Real-time monitoring of recombinant antibody breakthrough during Protein A affinity chromatography

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An on-line assay was developed to monitor antibody breakthrough in real time during Protein A affinity chromatography of recombinant antibodies. When loading cell culture fluid on to a Protein A affinity column, antibody breakthrough cannot be measured by UV absorbance because of the flow-through of UV-absorbing impurities. An assay using perfusion chromatography media with immobilized Protein A is a rapid, antibody-specific assay. It directly samples preparative column effluent, allowing real-time measurement of antibody breakthrough during loading of Protein A affinity chromatography. Breakthrough curves were generated for three column media at five flow rates, showing the effects of diffusion on the shape of the breakthrough curve. The breakthrough curves were used to measure dynamic capacity.

Introduction

Breakthrough curves are important in Protein A affinity chromatography for studying the effects of diffusion during loading and for measuring the dynamic capacity of the column [1]. Protein A affinity chromatography provides a technique for purifying recombinant antibodies because it can selectively bind antibodies in complex solutions such as cell culture fluid, allowing impurities to flow through [2–5]. No antibody is present in the column effluent until the dynamic capacity of the column is reached and antibody begins flowing through the column without binding [6]. The flow-through of antibody creates the breakthrough curve.

When loading purified antibody, the breakthrough curve can be monitored by UV absorbance. However, when loading cell culture fluid containing recombinant antibodies, antibody breakthrough cannot be measured using UV absorbance because impurities in the flow-through obscure UV detection of antibody breakthrough. Effluent from the column can be collected in fractions and assayed for antibody, but this is a slow procedure, and the lack of real-time data means that the end of breakthrough cannot be observed so the column must be greatly overloaded to ensure that complete breakthrough has occurred. A rapid, reliable on-line assay method could greatly speed bioprocess development by enabling fast evaluation of the critical operational parameters used to design Protein A affinity chromatography processes.

An on-line assay directly samples the preparative column effluent, and provides a quantitative measurement of antibody breakthrough in real time. The on-line assay must be rapid and antibody-specific. Methods such as flow-injection immunoassays [7–9], biosensors [10] and chromatographic assays [11,12] have been used for on-line analysis. Chromatographic assays are fast [13] and can be used to quickly assay recombinant proteins [14], and commercial instrumentation specifically designed to run on-line chromatographic assays is available [15]. Perfusion chromatography can produce protein analysis in as little as 20 s [16,17] and Protein A is antibody-specific, so the on-line assay uses Protein A immobilized on perfusion chromatography media, providing a very rapid antibody-specific assay [18].

Measuring antibody breakthrough on-line in real time rapidly evaluates the effects of diffusion on the breakthrough curve. The breakthrough curve can be used to measure dynamic capacity, the amount of antibody that can be loaded before antibody breakthrough occurs. Diffusion and dynamic capacity are affected by several factors, including column media and flow rate [19,20]. A study of antibody loading behaviour for three types of column media at five flow rates was performed by using an on-line assay to quickly, accurately and reliably measure antibody breakthrough.

Materials and methods

Materials

Poros A/M columns, Poros 50 A media and BioCAD chromatography instruments were from PerSeptive Biosystems (Framingham, MA, U.S.A.). Prosep A chromatography media was from Bioprocessing (Consett, Co. Durham, U.K.) and Sepharose A chromatography media was

Abbreviations used: C_t, antibody concentration in the column effluent (g/l); C_o, antibody concentration in the load (g/l); F, volumetric flow rate (l/h); t, time (h); V_c, column volume (l).

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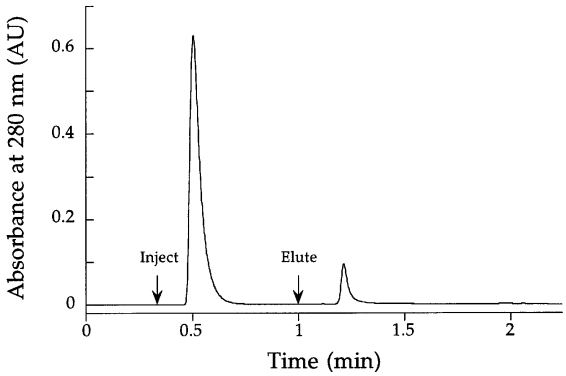

Figure 1 A chromatogram from the on-line assay

The eluted peak is antibody.

from Pharmacia (Uppsala, Sweden). Load material was cell culture fluid with cells and cell debris removed by tangential flow filtration, containing a monoclonal antibody with a human constant region produced in Chinese hamster ovary cells at a concentration of approximately 0.5 g/l. This load material was obtained from Genentech, Inc. (South San Francisco, CA, U.S.A.).

