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Real-time control of antibody loading during protein A affinity chromatography using an on-line assay

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Abstract

We show that an on-line chromatographic assay can reliably control antibody loading in real-time during protein A affinity chromatography purification of a recombinant antibody from clarified Chinese hamster ovary cell culture fluid. The on-line assay directly sampled preparative column effluent and provided real-time measurement of antibody breakthrough during loading. The on-line assay used protein A immobilized on perfusion chromatography media, equilibrated with phosphate-buffered saline at pH 7.2 and eluted with phosphate-buffered saline at pH 2.2. The assay reliably ended loading at 1% breakthrough with minimal yield loss. Reproducible yield and purity were obtained over 23 consecutive cycles. Yield remained constant while breakthrough capacity and the antibody concentration in the load changed.

Keywords: Process control; Preparative chromatography; Immobilized proteins; Antibodies; Proteins

1. Introduction

Recombinant monoclonal antibodies have important therapeutic applications, including the treatment of several types of cancer [1–5]. Protein A affinity chromatography provides a technique for purifying recombinant antibodies because it can selectively bind antibodies in complex solutions such as clarified cell culture fluid, allowing impurities to flow through [6–9]. When loading clarified 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. Since protein A affinity chromatography media are expensive, rather than using a column large enough to process a batch of antibody in a single cycle, typical process applications use a smaller column for several cycles to purify a single batch. Therefore in process chromatography, protein A affinity columns are typically loaded by volume after determining the antibody concentration in the load. A rapid, reliable on-line assay method could measure antibody breakthrough and stop loading when breakthrough occurs.

Methods such as flow-injection immunoassays [10–12], biosensors [13], and chromatographic assays have been used for on-line analysis. Chromatographic assays have been used for monitoring and controlling fermentation and cell culture processes by measuring product concentration in real-time [14–

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17], and for monitoring and controlling chromatography processes [18–22]. Chromatographic assays are particularly attractive for on-line use because they are fast and reliable [23–26], and instrumentation specifically designed to run on-line chromatographic assays is commercially available [15]. Perfusion chromatography can analyze proteins in as little as 20 s [24,27] 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 [16].

Loading by use of an on-line assay provides two important benefits over loading by volume: the column is always loaded to its breakthrough (or dynamic) capacity, and the antibody concentration in the load does not need to be measured before loading. Breakthrough capacity depends on many factors, including the type of protein A affinity chromatography medium, the antibody concentration in the load, the column temperature and column length, the buffer, conductivity and pH of the load, and the flow-rate [28–33]. If any of these variables change, the breakthrough capacity could also change. Also, the column may degrade over many uses, and its breakthrough capacity may decrease. Breakthrough capacity is often maximized in order to utilize as completely as possible the expensive protein A affinity chromatography medium. Using the on-line assay to stop loading ensures that the column is always loaded to its breakthrough capacity. The on-line assay can be used to stop loading independent of the antibody concentration in the load, so no assay needs to be run prior to loading. This allows the protein A affinity chromatography process to begin as soon as the clarified cell culture fluid is obtained, reducing production time.

In this paper, we describe an on-line chromatographic assay that can accurately quantify antibody concentration in 2.0 min. We show that an on-line assay has the speed and sensitivity necessary to accurately load to 1% breakthrough with minimal yield loss, that loading by use of the on-line assay is reliable over 23 consecutive cycles, and that the assay can reliably control loading when the breakthrough capacity or the antibody concentration in the load changes.

2. Experimental

2.1. Materials and instruments

Poros A/M columns (immobilized protein A, 20 μm particle) and BioCAD chromatography instruments were from PerSeptive Biosystems (Framingham, MA, USA). Prosep A chromatography medium (immobilized protein A, 80–120 μm irregular controlled-pore glass particle) was from Bioprocessing (Consett, UK). Load material was cell culture fluid with cells and cell debris removed by tangential flow filtration, containing a monoclonal antibody (IgG1) with a human constant region produced in Chinese hamster ovary cells at a concentration of approximately 0.73 g/l. This load material was obtained from Genentech (South San Francisco, CA, USA).

