4 Perfusion Affinity Chromatography for Rapid Antibody Separations

Robert L. Fahrner and Gregory S. Blank

Believe nothing because a wise man said it.
Believe nothing because it is generally held.
Believe nothing because it is said to be divine.
Believe only what you yourself judge to be true.
Lord Buddha

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1 Introduction

Perfusion chromatography is an important tool for rapid separations of biomolecules. Introduced more than a decade ago [1-3], perfusion chromatography media are polymeric (polystyrene-divinylbenzene) and contain large through-pores that completely penetrate the chromatographic bead. These large through-pores allow convective (or "perfusive") flow through the bead. Along the convective through-pores are smaller diffusive pores with short diffusive

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path lengths. The combination of convection and diffusion allows perfusion chromatography to be run at extremely high flow rates, in excess of 15,000 cm/h.

One of the most useful applications of perfusion chromatography is in affinity chromatography [10]. Because of the rapid adsorption/desorption rate for affinity ligands, they are well suited for use in perfusion chromatography. One of the most important applications of affinity chromatography is for the purification of antibodies. Antibodies, both full-length antibodies produced in Chinese hamster ovary (CHO) cells and antibody fragments produced in *E. coli*, are becoming more important as their usefulness in treating human diseases such as cancer is expanded [4, 5]. Affinity chromatography plays an important role in the analysis and purification of these antibodies. Protein A and protein G are bacterial cell wall proteins which bind specifically to antibodies with varying degrees of affinity depending on the species and subclass of the antibody. For human or humanized IgG, the affinity is typically very strong. Protein A and protein G affinity chromatography provide a technique for purifying recombinant antibodies because they can selectively bind antibodies in complex solutions, allowing impurities to flow through [6-9]. For assays, this produces high specificity for antibodies, and for preparative applications it produces an extremely high level of purification in a single step.

This chapter describes the use of rapid affinity chromatography for both analytical and preparative applications. Of particular concern is the use of "on-line" assays, where the assay directly samples the process stream and provides a real-time quantifiation of the antibody. On-line assays have been used extensively for rapid measurement of analytes in process streams [11-17], and we include extensive applications for both process monitoring and process control.

2 Materials

Equipment

Poros columns and media, BioCAD instruments and RPM software were obtained from PerSeptive Biosystems (Framingham, MA, USA). Load material for assays and preparative columns were obtained from Genentech, Inc. (South San Francisco, CA, USA). Load material was either a full-length recombinant humanized IgG1 produced in CHO cells, or a recombinant F(ab)'2 produced in *E. coli*. The HP1100 instrument was obtained from Hewlett-Packard (Mountain View, CA, USA).

Buffers

- PBS (phosphate-buffered saline) pH 7.2: 8 mM sodium phosphate, 2 mM potassium phosphate, 3 mM potassium chloride, 137 mM sodium chloride, pH 7.2 **(Buffer 1)**
- PBS pH 2.2: 8 mM sodium phosphate, 2 mM potassium phosphate, 3 mM potassium chloride, 137 mM sodium chloride, pH 2.2 **(Buffer 2)**
- Preparative equilibration buffer: 25 mM Tris, 25 mM NaCl, 5 mM EDTA, pH 7.1 **(Buffer 3)**
- Preparative elution buffer: 0.1 M acetic acid, pH 3.5 **(Buffer 4)**
- Preparative regeneration buffer: 2 M guanidine HCl, 10 mM Tris, pH 7.5 **(Buffer 5)**
- Preparative storage buffer: 50 mM sodium acetate, 2% benzyl alcohol, pH 5.0 **(Buffer 6)**

3 Methods

3.1 Analytical chromatography

All assays use 2.1 x 30 mm (inner diameter x length) Poros affinity columns (0.1 ml). Other column sizes may be used with equivalent results in terms of sensitivity and accuracy, but this small column produces the minimum analysis time because small buffer volumes are required for equilibration and elution due to the small column volume, and the antibody elutes with a small peak width due to the short column length. PBS pH 7.2 **(Buffer 1)** and PBS pH 2.2 **(Buffer 2)** are used as buffers for all assays. Other binding and elution buffers may be used if the PBS buffers cause precipitation or quantifiation difficulties. Good choices for alternate buffers are the preparative equilibration and elution buffers **(Buffers 3 and 4)**. We have found equivalent assay sensitivity and accuracy when running the assay from 500 to 15000 cm/h, so the actual flow rate used for the assay can be varied depending on the required assay speed and instrument limitations. For on-line assays, we have used the BioCAD chromatograph because it has been developed to run these rapid assays and has software (RPM) that is designed specifically to run on-line assays.

