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Technical Article

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Improved Resolution in the Separation of Monoclonal Antibody Isoforms Using Controlled pH Gradients in IEX Chromatography

The formation of controlled pISep pH gradients has been described in detail.[1] In essence, two nearly identical buffer compositions, one acidic and one basic, are mixed together in varying proportions to produce a titration curve smooth enough to allow precise fitting by a high-order polynomial. The polynomial equation incorporated into custom software can calculate protocols that are used to drive computer-controlled gradient LC pumps to form pH gradients of any desired shape and slope. In this paper, pISep cationic exchange (CEX) chromatography (**CryoBioPhysica, Inc.**, Rockville, MD) was used for the separation of IgG1 monoclonal antibodies (MAbs) on a nonporous weak cation exchange (WCX) stationary phase.

The isolation and purification of MAb variants that have differing degrees of glycosylation or other covalent modifications are of great potential in understanding the physiological and evolutionary role of post-translational processing in the function of the vertebrate immune system. Several investigators[2–5] have raised the possibility of enhanced antitumor activity by selective use of MAb isoforms. These proposals are based on the fact that antitumor activity centers around the induction of immune-cell mediated cytotoxicity, which is controlled by the strength of binding of the Fc constant region of IgG to receptors on the surface of immune effector cells.

This binding strength is greatly influenced by the extent and chemistry of the glycosylation of the Fc region and by variations in the amino acid sequence of that region. The rate at which MAbs are catabolized by target tumor cells may also be influenced by these changes, affecting the ability of the MAbs to sustain antitumor activity. Other studies have shown that mutations of key Fc amino acids have a significant effect on clearance rates and consequent tumor localization, an important component of MAb-based diagnosis and localization studies.

Variations in both glycosylation and amino acid substitution will generally alter the pH at which MAb isoforms elute from an ion exchange (IEX) column during a pH gradient separation. In cancer research and MAb expression studies, the use of controlled pISep pH gradients for IEX purification of MAb isoforms promises to increase the selectivity and resolution efficiency of the isolation procedures. In fact, by making it feasible to isolate and concentrate low-abundance isoforms currently not accessible by other chromatography procedures, pISep holds the possibility of identifying new mechanisms of antitumor activity. Maximizing the numbers of isoforms that can be isolated and purified for study will be critically important to these developments.

The effect of flattening of pISep pH gradients on chromatographic resolution was of special interest in this study. The retention factor, k, for each MAb isoform will vary depending on its degree of glycosylation and on the pH. As the pH gradient is flattened, the eluting isoforms spend more time in pH ranges wherein their k values are large but also differ significantly from each other. As a consequence, there is more time for prolonged differential movement of the isoforms down the column, leading to better resolution. The data presented here confirm that, with pISep, the flatter the pH gradient; the better the resolution; the larger the shift of the apparent cationic pI to a less alkaline pH; and the narrower the elution pH range of the isoforms, though at the expense of longer separation time. The physics of this phenomenon are explored in more detail in Ref. 1.

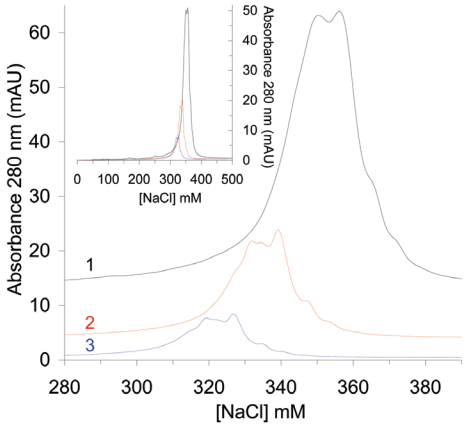

Figure 1 Effects of the salt gradient slope reduction on the chromatographic resolution of a CEX separation of the predominant, more alkaline isoforms of the monoclonal antibody IgG1. Slopes of the elution salt gradients—chromatogram 1: 10 mM CV^{-1}, chromatogram 2: 5 mM CV^{-1}, chromatogram 3: 2.5 mM CV^{-1}. Buffer A: 10 mM sodium acetate/acetic acid, pH 5; buffer B: 10 mM sodium acetate/acetic acid, pH 5, plus 500 mM NaCl, pH 5. Column: ProPac WCX 4 × 250 mm with column volume 3.124 mL, flow rate: 1 mL/min, injected IgG1: 240 µg, detection: 280 nm.