2.2. On-line assay and process control

The on-line assay (almost identical to a previously described assay [34]) used a 30 mm×2.1 mm Poros A/M column at room temperature. Buffer A was 8 m*M* sodium phosphate, 2 m*M* potassium phosphate, 3 m*M* potassium chloride, 137 m*M* sodium chloride, pH 7.2, and buffer B was 8 m*M* sodium phosphate, 2 m*M* potassium phosphate, 3 m*M* potassium chloride, 137 m*M* sodium chloride, pH 2.2. The assay was run on a BioCAD/RPM, and used two flow-rates, one for run (5.8 ml/min) and one for purge (50 ml/min). Detection was at 280 nm. The assay directly sampled the preparative column effluent using an in-line sampling valve, with an injection volume of 500 μl. The method was: purge 5 ml buffer A, run 3 ml buffer A, inject, run 2.5 ml buffer A, purge 5 ml buffer B, run 2 ml buffer B. The assay was 2.0 min long. The BioCAD/RPM software automatically integrates the antibody peak and displays the result immediately after the assay is finished. To enable process control, the BioCAD/RPM (running the on-line assay) would give a signal to the BioCAD (a separate instrument running the preparative chromatography) when the appropriate level of breakthrough was reached.

2.3. Off-line assays

The amount of host cell proteins (Chinese hamster ovary proteins) was determined by enzyme-linked immunosorbent assay (ELISA) using goat anti-(host cell proteins) antibodies [35].

The antibody concentration in both the load material and the purified pool (used to determine yield) was measured by an high-performance liquid chromatography (HPLC) assay. The assay used a 10 cm×0.46 cm I.D. Poros A/M column. The flow-rate was 2 ml/min, detection was absorbance at 280 nm, and the injection volume was 100 μl. Buffer A was 100 m*M* sodium phosphate, 250 m*M* sodium chloride pH 6.3, buffer B was 2% acetic acid, 100 m*M* glycine pH 3.0, and buffer C was 10% acetic acid. The method was: inject, wash for 2.5 min with 100% buffer A, gradient 100% buffer A to 100% buffer B over 2 min, hold 100% buffer B for 1 min, regenerate with 100% buffer C for 1 min, equilibrate 100% buffer A for 3 min. The total assay time was 9.5 min.

2.4. Semi-preparative chromatography

Semi-preparative chromatography used a 15 cm× 1 cm I.D. Prosep A column. Four buffers were used. Buffer A was 25 m*M* Tris, 25 m*M* NaCl, 5 m*M* EDTA, pH 7.1; buffer B was 25 m*M* Tris, 25 m*M* NaCl, 5 m*M* EDTA, 0.5 *M* tetramethylammonium chloride pH 7.0; buffer C was 0.1 *M* acetic acid, pH 3.5; and buffer D was 2 *M* guanidine·HCl, 10 m*M* Tris, pH 7.5. The column was equilibrated with five column volumes of buffer A, loaded, washed with 3 column volumes of buffer A, washed with 3 column volumes buffer B, washed with three column volumes of buffer A, eluted with five column volumes of buffer C, and regenerated with three column volumes of buffer D. All columns were equilibrated, washed, eluted, and regenerated at 550 cm/h. The columns were loaded at 550 cm/h except for the variable flow-rate experiments. The eluted peak was collected by absorbance at 280 nm. Chromatography was run on a BioCAD.

3. Results and discussion

For reliable process control, the on-line assay must be accurate, fast and sensitive. Because protein A binds human immunoglobulin G (IgG), the assay is accurate because the eluted peak is IgG. The assay must be fast enough that there will be little error in detecting breakthrough accurately. If the assay is too slow, significant breakthrough or even column saturation may occur before the assay detects any breakthrough. A 2 min long assay is fast enough for this application (allowing approximately 13 assays before breakthrough), but the assay speed could be increased by increasing the flow-rate or decreasing the injection volume and the associated wash volume. We found that a 0.5 ml injection was necessary to accurately measure antibody at a level of 0.0073 g/l (1% breakthrough, 3.65 μg antibody), and that a 2.5 ml wash was necessary to flush the 0.5-ml injection loop adequately. However, by optimizing the integration parameters, using a more sensitive detection method (such as fluorescence), or decreasing the instrument noise, a much smaller injection volume may be possible, thus increasing assay speed. In addition to decreasing assay time, the use of purges on the BioCAD instrument produces a flat baseline before and after the elution phase of the assay, ensuring reliable integration. The on-line assay requires absolutely reproducible peak integration, because for real-time process control there is no chance to reintegrate the peak and the failure to integrate a single assay correctly could result in control failure.

