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Microfluidic Chromatography for Early Stage Evaluation of Biopharmaceutical Binding and Separation Conditions

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Optimization of separation conditions for biopharmaceuticals requires evaluation of a large number of process variables. To miniaturize this evaluation a microfluidic column (1.5 µL volume and 1 cm height) was fabricated and packed with a typical process scale resin. The device was assessed by comparison to a protein separation at conventional laboratory scale. This was based upon measurement of the quality of packing and generation of breakthrough and elution curves. Dynamic binding capacities from the microfluidic column compared well with the laboratory scale. Microfluidic scale gradient elution separations also equated to the laboratory column three orders of magnitude larger in scale.

Keywords breakthrough; elution; ion exchange; microfluidics

INTRODUCTION

Ion exchange chromatography is a commonly used separation process within the biopharmaceutical industry. The charged stationary phase will interact, bind, and separate an oppositely charged macromolecule such as a protein or DNA present in the mobile phase. Cation exchange occurs where the stationary phase is negatively charged and the macromolecule in the mobile phase has a net positive charge, while the opposite situation occurs in the case of anion exchange chromatography (1,2). In assessing chromatographic performance, plate analysis combined with frontal and elution chromatography is commonly used (2). Frontal chromatography (or breakthrough) is the process by which the column becomes saturated with the biological macromolecule contained within the mobile phase, while elution chromatography involves the separation of solutes using a step or linear gradient, usually via increased salt concentration (2).

Biopharmaceuticals represent an internationally important and growing industry sector (3). This is especially highlighted in the field of chromatography where there are an ever increasing number of stationary phase materials available from suppliers each offering improved separations and recovery yields. During bioprocess development biopharmaceutical companies generally have restricted time and resources to analyse each of these resins for a specific purification process. Microfluidic scale chromatography columns, requiring minimal quantities of resin and product molecule, offer potential advantages in this respect. Additionally, many experimental variables such as protein concentration, buffer type, pH, flowrate, etc. may be investigated in systems that have the possibility to be used in parallel and in Lab-on-a-chip formats. Possibilities include sample preparation and eluate analysis on a single microfluidic chip (4).

There have been a number of approaches described in the literature recently to small-scale chromatography evaluation. The majority relate to 96-well microtiter filter plate technologies (micro-batch adsorption) (5) as well as resin-packed micro-pipette tips (micro-tip columns) (6,7). Additionally, more conventional mini columns have also been described (8). In each case parallelization of experimentation is achieved by integration with a laboratory liquid handling robot. Fig. 1 summarizes the available technologies (9) and also compares the scales of operation to the microfluidic approach established here.

Micro-batch adsorption is operated using a batch mode such that the resin is contained within the microwell plate while the remaining liquid is captured by using aspiration or filtration. Linear gradients cannot be performed using this technique; however, pseudo linear gradients have been developed using a series of increasing ionic strength batch adsorption steps (10). This approach has been applied to both breakthrough and elution chromatography yielding results that can compare favorably with laboratory scale columns (11–15). Micro-pipette tip systems also operate in batch mode; however, the solute containing fluid is moved in both directions through the resin bed by repeated automated aspiration and dispensing of the sample. This

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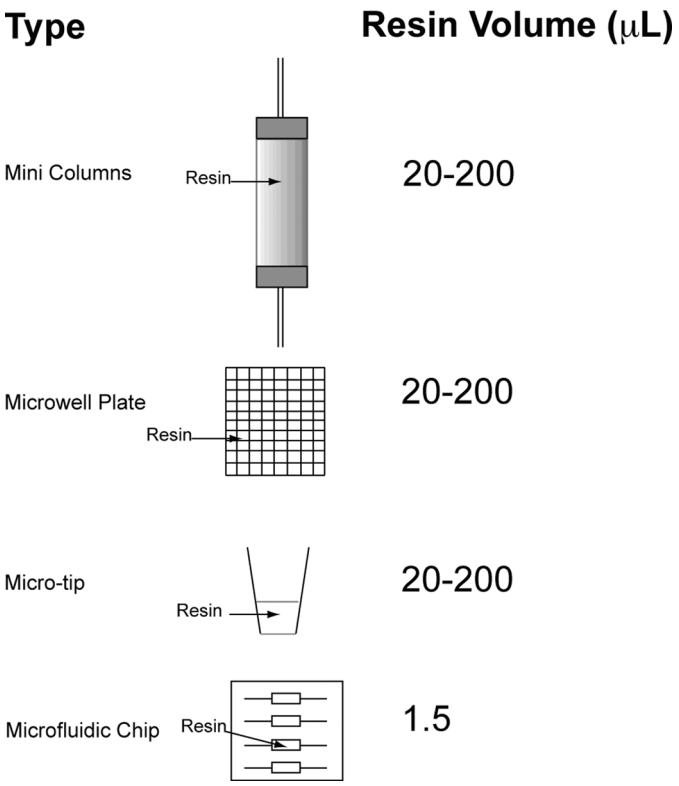