Off-line assays

Off-line assays can be run on any standard HPLC instrument (like the HP1100, which we use here). This example uses a protein G column for quantifiation of both antibodies and antibody fragments. A linear gradient is used to improve peak integration. Without the linear gradient (using a step elution), there is a slight baseline change that can interfere with accurate integration. The column is calibrated with a purified standard of known concentration. For a recombi-

nant F(ab)'2 with a 100 µl injection volume, we found a linear response from 0.01 mg/ml to 1 mg/ml. This range can be modified by adjusting the injection volume. Although the assay described below uses protein G for antibodies and antibody fragments, it can also use protein A in this configuration for analysis of full-length antibodies. Samples may be diluted with equilibration buffer to reduce the concentration so it is within the range of the assay or to adjust the pH of the sample to >6 for binding.

Protocol 1 off-line assay (Fig. 1)

1. System: Hewlett-Packard HP1100
2. Column: Poros G/M 2.1x30 mm
3. Flow rate: 3 ml/min
4. Injection: 100 µl
5. Detection: Absorbance at 280 nm
6. Method:

Time (min)	% PBS pH 7.2 (Buffer 1)	% PBS pH 2.2 (Buffer 2)	
0	100	0	
2	100	0	Equilibration
3.75	0	100	Linear gradient
4	0	100	Hold
4.01	100	0	Equilibration
6	100	0	

7. Integration: Peak width 0.005, Area Reject 15, Height Reject 0.08, Integration On at 2 min, Integration Off at 4 min.

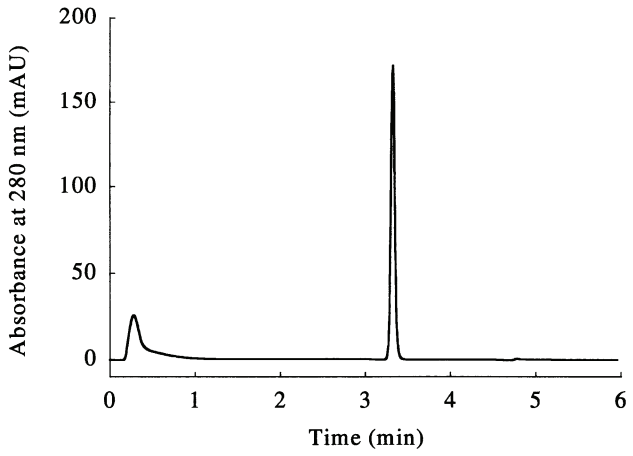

Figure 1 Chromatogram from the off-line protein G affinity assay (Protocol 1) analyzing a recombinant F(ab)'2 produced in *E. coli*.

On-line assays

For routine analysis the off-line assay is sufficient. However, when a large number of assays will be run on effluent from a preparative or process column or some other process stream, using on-line assays can be beneficial. On-line assays are more difficult to set up and run properly (primarily because of the time spent correcting hardware and software problems to ensure reliability), but once set up they can run thousands of assays with little user intervention. They are useful when a large number of runs need monitoring, where collecting and analyzing fractions would be prohibitive. Because the on-line assay injects samples directly from the process stream with no sample preparation, the assay can only be used when the process stream has a pH between approximately 5 and 9 to ensure sample binding after injection.

We have used the BioCAD chromatograph extensively for running on-line assays. We found several benefits with this instrument, such as software that is specifically designed to run on-line assays, a sample loader with multiple configurations, and the ability to run rapid assays in several configurations. The on-line assays described below use a basic protocol of switching between equilibration and elution buffers.

The only substantial difference between the on-line assays (Protocols 2 and 3) and the off-line assay (Protocol 1) is the use of purges on the BioCAD instrument for the on-line assays. 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. The purges are used for two reasons. First, the purges decrease assay time by rapidly filling the system dead volume with buffer. While a purge takes about 20 seconds at 50 ml/min, at the assay flow rate of 5.8 ml/min it takes over 1 minute for the buffer to fill the system dead volume. Second, the purge between injection/wash and elution allows more reproducible integration. The on-line assay requires absolutely reproducible peak integration, because for real-time analysis (for either process monitoring or control) there is no chance to reintegrate the peak. Purging gives the assay a flat baseline on either side of the peak for reliable integration, and the elution time for the antibody peak is defined by the purges before and after the elution.