Results and discussion

Comparisons were made between salt gradient and pISep pH gradient separations of an IgG1 MAb on a ProPac WCX-10 column (**Dionex**, Sunnyvale, CA). The apparent pI of the MAb was in the pH range 8.5–9.2 when eluted using pISep pH gradients. The salt fractionations were conducted at an isocratic pH of 5. All separations were carried out over pH ranges wherein the stationary phase is totally charged.

Figure 1 shows the changes in resolution of a salt gradient separation as a function of

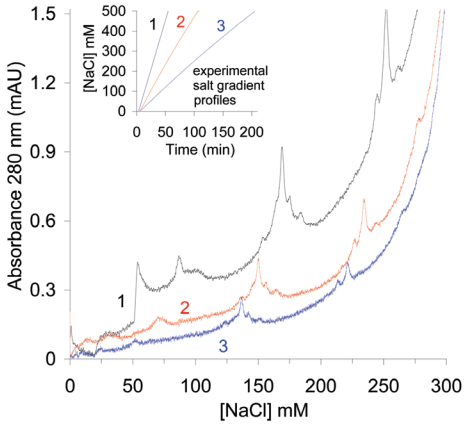

Figure 2 Effects of the salt gradient slope reduction on the chromatographic resolution of a CEX separation of the less abundant, more acidic isoforms of the monoclonal antibody IgG1. Slopes of the salt gradients presented in the inset—line 1: 10 mM CV^{-1}, line 2: 5 mM CV^{-1}, line 3: 2.5 mM CV^{-1}. Chromatograms 1, 2, and 3 illustrate IgG1 separations obtained with the salt gradients shown in the inset. Buffer A: 10 mM sodium acetate/acetic acid, pH 5; buffer B: 10 mM sodium acetate/acetic acid, pH 5, 500 mM NaCl, pH 5. Column: ProPac WCX 4 × 250 mm with column volume 3.124 mL, flow rate: 1 mL/min, injected IgG1: 240 µg, detection: 280 nm.

the slope of the gradient at pH 5. Despite the flattening of the gradient by a factor of 4 from 10 to 2.5 mM NaCl CV^{-1} (curve 3), the resolution of the predominant IgG1 isoforms seen at a slope of 10 mM NaCl CV^{-1} (curve 1) is not significantly improved. *Figure 2* illustrates the salt gradient separation of low-abundance IgG1 acidic isoforms as a function of the gradient slope. It is important to note that at the steepest gradient with a slope of 10 mM NaCl CV^{-1} (curve 1), about 12 acidic isoforms could be identified. At the flattest gradient, curve 3, with a slope of 2.5 mM NaCl CV^{-1}, three additional isoforms can be recognized but only as faint shoulders in the threshold of the main peak for a total of 15 acidic isoforms. Of these 15 isoforms, the resolution of nine is moderately improved by flattening the gradient.

Figure 3, curve 2 (in the inset) details the acidic to slightly alkaline pH region 5–8.1 of a pISep pH gradient separation with a rather steep slope of 0.25 pH units CV^{-1} from pH 5 to 9.5 (curve 1). The separation presented by curve 2 is similar to the salt gradient separations over the range 0–280 mM NaCl (shown in Figure 2), but it emphasizes the relative richness of the acidic isoform set revealed by the controlled pH gradient elution. About 35 acidic variants are identifiable, i.e., more than twice the number of isoforms seen in the flattest salt gradient fractionation (Figure 2, curve 3).