FIG. 1. A selection of the microscale chromatography technologies currently available. The first three are designed for use with a laboratory automation platform, while the fourth is the microfluidic chip presented here.

approach offers enhanced solute mass transfer under flow conditions and we have recently demonstrated its application to the miniaturization of virus-like particle purification (6). The more conventional mini columns (50, 100, 200 μL) resemble most closely standard laboratory chromatography columns. They are operated as part of a 96 microwell plate system where the mobile phase is pumped through the column in one direction. Such systems have been successfully used for the determination of dynamic binding capacity and elution chromatography of proteins (8).

While offering certain advantages none of these technologies truly operate as a chromatography column offering continuous mobile flow with either linear or gradient elution. Previously, we described the fabrication and flow characterization of a microfluidic chromatography column that has the necessary flow characteristics and which operates at at least a 20,000-fold smaller scale than standard laboratory scale columns. Dynamic binding capacities were generated that were comparable with those found within the literature (16) for laboratory scale columns. In this work study of the 1.5 μL microfluidic column is extended to both dynamic binding and separation studies with model protein solutions of either lysozyme or a mixture of hen egg white proteins. The column is packed with a polydisperse 6% agarose process resin, Sepharose 6FF, in order to resemble the columns used in the ultimate large scale applications. In this way the work is distinct from the small scale columns used in proteomics studies which are based on very small, monodisperse resins and which separate only digested protein fragments rather than whole proteins (17). When compared to results obtained on conventional laboratory scale columns the microfluidic column shows almost quantitiative agreement in dynamic binding capacity and gives a good indication of separation conditions.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma-Aldrich (Dorset, UK), apart from the fluorescein-5-isothiocyanate (FITC) which was purchased from Invitrogen (Paisley, UK), and were of the highest purity available. The chromatography resins, Sulfopropyl (SP) and Quaternary Amine Sepharose (Q) Fast Flow, were bought from GE Healthcare UK Ltd (Buckinghamshire, UK). Chicken eggs were purchased locally from Tesco (London, UK). All tubing, the connectors and the MilliGAT pump, were purchased from Presearch (Hampshire, UK). All buffers were prepared using analytical grade water (Millipore, Watford, UK).

Packing and HETP Measurements for the Laboratory Scale Columns

The 2 or 30 mL columns of Q or SP Sepharose Fast Flow (6% agarose) were gravity settled and flow packed at a velocity of 300 cm h^{-1} into an XK 16/20 Column to a height of 15 cm. To gravity pack, the bottom adaptor was placed on the bottom of the XK column then 30–35 mL of SP Sepharose Fast Flow in 20% (v/v) ethanol was poured into the column. The adaptor was opened to allow the ethanol to drip out. More matrix was then gently poured into the top of the column to ensure the height reached 17 cm. Once the matrix had formed a stable bed, the bottom adaptor was sealed and further addition of 20% (v/v) ethanol was added to the top of the column until a meniscus was formed. The top adaptor was connected to an AKTA Basic (GE Healthcare) and was placed at a 45° angle onto the top of the bed. Then, using a flowrate of 0.5 mL min^{-1} of ethanol to avoid air bubbles, the bottom adaptor was opened to ready the column for flow packing. The flowrate was slowly increased to 10 mL min^{-1} (300 cm h^{-1}) until the column had compressed to 15 cm, then the top adaptor was placed on top of the bed. The packing quality was then tested by producing a conductivity peak using a 100 μL sample loop filled with 2 M NaCl at 0.5 mL min^{-1}. The HETP and asymmetry of the peak were then calculated.