Because the assay must detect breakthrough before loading can stop, some yield loss will occur. Although the assay can accurately detect very small levels of breakthrough, there will always be some yield loss as antibody flows through the column. The amount of yield loss is related to the breakthrough curve. Breakthrough starts at 0% when all loaded antibody is bound to the column and ends at 100% when the column is saturated and all loaded antibody is flowing through the column [36]. With no mass transfer effects (ideal behavior), breakthrough would increase to 100% the instant that saturation capacity was reached, resulting in a vertical breakthrough curve. However, in non-ideal situations, the shape of the breakthrough curve results from the ability of the antibody to diffuse onto and into the chromatography media [37]. Diffusion changes the shape of the

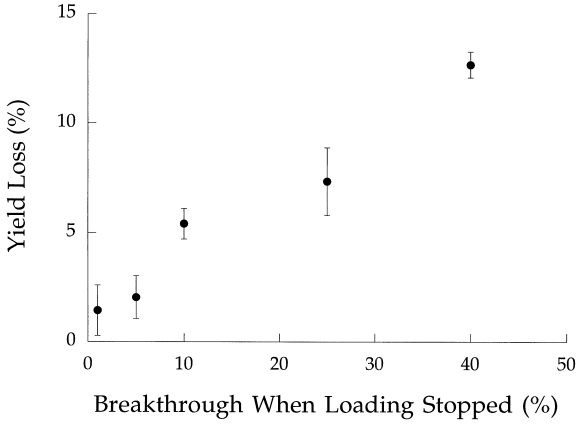

Fig. 1. The yield loss due to loading when using the on-line assay. Points are averages for three runs, and error bars are plus or minus one standard deviation. Yield loss was calculated against a control of the average of three runs loaded by volume to 13.6 g/l.

breakthrough curve [38], making it more shallow. Thus, the amount of yield loss will be determined by the level of breakthrough allowed before loading stops. We measured the yield loss at breakthrough levels from 1% to 40% (Fig. 1). The yield loss increased as the amount of breakthrough allowed before loading stopped increased. The precision of loading was approximately the same at 1% breakthrough as it was at 40% breakthrough as determined by yield. The standard deviations in Fig. 1 may be due to variations in the assay and not variations in the actual yield, since for each breakthrough level the amount of antibody loaded for the three replicate runs was nearly identical.

When used to stop loading at 1% breakthrough, the on-line assay reliably controlled loading over 23 consecutive cycles (Table 1) with no failures. Approximately 13 assays were run before breakthrough was detected. The low standard deviation for antibody loaded for runs controlled by the on-line assay (RSD 6%) shows that the assay can reproducibly end loading when the breakthrough capacity is reached. The data in Fig. 1 suggests that there will be a small yield loss (<2%) when loading to 1% breakthrough. However, we found that in this cycling study the observed yield loss was less than the error of the assay used to determine yield (Table 1). The yield loss was undetectable, with equal yield when the load was controlled by the on-line assay or by volume. A more accurate method of determining yield may be able to detect the yield loss, which is probably less that 5%. Purity (measured by the amount of host cell proteins) when loading by the on-line assay was the same as the purity when the column was loaded by volume (Table 1), indicating that the slight increase in load does not affect purity. Thus, the on-line assay can be used to reproducibly stop loading at 1% breakthrough, with minimal yield loss and comparable antibody purity.