Analytical chromatography

On-line analysis was performed by a 2.3 min perfusion affinity assay (Figure 1). The assay used a 30 × 2.1 mm Poros A/M (immobilized Protein A, 20 μm particle size) column at room temperature. Buffer A was 10 mM sodium phosphate/150 mM sodium chloride, pH 7.2, and buffer B was 10 mM sodium phosphate/150 mM sodium chloride, pH 2.2. Two flow rates were used: 20 ml/min for purge, 3.5 ml/min for run. Injection volume was 20 μl from the process stream using an in-line sampling T to directly sample the preparative column effluent. Detection was at 280 nm. The method was: purge 3.5 ml buffer A, run 15 column volumes of buffer A, inject, run 12 column volumes of buffer A, purge 3.5 ml buffer B, run 20 column volumes of buffer B. Chromatography was run on a BioCAD/RPM chromatograph.

On the BioCAD instrument, purging takes the column out of the flow path, allowing the increased purge flow rate to rapidly fill the flow path with buffer. After purging, the flow rate is decreased and the column is then placed back in-line. This purge/run method decreases assay time. The BioCAD/RPM software automatically integrates the antibody peak and displays the result immediately after the assay is finished.

Preparative chromatography

Preparative Protein A affinity chromatography used 0.66 cm-diameter columns. Columns were equilibrated with five column volumes of 25 mM Tris/25 mM NaCl/5 mM EDTA, pH 7.1, and eluted with three column volumes of 0.1 M acetic acid, pH 3.5. Media, flow rates and column lengths for breakthrough curves are given in the Figure legend (see below). All buffers and load material were filtered through a 0.22 μm filter prior to use. Chromatography was performed on a BioCAD chromatograph.

Results and discussion

The on-line assay measures antibody breakthrough in real time during loading, directly sampling the effluent from the preparative column. The assay is 2.3 min long (Figure 1), and the assay result is available as soon as the assay has finished running. For this study, a 2.3 min assay time was sufficient, but the assay time could be reduced by increasing the flow rate or by decreasing the equilibration time. The assay provides a fast method for real-time analysis of antibody breakthrough. The on-line assay was used to generate breakthrough curves, which then were used to measure dynamic capacity.

Breakthrough is often expressed as the fraction of loaded antibody that is in the column effluent, C_i/C_o, where C_i is the concentration of antibody in the column effluent and C_o is the concentration of antibody in the load. During breakthrough, C_i/C_o starts at 0 when all loaded antibody is bound to the column and ends at 1.0 when the column is saturated and all loaded antibody is flowing through the column [21]. Breakthrough curves can be plotted as C_i/C_o versus antibody loaded ($F \cdot C_o \cdot t/V_c$, where F is the volumetric flow rate, t is the load time, and V_c is the column volume), which allows dynamic capacity to be measured directly from the graph.

The shape of the breakthrough curve for affinity absorption results from the ability of the antibody to diffuse on to and into the chromatography medium. The rate of this diffusion is governed primarily by two mechanisms: film diffusion (mass transfer from the mobile phase to the surface of the particle) and pore diffusion (diffusion into the particle) [22]. With no mass transfer effects (ideal behaviour), C_i/C_o would increase to 1.0 the instant that saturation capacity was reached, resulting in a vertical breakthrough curve. Diffusion changes the shape of the breakthrough curve, distorting it from its ideal shape [23]. The shape of the breakthrough curve for $C_i/C_o < 0.5$ tends to be determined by film diffusion, and the shape of the breakthrough curve for $C_i/C_o > 0.5$ tends to be determined by pore diffusion [24].

Using the on-line assay, a study determined the effects of column media and flow rate on the shape of breakthrough curves (Figure 2). Each point on the breakthrough curves represents the results from a single assay, 2.3 min apart. When the flow rate was lower or the column length greater,