Figure 4 illustrates the changes in the chromatographic resolution of the more basic IgG1 isoforms eluted by pISep in the pH range 7.7–9.2 as a function of the slope of the pH gradient. At the steepest gradient with a slope of 0.25 pH units CV^{-1} (curve 1), the major isoforms are only modestly better resolved than the major isoforms separated by the flattest salt gradient (Figure 1, curve 3). When the slope of the pH gradient is decreased by half (Figure 4, curve 2), the four isoforms eluted between pH 8.1 and 8.7 (preceding the main peak) are much better resolved. The fine structure of the chromatogram in the pH range 7.8–8.1 (81–88 min in the inset) implies that there are as many as 15 or more isoforms revealed by pISep that are hidden in the steep threshold of the major peak in the salt gradient elution shown in Figure 1. Also in Figure 4, curve 2, the isoforms eluting from pH 8.4 to 8.8 are somewhat better resolved than the three isoforms that elute from pH 8.85 to 9 in curve 1 of Figure 4.

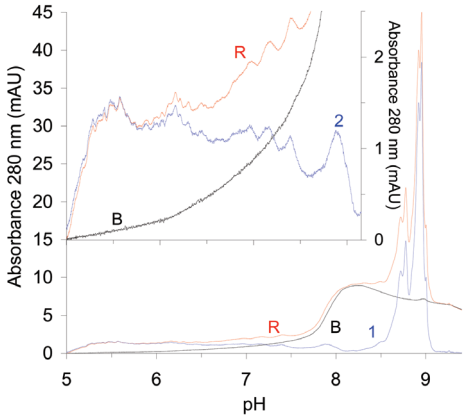

Figure 3 A CEX pISep pH gradient separation of the more acidic, less abundant isoforms of the MAb IgG1. Chromatograms 1 and 2 show baseline-corrected separation of IgG1 performed by a pH gradient with a slope of 0.25 pH units CV^{-1} from pH 5 to 9.5. Curves R (red) and B (black) are the experimental raw chromatogram and the experimental baseline. Buffer A: pISep, pH 2.4; buffer B: pISep, pH 10.9. Column: ProPac WCX 4 × 250 mm with column volume 3.124 mL, flow rate: 1 mL/min, injected IgG1: 110 µg, detection: 280 nm.

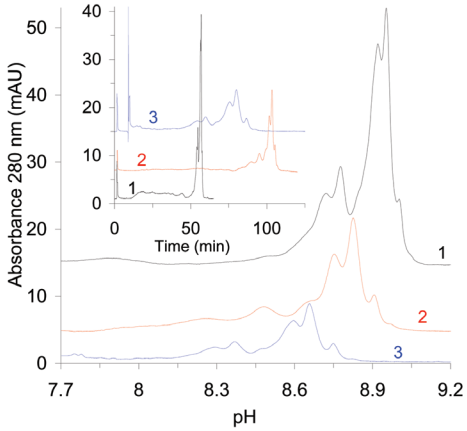

Figure 4 Effects of the pH gradient flattening on the chromatographic resolution of a pISep CEX separation of the predominant, more basic isoforms of the MAb IgG1. Slopes of the pH gradients used to obtain chromatograms 1, 2, and 3: 0.25, 0.125, and 0.042 pH units CV^{-1}, respectively. The main figure shows a part of the chromatograms detailing the separation of IgG1 in the pH range pH 7.7 to 9.2. The inset shows the whole chromatographic traces. Buffer A: pISep, pH 2.4; buffer B: pISep, pH 10.9. Column: ProPac WCX 4 × 250 mm with column volume 3.124 mL, flow rate: 1 mL/min, injected IgG1: 110 µg chromatograms 1 and 2 and 224 µg chromatogram 3, detection: 280 nm.

This illustrates an essential experimental fact that the improvement in resolution of each individual isoform is a unique nonlinear function of the pH gradient.