To study the ability of the on-line assay to accurately control loading when the breakthrough capacity changes, the assay was used to stop loading at several load flow-rates. An increased flow-rate will decrease breakthrough capacity. As the flow-rate during the load increased, breakthrough capacity decreased almost linearly (Fig. 2). The standard deviations for breakthrough capacity were very small, even for the runs at 550 cm/h, where 23 runs were performed. The yield remained constant as breakthrough capacity changed (Fig. 3). This study suggests that the on-line assay could reliably stop loading due to breakthrough capacity changing for any reason, such as column degradation or poor column packing. Thus the on-line assay could serve as an indicator of column performance, which may be important in large-scale production for detection of reduced column performance. This also suggests that the on-line assay could be used effectively during process development to accurately measure

Table 1
Results from study loading to 1% breakthrough by the on-line assay, compared to control runs loaded by volume[a]

	Antibody loaded (g/l)	Yield (%)	Host cell proteins (mg/g)
Load controlled by the on-line assay (n=23)	14.3±0.8	95.1±4.6	8.0±1.3
Loaded by volume (n=8)	13.6	93.3±4.3	8.3±1.4

[a] Antibody loaded is g antibody per l column volume, and the amount of host cell proteins in the eluted antibody pool is mg host cell proteins per g antibody. Values are averages plus or minus one standard deviation.

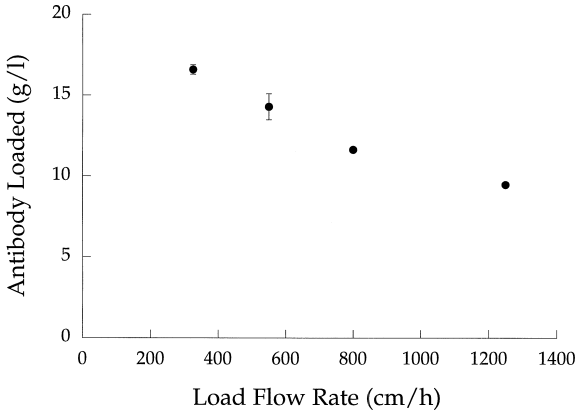

Fig. 2. Effect of varying load flow-rate on the amount of antibody loaded when using the on-line assay to stop loading at 1% breakthrough. Points are averages for three experimental runs (23 experimental runs at 550 cm/h), and error bars are plus or minus one standard deviation. The amount of antibody loaded is measured in g antibody per l column volume. The error bars for the two highest flow-rates are too small to see.

how varying process parameters affect breakthrough capacity, allowing rapid experimental evaluation of the critical operational parameters which can be used to design an efficient protein A chromatography process.

If the on-line assay is allowed to control loading as the concentration of the antibody in the load changes, the assay will have to stop loading at a predetermined level of antibody concentration rather than at a certain level of antibody breakthrough. Because the load material would not be assayed prior to loading, the appropriate concentration of antibody in the preparative column effluent for 1% breakthrough cannot be calculated. In a study of varying antibody concentration in the load, the assay was able to reliably and accurately control loading when the antibody concentration in the load changed. The volume loaded increased as the concentration of antibody in the load decreased (Fig. 4), while the yield (Fig. 5) did not change significantly.

Determining if this approach to process control would be effective in large-scale antibody production will require further analysis. During this study with 50 process control runs, no control failures were observed, and yield and purity remained constant. However, in large-scale production a control failure could cost several hundred thousand dollars due to lost product, so the failure rate will need to be more closely studied. A complete economic analysis would need to include the cost of running, maintaining, and validating the on-line assay. This cost would be offset by the reduction in cost for the quality control department to assay the load material for volumetric loading, the cost of holding load material while the assay is being run (including the loss in production time), and any product loss that may

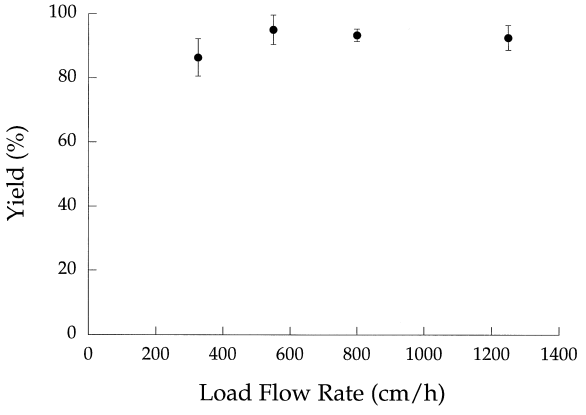

Fig. 3. Effect of varying load flow-rate on the yield of antibody in the eluted antibody pool when using the on-line assay to stop loading at 1% breakthrough. Points are averages for three experimental runs (23 experimental runs at 550 cm/h), and error bars are plus or minus one standard deviation.