Laboratory Column Breakthrough Measurements

Each SP Sepharose 6FF column was equilibrated with 5 Column Volumes (CVs) of 0.05 M sodium phosphate buffer pH 5.5 (start buffer). This buffer system was selected to have a large buffering pH range; however, at pH 5.5 it is limited, and to ensure capacity results were not impacted by this selection they were validated against a sodium acetate system. Lysozyme (1 mg mL^{-1}) from egg white in the same buffer was continually pumped through at either 9, 7.5, 5, or 2 mL min^{-1} (270, 225, 150, and 60 cm h^{-1} calculated on the basis of an XK 16/20 column). When breakthrough was achieved, the column was washed with 5 CVs of the start buffer, and then the lysozyme was eluted from the column using 5 CVs of 0.05 M sodium phosphate and 1 M NaCl buffer (elution buffer) at the same pH. The column was then regenerated with 5 CVs of 1 M sodium hydroxide and equilibrated with the start buffer. Dynamic binding capacity was then calculated at 5, 10, and 100% capacity by calculating the area under the breakthrough curve, subtracting the mass of protein adsorbed from that in the feed and then dividing the result by the column volume.

Laboratory Column Elution Measurements

Egg white was separated from the egg yolk and 1 in 2 diluted using 0.05 M Tris/HCl pH 8. The mixture was stirred overnight at 4°C to remove glycosylated proteins by precipitation, then centrifuged at 10000 RPM for 30 minutes (18–20).

Each Q Sepharose 6FF column was equilibrated with 5 CVs of 0.05 M Tris/HCl pH 8 and then run isocratically at 2, 5, 7.5, or 9 mL min^{-1} (linear velocities of 60, 150, 220, and 270 cm h^{-1}). For the 2 mL column, injection volumes of 0.13, 0.33, 0.67, and 1.33 mL were used while for the 30 mL column, volumes of 2, 5, 10, and 20 mL were used. Two CVs were used for the injection process using either standard loops with PEEK tubing or for larger volumes, a superloop. The superloop is a pressurized cylinder which can be connected to the pump as a standard loop. It is used for injections of 10 mL and above. Fifteen CVs were used for the linear gradient based on 0.05 M Tris/HCl, 0.3 M NaCl pH 8 and then 5 CVs were used for the final wash step in which the elution buffer was used. The column was then re-equilibrated for 5 CVs using 0.05 M Tris/HCl pH 8. Experiments were also performed using a linear gradient of 0.05 M Tris/HCl, 0.3 M NaCl pH 8 over 150 CVs to facilitate comparison with the microfluidic column results.

Microfluidic Column Fabrication and Operation

Details of microfluidic column fabrication are described in our previous work (16). Briefly, the glass chip was fabricated using standard photolithography and wet etching techniques. The column length, width, and depth were constant at 10 mm, 1000 μm, and 150 μm. The packing procedure involved the dilution of a 20% (v/v) slurry of resin in 20% (v/v) aqueous ethanol. Beads were sieved using 38 and 106 μm (VWR Leicestershire, UK) mesh to reduce the size range in order to prevent blockages. Beads were packed into the microfluidic column using a 2 mL plastic syringe with an adapted micropipette tip. The matrix was manually pressurized into the packing chamber and held in position due to a keystoning effect. The quality of the packed microfluidic column was then checked using a Leica DMRA2 microscope (Leica Microsystems, Milton Keynes UK) and QWin Software to ensure that there were no resin particles that blocked the inlet and outlet channels. MilliGAT pumps were used throughout this work in the experimental setup outlined in Fig. 2. These pumps are able to accurately pump from $0.6-6 \times 10^6$ μL min^{-1} and can accurately dispense 10 μL of liquid. A small internal volume Rheodyne Valve was used for all injections into the microfluidic column.