Finally, the almost invisible deviation in the back shoulder of the main peak at pH 9.05 in curve 1 is clearly resolved as an isoform at pH 9 in curve 2. Further analysis of curve 3 in Figure 4 highlights an important virtue of the pISep pH gradient technique: the continuing improvement in separation resolution as the gradient slope is progressively reduced. The two main isoforms at pH 8.76 and 8.83 and the minor isoforms at pH 8.9 and 8.98 in curve 2 are better resolved at pH 8.6, 8.67, 8.76, and 8.83 in curve 3. In this experiment, to shorten the run time, loading and binding of the IgG1 were initiated at pH 5 followed by a column wash and equilibration at pH 5, then stepping to pH 7.8 to begin a pH gradient elution ending at pH 9.5 with a slope of 0.042 pH units CV^{-1} (see inset in *Figure 5*, curve 3). This stepping protocol illustrates the flexibility of the pISep technique, i.e., it enables not only formation of controlled pH gradients, but also permits step changes from any pH to any other pH within the

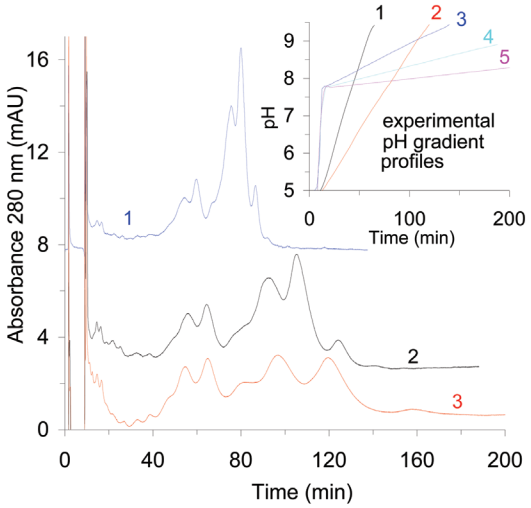

Figure 5 Effects of very flat pH gradients on the chromatographic resolution of the pISep CEX separation of the predominant, more basic isoforms of the MAb IgG1. Slopes of the pH gradients used to obtain chromatograms 1, 2, and 3: 0.042, 0.02, and 0.01 pH units CV^{-1}, respectively. The main figure details the separations of IgG1 in the pH range pH 7.7 to 9.2 (from 0 to 200 min). The inset shows the experimental pH gradients used to obtain the chromatograms presented in Figures 4 and 5. Lines 1, 2, 3, 4, and 5: pH gradients with slope of 0.25, 0.125, 0.042, 0.02, and 0.01 pH units CV^{-1}. Buffer A: pISep, pH 2.4; buffer B: pISep, pH 10.9. Column: ProPac WCX 4 × 250 mm with column volume 3.124 mL, flow rate: 1 mL/min, injected IgG1: 224 µg chromatogram 1, 456 µg chromatogram 2, and 651 µg chromatogram 3; detection: 280 nm.

pH limits of the acidic and alkaline pISep buffers in the two LC reservoirs.

In Figure 5 the influence of the pH gradient slope on the resolution is extended to pISep fractionations with very flat pH gradients. Here, chromatogram 1 is identical to chromatogram 3 of Figure 4, and the other two chromatograms show pH gradient separations at slopes of 0.02 pH units CV^{-1} (curve 2) and 0.01 pH units CV^{-1} (curve 3). Two effects of the reduced slopes stand out. First, the major peaks continue to be better resolved down to the lowest slope, and the UV detection sensitivity is high enough to suggest that further slope reduction could continue to improve the separation of the major peaks. Second, some of the components of the rather rich amalgam of less alkaline isoforms that are poorly resolved in the steepest gradient appear to be better resolved in the flatter gradients.

Numerous MAb separations not shown here also confirm that pISep allows one to scout both isoform resolution and shifts in the apparent pIs of closely eluting isoforms merely by varying the loading pH and the slope of the step(s) preceding the initiation of the pH gradient.[6] The elution order of the isoforms remains unaffected by these manipulations.

In conclusion, these IgG1 salt and pH gradient CEX data clearly demonstrate that, unlike flattening of a salt gradient, flattening of a pISep pH gradient is a simple and practical strategy that substantially improves the selectivity and resolution of MAb isoforms.

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