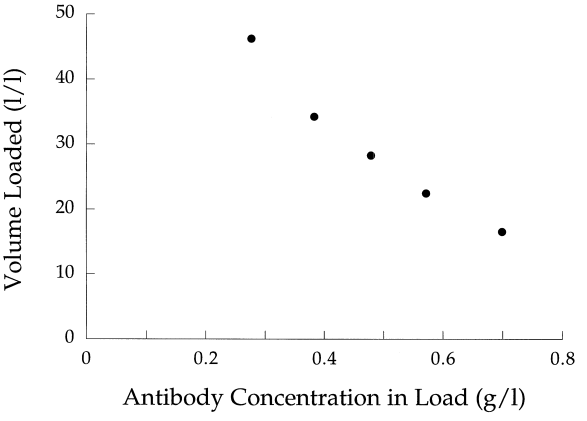

Fig. 4. The effect of varying antibody concentration in the load on the volume of antibody loaded when loading to 0.0075 g/l of antibody in the preparative column effluent using the on-line assay. The amount of antibody loaded is measured in l of antibody per l of column volume.

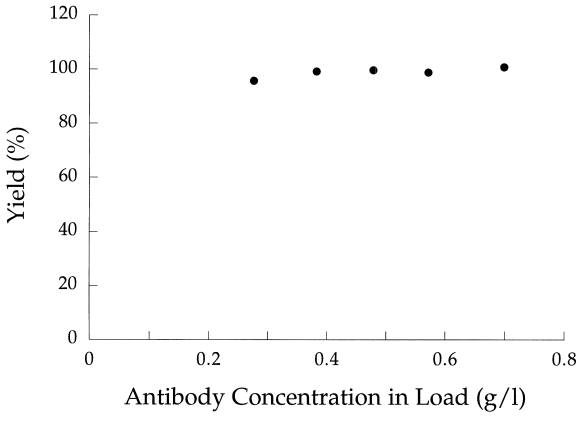

Fig. 5. The effect of varying antibody concentration in the load on the yield of antibody in the eluted antibody pool when loading to 0.0075 g/l of antibody in the preparative column effluent using the on-line assay.

occur due to undetected breakthrough capacity changes in the column.