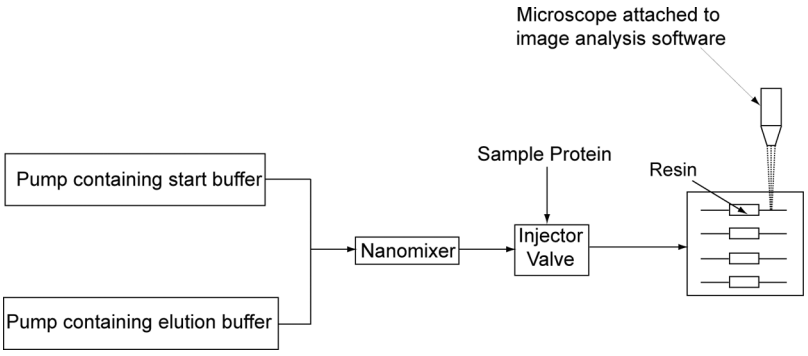

FIG. 2. Schematic diagram of the experimental system used for microfluidic elution chromatography. Two precision MilliGAT pumps were used to pump the start buffer (0.05 M Tris/HCl pH 8) and elution buffer (0.05 M Tris/HCl/0.3 M NaCl pH 8) through the nanomixer and injection valve and into the microfluidic column containing Q Sepharose FF beads. A sample of 1 μL of 1 mg mL^{-1} lysozyme, 3.6 mg mL^{-1} conalbumin and 15 mg mL^{-1} ovalbumin in start buffer was injected onto the microfluidic column once equilibrated with start buffer (20% (v/v) of each protein was fluorescently labelled using FITC).

All protein solutions contained 20% (v/v) of the protein fluorescently labelled using fluorescein-5-ithocyanate (FITC). The preparation of the fluorescently labelled proteins was performed using the manufacturer's instructions with slight modifications (21) as described previously (16). Leica QWin software was used as the image analysis software where fluorescence quantification was analyzed by altering the resolution time depending on the level of fluorescence. A time interval programe was developed to record the average fluorescence intensity in the outlet channel every 3 seconds (16).

Breakthrough measurements occurred using $1\,mg\,mL^{-1}$ lysozyme in 0.05 M sodium phosphate buffer pH 5.5 (start buffer). The buffer was pumped at linear velocities of 60, 150, 220, and $270\,cm\ h^{-1}$ (1.33, 3.67, 5.33, and $6.67\,\mu L\ min^{-1}$ respectively) calculated for the microfluidic packed bed using the crosssectional area of the column. Column dead volume was analyzed using $1\,mg\,mL^{-1}$ lysozyme in 0.05 M Tris/HCl pH 8 using Quarternary amine (Q Sepharose FF) beads. Dynamic protein binding capacity was calculated at 5, 10, and 100% breakthrough. All data was normalized in terms of c/c_o where c is the lysozyme concentration in the effluent at each time point and c_0 is the highest value of recorded effluent concentration. Equation (1) was used to calculate the binding capacity (Q) (22):

$$Q = \frac{\left(M_{in} - V_{retained}\int_{c_{in}}^{c_o} dc\right)}{V_{beads}} \times \frac{\varepsilon_m}{\varepsilon_s} \qquad (1)$$

Here M_{in} is the total protein mass into the column, $V_{retained}$ and V_{beads} are the volume retained through the system and the volume of the beads packed in the column respectively and ε_s and ε_m are the voidages in a standard column and in the microfluidic column respectively. The voidage was normalized in this way to allow comparison with larger scale published results. Data presented is the average of at least three breakthrough curves determined at different flowrates.

Microfluidic Column HETP Measurements

The experimental procedure used was the same as for the breakthrough measurements, except the fluorescent light microscope detection occurred at either end of the microfluidic column which now contained sieved Q Sepharose Fast Flow beads (average diameter of 70 μm). The variance and HETP (23) were then calculated from the intensity readings produced. A volume of 0.4 μL (the smallest volume available) of $1\,mg\,mL^{-1}$ lysozyme (20% (v/v) fluorescently labelled) in 0.05 M Tris/HCl pH 8 was injected into the microfluidic column. The method was validated against the laboratory scale method described earlier to ensure no interactive effects between the protein and column.

