–8.5	2	100	0
0.5–8	2	100–0	0–100
8–8.5	2	0	100
8.5–10	2	100	0

Results

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.

The separation of proteins with different pI values by pH gradient was demonstrated in Figure 1. The protein mixture contained lentil lectin with three isoforms of lectin-1 (pI 7.8), lectin-2 (pI 8.0), lectin-3 (pI 8.2), trypsinogen (pI 8.7), ribonuclease A (pI 9.3), and cytochrome C (pI 10.2). The concentration of these proteins were 3 mg/mL, 2 mg/mL, 3 mg/mL and 2 mg/mL respectively. Figure 1 showed the multiple UV traces of chromagrams recorded from a series injections of the protein mixture at 2 µL, 4 µL, 8 µL, 16 µL, 32 µL, 50 µL, and 100 µL. The peak area and peak width at half height (PWHH) were plotted against the sample loading in Figure 2a, 2b, 2c and 2d for lentil lectin-1, trypsinogen, ribonuclease A, and cytochrome C respectively. Trypsinogen, ribonuclease A, and cytochrome c exhibited very linear response up 100 µL of sample loading while lentil lectin-1 showed linear response up to 50 µL of sample loading. The separation of these proteins maintained throughout the range of 20 µg to 1,000 µg total protein loading.

FIGURE 1. Chromatogram of a protein mixture separated using a 30-min linear pH gradient on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. Protein names are labeled for each peak. The protein concentrations in the sample mixture were: lentil lectin, 3 mg/ml; trypsinogen, 2 mg/ml; ribonuclease A, 3 mg/ml; cytochrome C, 2 mg/ml. An increasing amount of samples were loaded onto the column using the following injection volumes: 2 µL, 4 µL, 8 µL, 16 µL, 32 µL, 50 µL, and 100 µL. The pH trace is colored in blue.

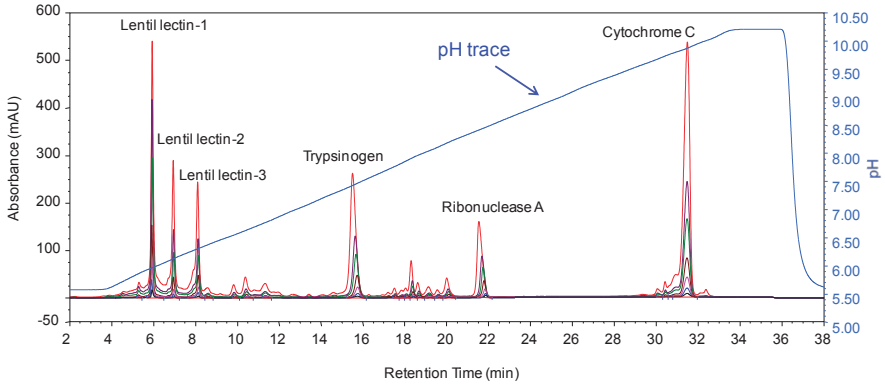


FIGURE 2. Plots of peak area and PWHH versus sample loading. Peak area is plotted on the primary Y-axis and the PWHH is plotted on the secondary Y-axis. (a) lentil lectin; (b) trypsinogen ; (c) ribonuclease A; (d) cytochrome C.

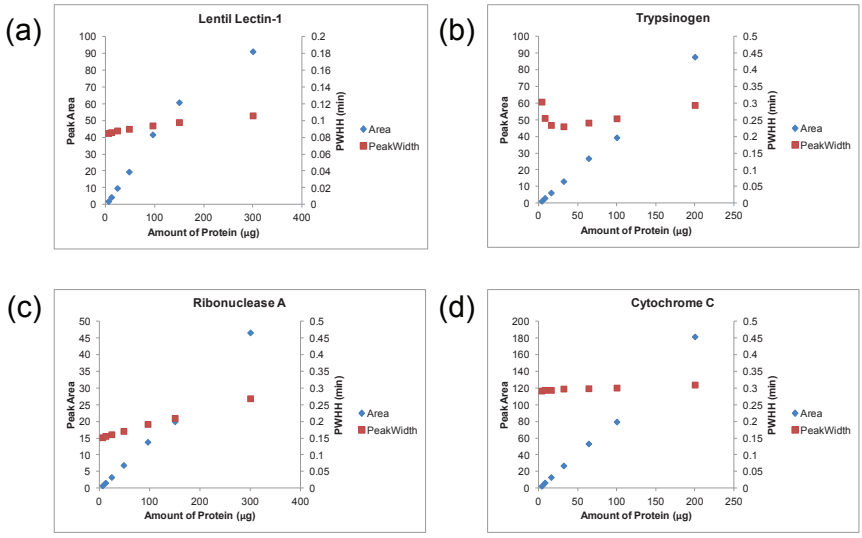
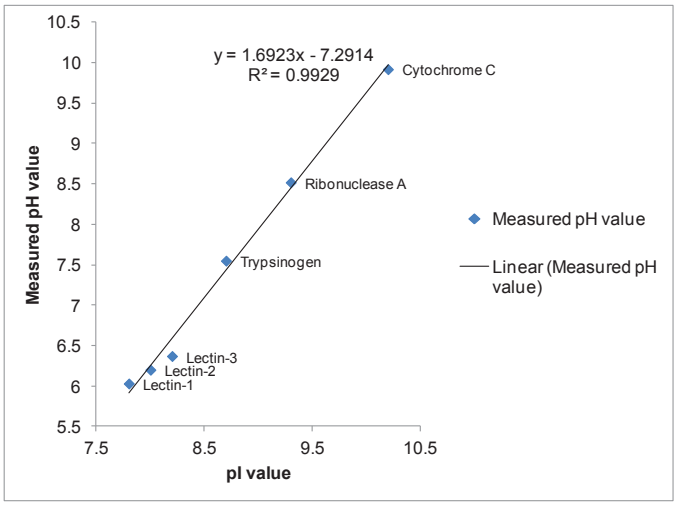