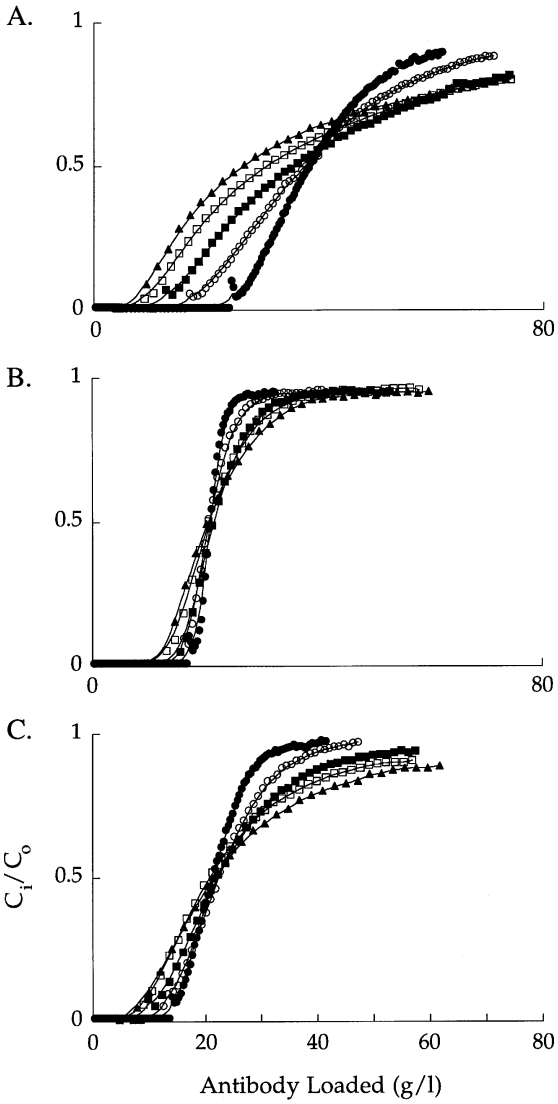

Figure 2 Breakthrough curves measured by the on-line assay

On all three graphs, the horizontal axis is antibody loaded ($F \cdot C_o \cdot t/V_c$) in g of antibody/l of column volume, and the vertical axis is C_i/C_o. The column length was 10 cm for all media. (**A**) Sepharose A, (**B**) Poros 50 A, (**C**) Prosep A. Flow rates were: ●, 200 cm/h; ○, 400 cm/h; ■, 600 cm/h; □, 800 cm/h; and ▲, 1000 cm/h.

more assays were run during breakthrough. In certain cases, breakthrough approached $C_i/C_o = 1.0$ asymptotically, so not all curves were run to $C_i/C_o = 1.0$. At lower flow rates the breakthrough curves become almost vertical, approaching ideal behaviour. At higher flow rates the slope of the breakthrough curve became shallow because the antibody exited the column before it could diffuse to the binding sites.

The breakthrough curves for each chromatography medium have different shapes, due to differences in the diffusion characteristics of the chromatography media. For Sepharose A (Figure 2A), at higher flow rates the shape of

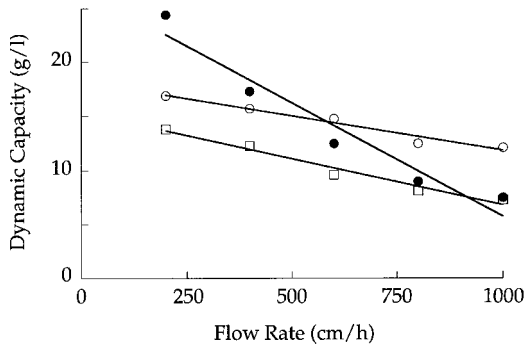

Figure 3 Dynamic capacity measured at $C_i/C_o = 0.05$ on the breakthrough curves from Figure 2, in g of antibody/l of column volume

Media were: ●, Sepharose A ($y = 27\text{-}0.021x$, $R = 0.97$); ○, Poros 50 A ($y = 18\text{-}0.0064x$, $R = 0.98$); and □, Prosep A ($y = 15\text{-}0.0086x$, $R = 0.98$).

the breakthrough curve was not symmetrical around $C_i/C_o = 0.5$, indicating that a relatively large amount of antibody has to be loaded before saturation is reached due to slow pore diffusion. At higher flow rates, only the most accessible binding sites are loaded before breakthrough occurs. Poros 50 A (Figure 2B) has breakthrough curves that are symmetrical around $C_i/C_o = 0.5$, indicating equal contributions of pore and film diffusion and relatively short diffusion path lengths in the pores. The breakthrough curves for Prosep A (Figure 2C) have high slope when $C_i/C_o < 0.5$, but the slope decreases when $C_i/C_o > 0.5$ at higher flow rates due to slow pore diffusion, indicating that some binding sites are deep in the media.

The differences in diffusion characteristics produce differences in dynamic capacity, which was measured as the amount of antibody loaded at $C_i/C_o = 0.05$. This is the smallest breakthrough level that can be accurately measured with the on-line assay using a 20 μl injection, but a lower breakthrough level can be measured by increasing the injection volume. Dynamic capacity was found to be linearly related to flow rate (Figure 3). Sepharose A has a high dynamic capacity at low flow rates, but dynamic capacity drops off quickly as flow rate increases because only the most accessible binding sites are filled. Poros 50 A and Prosep A both have shallower slopes than Sepharose A, and Poros 50 A has a higher dynamic capacity than Prosep A at all flow rates studied. When breakthrough curves are sharp, there is little additional capacity that can be gained by loading past breakthrough. When breakthrough curves are shallow or are not symmetrical, there is significant additional capacity which can be loaded at the expense of antibody loss in the flow-through.