- Process monitoring

This assay can be used for a variety of applications where real-time quantifiation of antibody concentration is beneficial. One important application is generating antibody breakthrough curves in real time (see Section 5.1). It can also be used for monitoring the elution of antibodies from columns when absorbance cannot effectively monitor antibody, or to monitor the antibody concentration in tanks or fermentors.

Protocol 2 on-line assay for process monitoring (Fig. 2)

1. System: BioCAD/RPM
2. Column: Poros 2.1 x 30 mm A/M
3. Flow rate: run 3.5 ml/min, purge 20 ml/min
4. Injection: 20 µl
5. Detection: absorbance at 280 nm
6. Method: purge 3.5 ml PBS pH 7.2 **(Buffer 1)**, run 15 column volumes of PBS pH 7.2, inject, run 12 column volumes of PBS pH 7.2, purge 3.5 ml PBS pH 2.2 **(Buffer 2)**, run 20 column volumes of PBS pH 2.2 **(Buffer 2)**.

- Process control

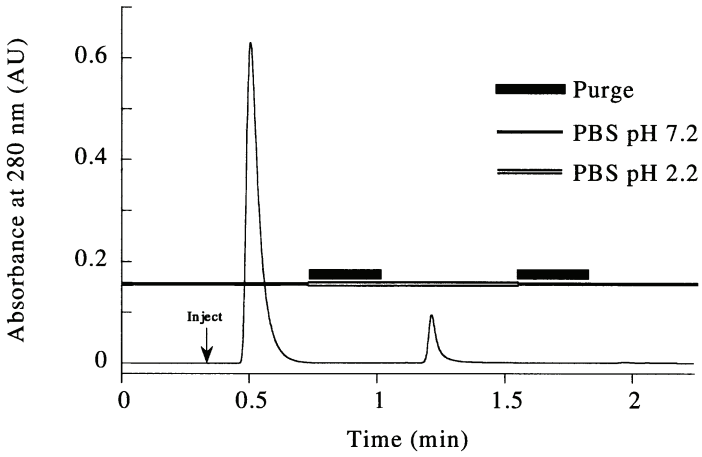

Figure 2 Chromatogram from the on-line protein A affinity assay (Protocol 2) analyzing a recombinant full-length antibody produced in Chinese hamster ovary cells.

This assay may be used to control loading during chromatography where the breakthrough of antibody cannot be seen by absorbance alone–for example, when loading clarified cell culture fluid onto a protein A affinity column. This assay is more sensitive than the assay used for process monitoring, and it can reliably detect antibody concentrations of <0.003 g/l in the process stream. In a recent study, over 1,000 assays were run in real time, with no errors in real-time peak integration [17].

An important factor in designing this assay was when to stop the post-injection wash and begin the purge of elution buffer. As the wash volume increases, the eluted peak area becomes smaller because the tail of the wash peak elutes as a sharp peak just in front of the actual antibody peak (Fig. 3). Both elution peaks are integrated as a single peak because it would be difficult to reliably integrate them separately. Although increasing the wash volume could slightly increase sensitivity, we found that increasing the wash volume beyond 2.5 ml provided little benefit in either accuracy or sensitivity. Using the conditions described below, the assay can reliably quantify 0.5% breakthrough in 2.0 minutes.

Protocol 3 on-line assay for process control (Fig. 3)

1. System: BioCAD/RPM
2. Column: 2.1x30 mm Poros A/M
3. Flow rate: 5.8 ml/min run, 50 ml/min purge
4. Injection: 500 µl (500 µl loop overfilled with a 1000 µl injection)
5. Detection: absorbance at 280 nm
6. Method: below is the method as written on a BioCAD:

	General Settings: Set Detectors Offline
	General Settings: Set Column 1 Inline
	General Settings: UV Detector Wavelength = 280 nm
	General Settings: Flow Rate = 5.8 ml/min
	General Settings: Turn UV Detector Lamp On
0.00 min	Method Start
0.00 min	[01] Load Block
0.00 min	(A) Equil
0.00 min	0.00 ml Load through Pump 100% A: PBS pH 7.2
0.52 min	3.00 ml Load through pump 100% A: PBS pH 7.2
0.52 min	(B) Inject
0.52 min	0.00 min Load sample into loop: 1000 µl
0.77 min	0.25 min Inject sample
0.77 min	[02] Wash Block
0.77 min	(A) Wash
0.77 min	0.00 ml Set Solvent Blend 100% A: PBS pH 7.2
1.20 min	2.50 ml Set Sample Loop to Load Position*
1.20 min	2.50 ml End Solvent Blend 100% A: PBS pH 7.2
1.20 min	[03] Elute Block
1.20 min	(A) Purge Elute
1.20 min	0.00 ml Set Column Offline
1.20 min	0.00 ml Set Solvent Blend 100% B: PBS pH 2.2
1.20 min	0.00 ml Set Flow Rate = 50.00 ml/min
1.30 min	5.00 ml Set Flow Rate = 5.8 ml/min
1.38 min	5.48 ml Set Column 1 Inline
1.38 min	(B) Elute
1.38 min	0.00 CV Set Solvent Blend 100% B: PBS pH 2.2
1.74 min	20.00 CV End Solvent Blend 100% B: PBS pH 2.2
1.74 min	[04] Equil Block
1.74 min	(A) Purge Elute
1.74 min	0.00 ml Set Column Offline
1.74 min	0.00 ml Set Solvent Blend 100% B: PBS pH 2.2
1.74 min	0.00 ml Set Flow Rate = 50.00 ml/min
1.84 min	5.00 ml Set Flow Rate = 5.8 ml/min
1.92 min	5.48 ml Set Column 1 Inline
1.92 min	(B) Equil
1.92 min	0.00 min Set Solvent Blend 100% A: PBS pH 7.2
1.92 min	0.00 min Zero UV Detector*
1.92 min	0.00 min End Solvent Blend 100% A: PBS pH 7.2

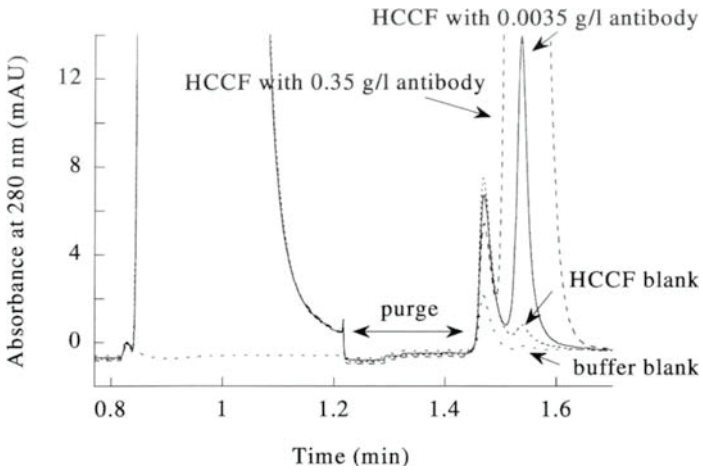

Figure 3 Overlaid chromatograms from the on-line protein A affinity assay for process control (Protocol 3). Several concentrations of a recombinant full-length antibody in HCCF (harvested cell culture fluid) were injected, as well as a buffer blank. The flow-through peak rises to >2000 mAU and the antibody peak for the 0.35 g/l injection rises to 600 mAU. In this extremely magnified view, the elution profile for the 0.0035 g/l peak can be seen.

Approximately 0.25 min can be eliminated by pumping the preparative column eluent through the sample loop and eliminating the need to pull the sample into the loop with the syringe pump. We use the syringe pump on the instrument because it gives greater control over injections. The loop is overfilled to ensure an accurate injection volume. This method, when programmed as shown, will transfer well to the RPM software on the BioCAD/RPM system.

7. Integration. On the BioCAD, the integration parameters listed below will produce reliable integration of the peak. Note that the integrator begins timing when the sample injection occurs, so integration is performed from 0.5 min until 1.2 min.