FIGURE 3. A graph plotting the measured pH values for six protein component peaks as a function of the corresponding pI value. The measured pH values of all six components were exported from the same experiment shown in Figure 1.



Linear correlation of elution pH versus protein pI value

An analysis was performed to show that there is a correlation between the elution pH versus 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, with R^2 value at 0.9929. Since MAb molecules within the IgG1 class share most of the protein sequence, we expect to see a more linear correlation of the MAb elution pH versus their pI. Figure 4 showed pH gradient separation of charge variants from six MAbs with pI values ranging from 7 to 10. Figure 5 is a plot of the elution pH of each MAb major variant versus the MAb pI value. The linear fit yielded a R^2 value at 0.9988. 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 MAb based on the peak retention time and elution pH.

FIGURE 4. Chromatogram of six MAbs separated using a 30-min linear pH gradient on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. The UV traces are corresponding to the MAbs with the following pI : 7.2 (black), 7.6 (blue), 7.8 (pink), 8.3 (brown), 9.0 (green), and 10.0 (purple). The pH trace is colored in blue.

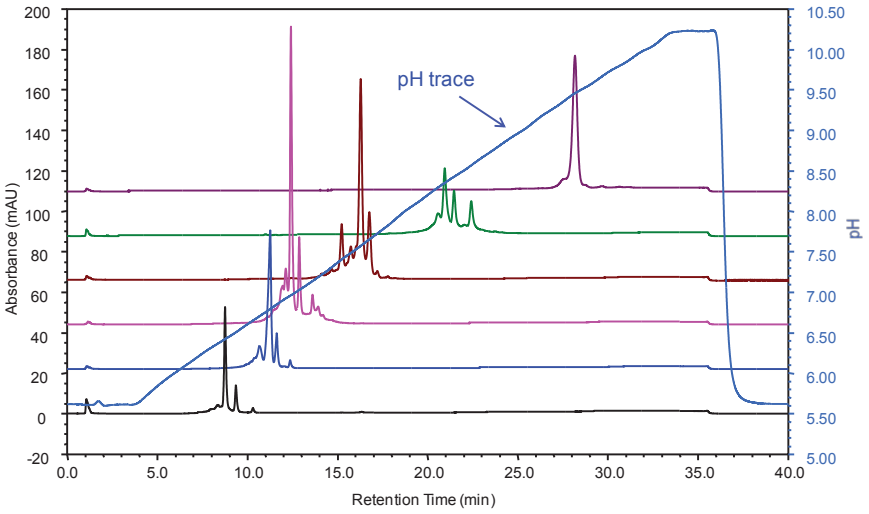
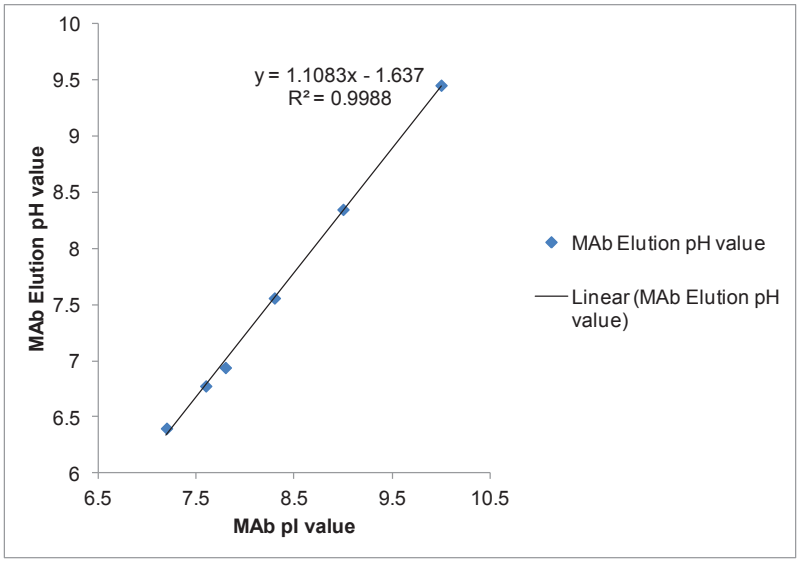