Other factors may affect the dynamic capacity of Protein A affinity chromatography, including the antibody concentration in the load, buffer type, temperature, pH and column length [20,25–28]. Using the on-line assay, these

factors can be rapidly assessed for their impact on antibody breakthrough to aid in bioprocess development.

The on-line assay measures antibody breakthrough in real time during loading of preparative Protein A chromatography, leading to a fast evaluation of diffusion characteristics and measurement of dynamic capacity. The assay is fast, reliable and accurate enough to generate breakthrough curves and to measure dynamic capacity. This approach allows rapid experimental evaluation of the critical operational parameters that can be used to design Protein A chromatography processes.

References

1 Chase, H. A. (1984) J. Chromatogr. **297**, 179–202
2 Surolia, A., Pain, D. and Khan, M. I. (1982) Trends Biochem. Sci. **7**, 74–76
3 Ey, P. L., Prowse, S. J. and Jenkin, C. R. (1978) Immunochemistry **15**, 429–436
4 Reis, K. J., Boyle, M. D. P. and Ayoub, E. M. (1984) J. Clin. Lab. Immunol. **13**, 75–80
5 Lindmark, R., Thoren-Tolling, K. and Sjoquist, J. (1983) J. Immunol. Meth. **62**, 1–13
6 Arnold, F. H., Blanch, H. W. and Wilke, C. R. (1985) Chem. Eng. J. **30**, B9–B23
7 Middendorf, C., Schulze, B., Freitag, R., Scheper, T., Howaldt, M. and Hoffmann, H. (1993) J. Biotechnol. **31**, 395–403
8 van der Pol, J. J., de Gooijer, C. D., Biselli, M., Wandrey, C. and Tramper, J. (1996) Trends Biotechnol. **14**, 471–477
9 Nilsson, M., Hakanson, H. and Mattiasson, B. (1992) J. Chromatogr. **597**, 383–389
10 Mulchandani, A. and Bassi, A. S. (1995) Crit. Rev. Biotechnol. **15**, 105–124
11 Hunt, A. J., Lynch, P. D., Londo, T., Dimond, P., Gordon, N. F., McCormack, T., Schutz, A., Percoskie, M., Cao, X., McGrath, J. P., Putney, S. and Hamilton, R. A. (1995) J. Chromatogr. A **708**, 61–70
12 Chase, H. A. (1986) Biosensors **2**, 269–286
13 Nugent, K. and Olson, K. (1990) BioChromatography **5**, 101–105
14 Olson, K. C. and Gehant, R. L. (1992) Biotechnol. Progr. **8**, 562–566
15 Ozturk, S. S., Thrift, J. C., Blackie, J. D. and Naveh, D. (1995) Biotechnol. Bioeng. **48**, 201–206
16 Afeyan, N. B., Fulton, S. P., Gordon, N. F., Mazsaroff, I., Varaday, L. and Regnier, F. E. (1990) BioTechnology **8**, 203–206
17 Fulton, S. P., Afeyan, N. B., Gordon, N. F. and Regnier, F. E. (1991) J. Chromatogr. **547**, 452–456
18 Paliwal, S. K., Nadler, T. K., Wang, D. I. C. and Regnier, F. E. (1993) Anal. Chem. **65**, 3363–3367
19 Horstmann, B. J. and Chase, H. A. (1989) Chem. Eng. Res. Des. **67**, 243–254
20 Van Sommeren, A. P. G., Machielsen, P. A. G. M. and Gribnau, T. C. J. (1992) Prep. Biochem. **22**, 135–149
21 Johnston, A., Mao, Q. M. and Hearn, M. T. W. (1991) J. Chromatogr. **548**, 127–145
22 Cowan, G. H., Gosling, I. S., Laws, J. F. and Sweetenham, W. P. (1986) J. Chromatogr. **363**, 37–56
23 Mao, Q. M., Prince, I. G. and Hearn, M. T. W. (1995) J. Chromatogr. A **691**, 273–283
24 Helfferich, F. G. and Carr, P. W. (1993) J. Chromatogr. **629**, 97–122
25 Kamiya, Y., Majima, T., Sohma, Y., Katoh, S. and Sada, E. (1990) J. Ferment. Bioeng. **69**, 298–301
26 Tu, Y. Y., Primus, F. J. and Goldenberg, D. M. (1988) **109**, 43–47
27 Yamamoto, S. and Sano, Y. (1992) J. Chromatogr. **597**, 173–179
28 Katoh, S., Kambayashi, T., Deguchi, R. and Yoshida, F. (1978) Biotechnol. Bioeng. **20**, 267–280

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