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References

[1] P. Carter, L. Presta, C.M. Gorman, J.B.B. Ridgway, D. Henner, W.L.T. Wong, A.M. Rowland, C. Kotts, M.E. Carver, H.M. Shepard, Proc. Natl. Acad. Sci. USA 89 (1992) 4285.
[2] B. Bodey, H.E. Kaiser, R.H. Goldfarb, Anticancer Res. 16 (1996) 517.
[3] D.R. Anderson, A. Grillo-Lopez, C. Varns, K.S. Chambers, N. Hanna, Biochem. Soc. Trans. 25 (1996) 705.
[4] D.L. Longo, Curr. Opin. Oncol. 8 (1996) 353.
[5] J. Baselga, D. Tripathy, J. Mendelsohn, S. Baughman, C.C. Benz, L. Dantis, N.T. Sklarin, A.D. Seidman, C.A. Hudis, J. Moore, P.P. Rosen, T. Twaddell, I.C. Henderson, L. Norton, J. Clin. Oncol. 14 (3) (1996) 737.
[6] A. Surolia, D. Pain, M.I. Khan, Trends. Biochem. Sci. 7 (1982) 74.
[7] P.L. Ey, S.J. Prowse, C.R. Jenkin, Immunochemistry 15 (1978) 429.
[8] K.J. Reis, M.D.P. Boyle, E.M. Ayoub, J. Clin. Lab. Immunol. 13 (1984) 75.
[9] R. Lindmark, K. Thoren-Tolling, J. Sjoquist, J. Immunol. Methods 62 (1983) 1.
[10] C. Middendorf, B. Schulze, R. Freitag, T. Scheper, M. Howaldt, H. Hoffmann, J. Biotechnol. 31 (1993) 395.
[11] J.J. van der Pol, C.D. de Gooijer, M. Biselli, C. Wandrey, J. Tramper, Trends Biotechnol. 14 (1996) 471.
[12] M. Nilsson, H. Hakanson, B. Mattiasson, J. Chromatogr. 597 (1992) 383.
[13] A. Mulchandani, A.S. Bassi, Crit. Rev. Biotechnol. 15 (No. 2) (1995) 105.
[14] H. Lundstrom, M. Brobjer, B. Osterlof, T. Moks, Biotechnol. Bioeng. 36 (1990) 1056.
[15] S.S. Ozturk, J.C. Thrift, J.D. Blackie, D. Naveh, Biotechnol. Bioeng. 48 (1995) 201.
[16] S.K. Paliwal, T.K. Nadler, D.I.C. Wang, F.E. Regnier, Anal. Chem. 65 (1993) 3363.
[17] C. Turner, M.E. Gregory, N.F. Thornhill, Biotechnol. Bioeng. 44 (1994) 819.
[18] H.A. Chase, Biosensors 2 (1986) 269.
[19] A.J. Hunt, P.D. Lynch, T. Londo, P. Dimond, N.F. Gordon, T. McCormack, A. Schutz, M. Percoskie, X. Cao, J.P. McGrath, S. Putney, R.A. Hamilton, J. Chromatogr. A 708 (1995) 61.
[20] G. Thevenon-Emeric, F.E. Regnier, Anal. Chem. 63 (1991) 1114.
[21] R.E. Cooley, C.E. Stevenson, Process Control Qual. 2 (1992) 43.
[22] R.L. Fahrner, P.M. Lester, G.S. Blank, D.H. Reifsnyder, J. Chromatogr. A 827 (1999) 37.
[23] K. Nugent, K. Olson, BioChromatography 5 (No. 2) (1990) 101.
[24] N.B. Afeyan, S.P. Fulton, N.F. Gordon, I. Mazsaroff, L. Varaday, F.E. Regnier, Bio/Technology 8 (1990) 203.
[25] S.K. Paliwal, T.K. Nadler, F.E. Regnier, Trends Biotechnol. 11 (1993) 95.
[26] K.C. Olson, R.L. Gehant, Biotechnol. Prog. 8 (1992) 562.
[27] S.P. Fulton, N.B. Afeyan, N.F. Gordon, F.E. Regnier, J. Chromatogr. 547 (1991) 452.
[28] Y. Kamiya, T. Majima, Y. Sohma, S. Katoh, E. Sada, J. Ferment. Bioeng. 69 (No. 5) (1990) 298.
[29] Y.Y. Tu, F.J. Primus, D.M. Goldenberg, J. Immunol. Methods 109 (1988) 43.
[30] A.P.G. Van Sommeren, P.A.G.M. Machielsen, T.C.J. Gribnau, Prep. Biochem. 22 (No. 2) (1992) 135.
[31] S. Katoh, T. Kambayashi, R. Deguchi, F. Yoshida, Biotechnol. Bioeng. 20 (1978) 267.
[32] G. Schuler, M. Reinacher, J. Chromatogr. 587 (1991) 61.
[33] P. Fuglistaller, J. Immunol. Methods 124 (1989) 171.
[34] R.L. Fahrner, G.S. Blank, Biotechnol. Appl. Biochem. 29 (1999) 109.
[35] A.B. Chen, J. Biotechnol. Healthcare 3 (No. 1) (1996) 70.
[36] A. Johnston, Q.M. Mao, M.T.W. Hearn, J. Chromatogr. 548 (1991) 127.
[37] G.H. Cowan, I.S. Gosling, J.F. Laws, W.P. Sweetenham, J. Chromatogr. 363 (1986) 37.
[38] Q.M. Mao, I.G. Prince, M.T.W. Hearn, J. Chromatogr. A 691 (1995) 273.