Microfluidic Column Elution Measurements

A 1.5 μL microfluidic column packed with sieved Q Sepharose Fast Flow was equilibrated with 5 CVs of 0.05 M Tris/HCl pH 8 (start buffer). A concentration of $1\,mg\,mL^{-1}$ lysozyme, $3.6\,mg\,mL^{-1}$ conalbumin, and $15\,mg\,mL^{-1}$ ovalbumin proteins (20% (v/v) of each labelled with FITC) was pumped through in the start buffer. Elution was carried out using the same method outlined in the laboratory column elution measurements. The peaks were monitored using the Leica fluorescent microscope as shown in Fig. 2. In order to achieve the gradient, $0.2\,mg\,mL^{-1}$ fluorescently labelled lysozyme was used in place of the elution buffer allowing the monitoring of the gradient using the Leica microscope. The gradient used for the separations was 0.05 M Tris/HCl pH 8 with 0.3 M NaCl over 150 CVs over 16 step changes.

RESULTS AND DISCUSSION

Determination and Assessment of Column Packing

Initial experiments focused on measurement of the HETP of the three columns in order to assess the quality of resin packing. The HETP for the 30 mL (15 cm height) and 2 mL (1 cm height) columns were calculated to be 389.9 and 350.7 μm at a linear velocity of $30\,cm\ h^{-1}$ while the variance (σ^2) for each was 1.83 and $0.10\,mL^2$. The variance for the 2 mL column gave a lower plate number due to the reduced height and increased complexity involved in packing the column. The asymmetry of the 30 and 2 mL columns were 0.65 and 2 respectively. Ideal peak symmetry should be achieved for a well-packed chromatography column. The values reported here display under and over packing respectively; however, the results produced were adequate for comparisons with the microfluidic column (24).

Table 1 displays the measured peak asymmetry, the calculated variance of the peak, the HETP and the HETP/d_p (diameter of the resin particle) as a function of mobile phase linear velocity for the 1.5 μL microfluidic column. Values were calculated from the width at 50% of the maximum height of the peak using the standard plate analysis (23). Although the peaks used for analysis were not Gaussian, due to tailing, standard peak analysis was considered useful as a method to compliment the confocal microscopy analysis developed previously *16*. The asymmetry values for all peaks were two to three times the value of a Gaussian peak (unity). The variance calculated over the column was smaller than expected since laminar flow and diffusive mixing throughout the extra-column system appear to be the dominating factor. The volume of the adjacent equipment was 2 μL, compared to the volume of the packed bed which was 1.5 μL, producing dispersed peaks. The data presented, however, does show that variance and HETP may still be calculated within a

TABLE 1
Influence of mobile phase linear velocity on microfluidic chromatography column performance. Peak symmetry, σ^2 and the HETP and HETP/d_p determined using 0.4 μL of 0.2 mg mL^{-1} fluorescently labelled lysozyme in 0.05 M Tris/HCl buffer pH 8 (non-binding conditions). Asymmetry and σ^2 were calculated from the values obtained before and after the packed microfluidic column. Errors represent one standard deviation around the mean

Linear velocity (cm h^{-1})	σ^2 (mL^2)	HETP (μm)	HETP/d_P	Asymmetry
60	$1.41 \times 10^{-6} \pm 4.35 \times 10^{-7}$	945 ± 290	13.52 ± 4.16	2.98 ± 0.56
105	$1.44 \times 10^{-6} \pm 1.70 \times 10^{-7}$	1050 ± 175	15.00 ± 2.52	2.64 ± 0.31
150	$1.21 \times 10^{-6} \pm 0$	1035 ± 35.30	14.79 ± 0.50	2.90 ± 0.36
220	$1.09 \times 10^{-6} \pm 2.74 \times 10^{-7}$	970 ± 95.80	13.88 ± 1.37	3.05 ± 0.30
270	$1.12 \times 10^{-6} \pm 0$	1010 ± 0	14.44 ± 0	3.10 ± 0.22

microfluidic system. In Table 1, σ^2 can be considered to be constant at around 1.5×10^{-6} mL^2.

The HETP data are also presented in Table 1. As the variance is directly proportional to the HETP (23) a similar level of error is observed. The value for the HETP can be considered to be around 1 mm, while the HETP/d_p is around 14. In conventional terms, this value would be indicative of poor packing especially when compared with the laboratory scale 15 cm column (experimentally determined to have a HETP/d_p of 4). However, the 2 mL column produced a similar value to the microfluidic column due to similar heights.