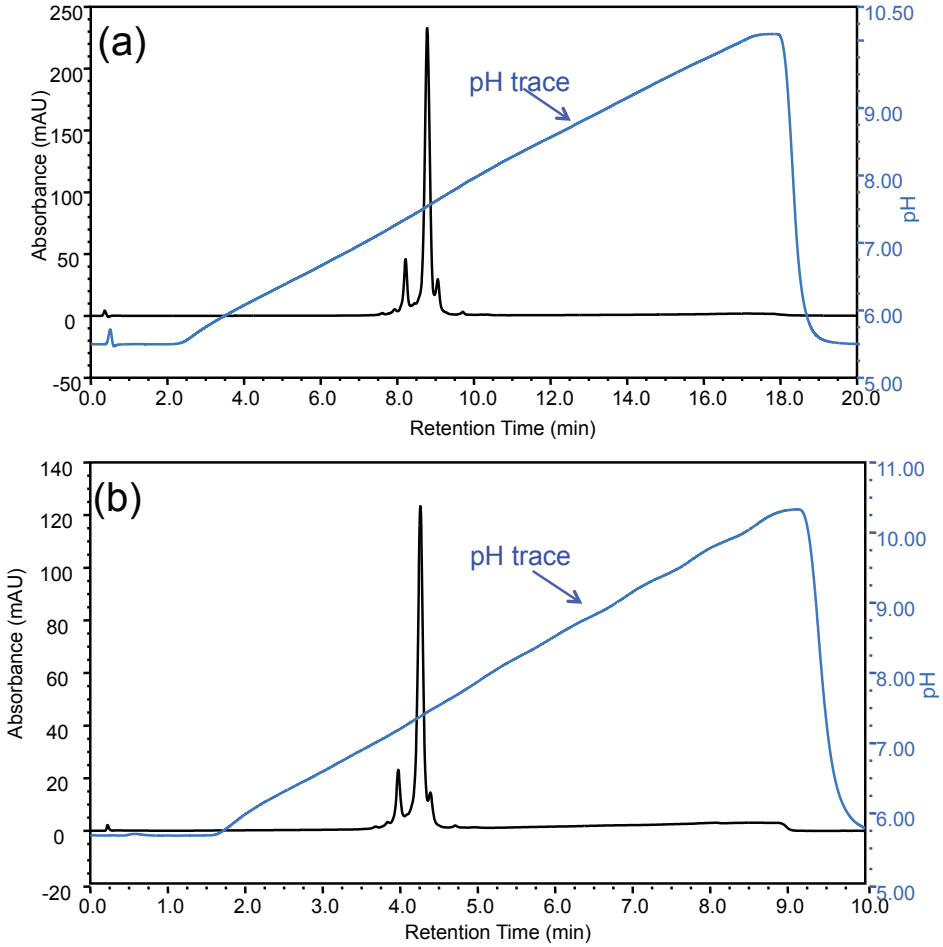
FIGURE 5. A graph plotting the elution pH values for six MAbs as a function of the corresponding pI value. The measured pH values of the major variant from all six MAbs were exported from the same experiment shown in Figure 4.



Fast pH Gradient Separation

The pH gradient method is fast and rugged. Figure 6 showed pH gradient runs on a MAbPac SCX-10, 5 µm, 4 × 50 mm column. Using 1 mL/min flow rate, MAb variants separation can be achieved within 15 min with a total run time of 20 min (figure 6a). Using 2 mL/min flow rate, MAb variants separation can be achieved within 7.5 min with a total run time of 10 min (figure 6b). In both cases, the linearity of the pH gradient maintained.

FIGURE 6. Fast MAb variant separation using pH gradient on a MAbPac SCX-10, 5 µm, 4 × 50 mm column. (a) 15-min gradient method; (b) 7.5-min gradient method. The MAb sample concentration was 5 mg/mL.



Conclusions

- Using pH gradient method, linear signal response is achieved with up to 300 µg protein loading;
- Using pH gradient method, the elution pH values of MAbs are in linear relationship with their corresponding pI values;
- Using pH gradient method, fast separation of MAb charge variants is achieved within a 10-min cycle time.

References

1. Lin, S., Baek, J., Decrop, W., Rao, S., Agroskin, Y., and Pohl, C. Development of a Cation-Exchange pH Gradient Separation Platform. Presented at 39th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Amsterdam, The Netherlands, June 16-20, 2013.

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