Ev#	Event	Start Time	Stop Time	Value
1	Slope	0.000	0.000	1000
2	Fine Slope	0.000	0.000	1000
3	Width	0.000	0.000	20
4	Integration Off	0.000	0.500	0
5	Minimum Area	0.000	0.000	100000
6	Integration Off	1.200	2.000	0

8. Process control. If two BioCAD instruments are available, they can be easily hooked together to enable real-time process control to stop loading when breakthrough occurs. To enable process control with one BioCAD (running the preparative chromatography) and one BioCAD/RPM (running the on-line assay), make the following connections between the auxiliary ports on the two instruments:

BioCAD/RPM (assay system)		BioCAD (preparative system)
7 (ground)	to	12 (ground)
8 (start)	to	10 (aux out 1)
9 (hold)	to	11 (aux out 2)
12 (ground)	to	2 (ground)
11 (aux TTL out 2)	to	1 (aux in)

On the BioCAD/RPM, when concentration > 0.01 g/l, set channel 2 (aux TTL out 2) high. On the BioCAD, set a threshold during the load that ends the segment when the auxiliary input > 0.75 volts. When the load begins, the BioCAD is set to give an auxiliary out event which starts the BioCAD/RPM. After loading, the BioCAD gives another auxiliary out event which stops the BioCAD/RPM. (See Protocol 4 for the preparative method).

3.2 Preparative chromatography

The 20 μm Poros medium (A/M) can be used preparatively simply by loading a larger amount of antibody onto the column, with loads typically >25 g/l. (Note that loads for preparative columns are typically stated in g/l, grams of antibody per liter of column volume.) Although this is efficient for small-scale purification of <100 mg, at larger scales it requires a large high-pressure system. The Poros protein A affinity chromatography medium also comes in 50 μm particle size, which can be run using low-pressure pumps and is scaleable to preparative or process scale. Because of its larger particle size, it does not produce as much convective flow as the 20 μm medium, and flow rates above 1000 cm/h generally produce poor peaks and lowered capacity.

Protocol 4 preparative chromatography (Fig. 4)

1. System: BioCAD
2. Column: 1.6 cm x 10 cm Poros 50 A
3. Flow rate: 500 cm/h (17 ml/min). The pressure drop on a 10 cm bed height column with a flow rate of 500 cm/h is approximately 1 bar, well within the limits of most preparative and process columns. The normal operation flow rate can be from 50 to 1000 cm/h.

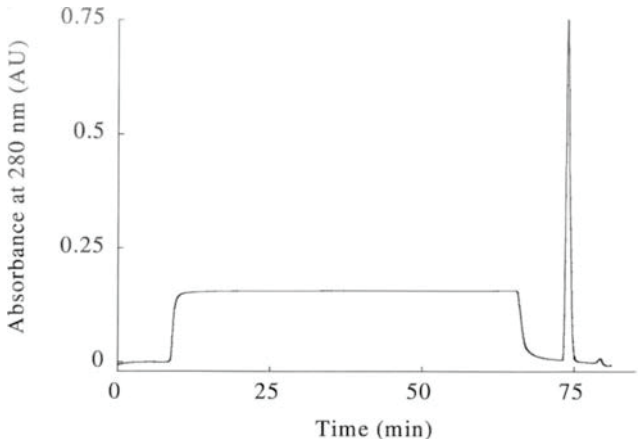

Figure 4 Chromatogram from the preparative chromatography method (Protocol 4), purifying a recombinant full-length antibody.

4. Load: approximately 15–20 g/l for human IgG
5. Detection: absorbance at 280 nm
6. Method:

Buffer	Duration
Equilibration **(Buffer 3)**	5 column volumes (CV)
Load	15–20 g/l
Wash with equilibration buffer (Buffer 3)	6 CV
Elution **(Buffer 4)**	5 CV, collect peak 0.1 to 0.1 AU
Regeneration **(Buffer 5)**	2 CV
Storage **(Buffer 6)**	5 CV

7. For programming on a BioCAD chromatograph, with process control enabled by linking it with a BioCAD/RPM, the following BioCAD method is used:

(In the method, A is **Buffer 3**, C is **Buffer 4**, D is **Buffer 5** and E is the load material.)