The voidage in the microfluidic column was 0.55 (as calculated and presented previously (16)) which implies that there are more voids present in the microfluidic packed system than in a standard column. When considering the assessment of microfluidic column packing the plate test results are best interpreted in conjunction with a three dimensional image of the packing, as shown within Fig. 3. In practice the packing was good enough for the binding and elution studies given that there was only 1.5 μL of packing and that on average it is only 2 beads high.

Laboratory Scale Column Breakthrough Profiles

As a basis for later comparison with the microfluidic column, Fig. 4 displays the breakthrough curves for all 4 flowrates as a function of the volume retained for (a) the 30 mL (15 cm height) column and (b) the 2 mL (1 cm height) column. Breakthrough curves for the 30 mL column (Fig. 4(a)) are consistent and independent of mobile phase linear velocity. They show near perfect representations of breakthrough curves with a constant pattern front (25,26). This was expected and is due to a number of factors including the use of low concentrations of a model protein and the fact that lysozyme is a protein with a high isoelectric point (11.1) that adsorbs with high affinity (27).

For the 2 mL column (Fig. 4(b)) there is some variation of the breakthrough curves with increasing linear velocity; however, in general the flow rates are consistent. At the higher flowrates the residence time in the column would be reduced leading to proportionally less time for the protein to interact with the matrix particles. Additionally, as shown in Table 2 there was a decreased number of theoretical plates. However, the breakthrough curves are highly consistent and accurate over the range required.

The breakthrough curves determined here in both the laboratory scale columns are similar to those described in the literature (28,29) using lysozyme with the same Sepharose FF resin. The reported values of maximum dynamic binding capacity were also similar to those calculated and reported here in Table 1 (~120 mg mL matrix^{-1}).

Microfluidic Scale Column Breakthrough Profiles

In our previous work on chip fabrication and analysis of fluid flow within the column initial breakthrough studies

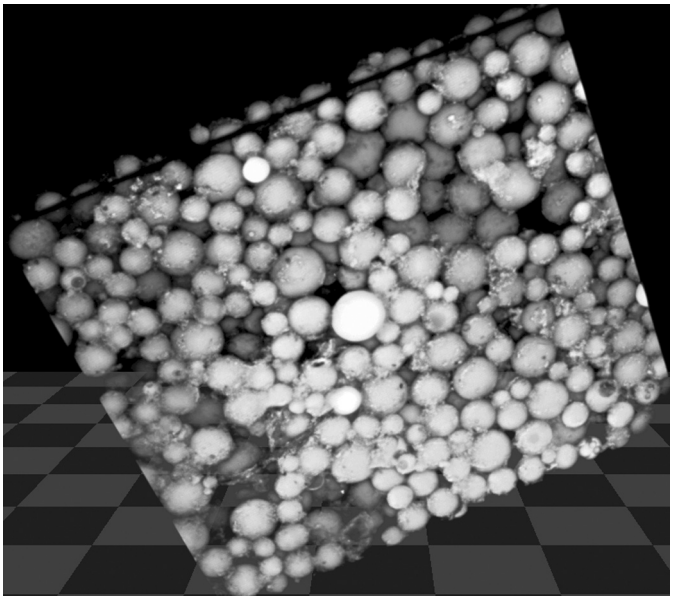

FIG. 3. Confocal microscope image of a section of the microfluidic packed bed rendered in a 3 dimensional plane. The perspective is of a 45° angle from the top of the bed (average particle diameter 70 μm).