	General Settings: Set Detectors Offline
	General Settings: Set Column 1 Inline
	General Settings: UV Detector Wavelength = 280 nm
	General Settings: Flow Rate = 500 cm/hr
	General Settings: Turn UV Detector Lamp On
0.00 min	Method Start
0.00 min	[01] Equil Block
0.00 min	(A) Equil
0.00 min	0.00 CV Set Solvent Blend 100% A: Equil
0.30 min	0.25 CV Auxiliary Out Event*
6.00 min	5.00 CV Zero UV Detector
6.00 min	5.00 CV End Solvent Blend 100% A: Equil
6.00 min	[02] Load Block
6.00 min	(A) Load 1
6.00 min	0.00 ml Load Through Pump 100% E: Load
13.64 min	50.00 ml Load Through Pump: 100% E: Load
13.64 min	Thresholds [UV > 0.05 AU, Delay 0.00 ml]
13.64 min	(B) Load 2
13.64 min	0.00 CV Load Through Pump 100% E: Load
13.88 min	0.20 CV Auxiliary Out Event*
14.24 min	0.50 CV Load Through Pump 100% E: Load
14.24 min	(C) Load 3
14.24 min	0.00 ml Load Through Pump 100% E: Load
128.83 min	750 ml Load Through Pump 100% E: Load
128.83 min	Thresholds [Aux > 0.75 Volts, Delay 0.00 ml]
128.83 min	[03] Wash Block
128.83 min	(A) Wash
128.83 min	0.00 CV Set Solvent Blend 100% A: Equil
136.03 min	0.00 CV Auxiliary Out Event*
136.03 min	6.00 CV End Solvent Blend 100% A: Equil
136.03 min	[04] Elute Block
136.03 min	(A) Elute 1
136.03 min	0.00 CV Set Solvent Blend 100% C: Elution
144.43 min	7.00 CV End Solvent Blend 100% C: Elution
144.43 min	Thresholds [UV > 0.100 AU, Delay 0.00 ml]
144.43 min	(B) Elute 2
144.43 min	0.00 CV Set Solvent Blend 100% C: Elution
144.43 min	0.00 CV Collect Fraction*
156.43 min	10.00 CV End Solvent Blend 100% C: Elution
156.43 min	Thresholds [UV < 0.080 AU, Delay 0.00 ml]
156.43 min	(C) Elute 3
156.43 min	0.00 ml Set Solvent Blend 100% C: Elution
157.96 min	10.00 ml Divert to Waste*
157.96 min	10.00 ml End Solvent Blend 100% C: Elution
157.96 min	[05] Clean Block
157.96 min	(A) Regeneration
157.96 min	0.00 CV Set Solvent Blend 100% D: Regen
160.36 min	2.00 CV End Solvent Blend 100% D: Regen
160.36 min	[06] Equil Block
160.36 min	(A) Equil
160.36 min	0.00 CV Set Solvent Blend 100% A: Equil
162.76 min	2.00 CV Pump Off*
162.76 min	2.00 CV End Solvent Blend 100% A: Equil

4 Troubleshooting

- *The off-line assay will not integrate properly.* A slight baseline shift occurs between the equilibration and elution buffers, and this shift may interfere with integration. Using a linear gradient for elution will minimize the effect of this shift. Different systems may need different gradient lengths to ensure reliable integration. Increasing the gradient length may resolve integration issues.
- *The on-line assay will not integrate properly.* Ensure that the program is written exactly as in the protocols, and that the correct integration parameters are used. See Protocol 3 for integration parameters. The time for "integration start" and "integration end" events is calculated from the injection and not from the start of the method. Make sure there is sufficient wash volume (>5 times the loop volume) to completely wash the sample out of the loop. Any leftover sample will be integrated with the elution peak after the purge.
- *There is no elution peak on the assay.* Make sure that the antibody bound, and that the sample has antibody in it. For protein A and protein G affinity chromatography, the pH of the sample should be between pH 6 and pH 9 to bind properly. For the off-line assay, dilute the sample 2x or 3x with the equilibration buffer prior to loading. For the on-line assay, make sure the process stream is at the correct pH. In addition to constraints on pH, the protein G affinity assay often requires a high ionic strength to properly bind (>100 mM).
- *Yield is low for the preparative method.* First ensure that the load material contains antibody. If it does, the most common cause of low yield is antibody flowing through during the load. The load amount of 15–20 g/l is for human IgG1, and other subclasses and species of antibodies will have less capacity, sometimes as low as 1–2 g/l. If the capacity is lower, simply load less antibody. Another problem can be accurate collection of the elution peak. The peak can elute in about 1 column volume, and this rapid, concentrated elution is sometimes difficult to collect. Ensure that the instrument is programmed to start and stop collection at a relatively low absorbance (0.1 to 0.1 AU is common), and take into account the dead volume between the detector and the collector.
- *The backpressure on the assay column constantly increases.* The PBS buffers can sometimes cause sample precipitation. If this is the case, try using the equilibration and elution buffers for the preparative method. These buffers are much less likely to cause problems with the sample. Also, ensure that all buffers are filtered through a 0.2 μm filter before use.
- *The assay is too slow.* Some applications require an assay faster than the 2 min assay we have described in this chapter. Increasing the flow rate or decreasing the injection volume (and associated loop wash) can reduce assay time. There are also several other options, such as running "sub-

tractive" assays and configuring the BioCAD for faster buffer introduction. Other methods may be found at the PerSeptive website (www.pbio.com).

- *The syringe pump injects air.* When using the syringe pump to pull a sample from a process stream for the on-line assay, it must often pull through a long length of tubing, which can cause cavitation in the syringe. Using larger inner diameter tubing or shorter tubing length can help. If the process stream is too viscous to pull through the tubing without cavitation, use a separate sample pump (such as a peristaltic pump) to pump the sample into the loop.

5 Applications

5.1 Breakthrough curves

Breakthrough curves are important in chromatography for studying the effects of mass transfer during loading and for measuring the breakthrough capacity of the column [18]. No antibody is present in the column effluent until the breakthrough capacity of the column is reached and antibody begins flowing through the column without binding [19]. 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. The perfusion affinity chromatography assays can be used to measure breakthrough curves when antibody breakthrough cannot be measured by absorbance.

Using the off-line assay to generate a breakthrough curve (Fig. 5).
When loading a semi-purified recombinant F(ab)'2 onto an ion-exchange column, the breakthrough can be monitored by collecting fractions and assaying it with the protein G assay (Protocol 1). Breakthrough is often expressed as the fraction of loaded antibody that is in the column effluent, C/C_0, where C is the concentration of antibody in the column effluent and C_0 is the concentration of antibody in the load. During breakthrough, C/C_0 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. Breakthrough curves can be plotted as C/C_0 vs cumulative antibody loaded ($F \bullet C_0 \bullet t/V_c$, where F is the volumetric flow rate, t is the load time, and V_c is the column volume), which allows breakthrough capacity to be measured directly from the graph.

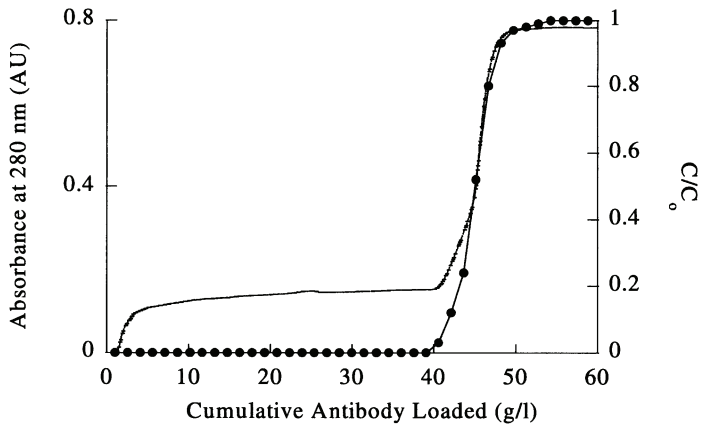

Figure 5 Breakthrough curve for a partially purified recombinant F(ab)'2 loaded onto an cation exchange (Poros 50 HS) column at pH 4.0. Fractions were collected and diluted 3x with PBS pH 7.2 (Buffer 1) before injection onto the off-line protein G affinity assay. The dashed line is absorbance and the solid line with circles is C/C_0.

Using the on-line assay to generate a breakthrough curve (Fig. 6)

The off-line assay is useful when only a few breakthrough curves are generated. 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 often be greatly overloaded to ensure that complete breakthrough has occurred. Once it is set up and running, the on-line assay can be used to quickly generate breakthrough curves for a variety of loading conditions. For example, one of the process variables that most strongly affects the breakthrough curves is flow rate, and the on-line assay can quickly measure breakthrough curves at varying flow rate (Fig. 6). When the flow rate increases from 200 cm/h to 1000 cm/h, the breakthrough curve becomes shallower and the antibody begins breakthrough sooner. To use the preparative column most effectively, the column is loaded to its breakthrough capacity (capacity at 1% or 5% breakthrough), which allows full

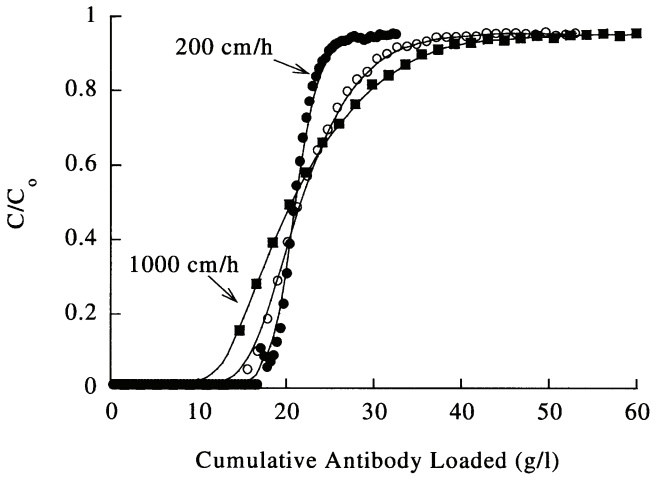

Figure 6 Breakthrough curves for a recombinant antibody loaded onto a Poros 50 A (protein A, 50 μm particle size) column. The column length was 10 cm. Three flow rates were used: ● 200 cm/h, ○ 600 cm/h, ■ 1000 cm/h.

utilization of available binding sites while allowing very little antibody to flow through. From the breakthrough curve, breakthrough capacity can be measured easily.

If the on-line assay is run with the RPM software, the software will export data to a Microsoft Excel spreadsheet. These data are in the form of time vs. concentration. Using the formula above for cumulative antibody loaded, the time can be converted into cumulative antibody loaded. By dividing the concentration data from the RPM software by the concentration of antibody in the load, concentration can be converted to C/C_0. These converted spreadsheet data can then be graphed and the breakthrough curve can be seen.

5.2 Using the on-line assay and the preparative method together (Fig. 7)

Using the on-line assay for process control (Protocol 3) and the preparative method (Protocol 4) together allows antibody loading to stop when breakthrough occurs. This provides several benefits. First, the column is always loaded to its breakthrough capacity, fully utilizing all available binding sites. Second, by adjusting the conditions used to run the preparative method, the effect of the changing conditions on breakthrough capacity can be quickly measured. For example, as flow rate increases, breakthrough capacity decreases (Fig. 7), and this can be quickly measured by ending the load at 1% breakthrough and measuring the volume loaded. Finally, 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.

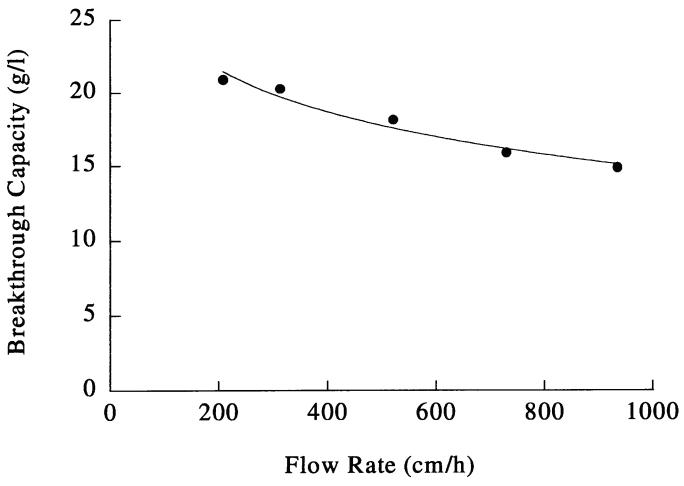

Figure 7 The effect of flow rate on breakthrough capacity for a recombinant antibody loaded onto a 10 cm length Poros 50 A column. Breakthrough capacity was measured at 1% breakthrough using the on-line assay (Protocol 3) and the preparative method (Protocol 4).

6 Remarks and conclusions

Perfusion chromatography lends itself well to running affinity methods, for both analytical and preparative separations of antibodies and antibody fragments. The speed of perfusion chromatography coupled with the accuracy and sensitivity of affinity chromatography allow new types of analysis to be done. Using rapid on-line assays makes it possible to quickly generate many breakthrough curves to study the loading behavior of antibodies.

We have described the application of the assay to chromatography monitoring and control, but the on-line assay can also be used for quantifying antibody in any type of process stream, including fermentors, tanks and filtration systems. Although we have described the use of protein A and protein G affinity methods, any molecule can be analyzed if an appropriate affinity ligand is available. This makes this method widely applicable to real-time analysis of many process streams.

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