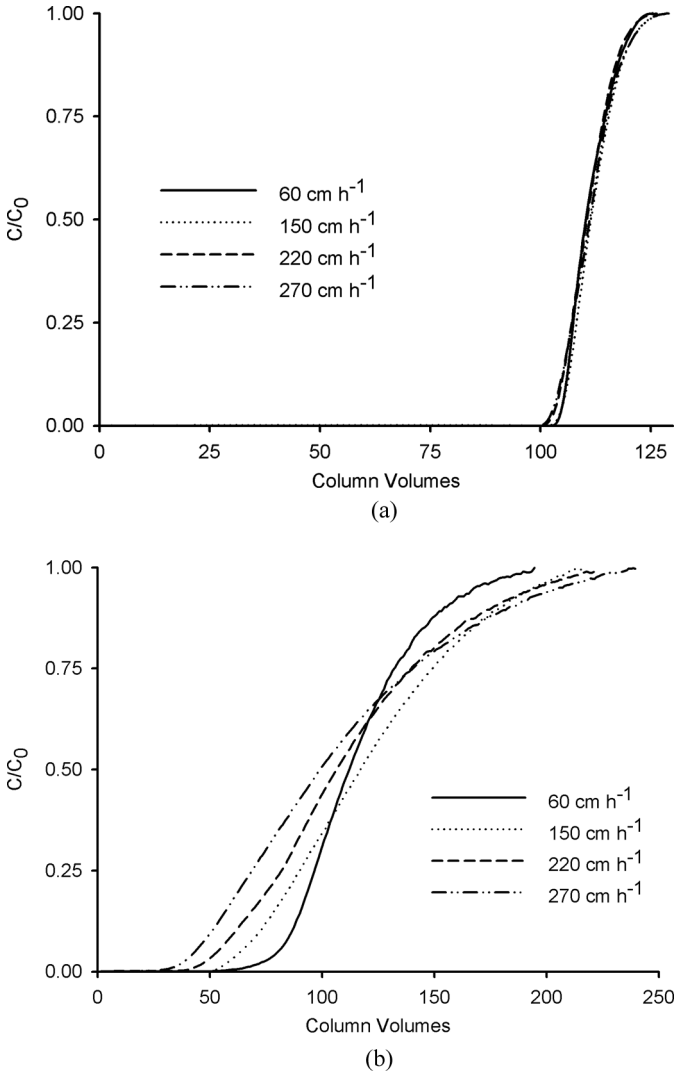

FIG. 4. Influence of mobile phase velocity on dynamic binding capacity of laboratory scale chromatography columns. Breakthrough curves of 1 mg mL^{-1} lysozyme using (a) 30 mL (15 cm height) and (b) 2 mL (1 cm height) XK 16/20 columns containing SP Sepharose Fast Flow in 0.05 M sodium phosphate pH 5.5.

on the microfluidic column were also reported. Here comparisons are made between breakthrough profiles determined on the microfluidic column and the two laboratory scale columns at an identical linear velocity of 270 cm h^{-1}. The voidage for the 1.5 μL column was 0.55 as opposed to 0.4 which was present in the standard laboratory columns (as is also standard in general chromatography columns) (16). Instead of the standard column volumes employed in previous figures, the resin column volumes was used where the column volume was multiplied by the bed voidage, so as to standardize the results.

As shown in Fig. 5, the microfluidic and the 2 mL laboratory scale columns are very similar in terms of breakthrough profile shape and the column residence time. This agreement is considered excellent given the 1300-fold difference in scale and is probably related to the columns having the same length. The breakthrough curve for the 30 mL column is a near perfect representation of a breakthrough curve which was considered to be an excellent basis to make comparisons with other scales.

Table 2 presents a comparison of the calculated maximum and 5% dynamic binding capacities for both laboratory scale columns and the microfluidic column at different mobile phase linear velocities. Values of the maximum binding capacity are in reasonable agreement between the microfluidic and laboratory scale, as suggested by the breakthrough profiles shown in Fig. 5. Although the shape of the breakthrough curve was different, the calculated maximum binding capacity for the 30 mL column was similar to the microfluidic column.

Calculated dynamic binding capacities (at 5% capacity) were not as similar between the various scale columns as were the maximum binding capacities. This may have been due to non-specific binding and channelling through the column (16). Overall, the capacities calculated are in reasonable agreement with previously published data and laboratory scale data (28,29). This provides a further confirmation that the microfluidic chromatography column does offer a useful methodology for the determination of breakthrough curves and binding capacities using considerably reduced quantities of material.

TABLE 2
Comparison of maximum and 5% dynamic binding capacities for the microfluidic and laboratory scale columns

Dynamic binding capacity (mg mL matrix^{-1})	Linear velocity (cm h^{-1})											
	1.5 μL Column				2 mL Column				30 mL Column			
	60	150	220	270	60	150	220	270	60	150	220	270
5%	140	144	50.3	55.7	79.8	57.3	53.5	39.6	105	105	103	103
Maximum	160	175	96.4	117	116	121	113	108	116	112	104	111

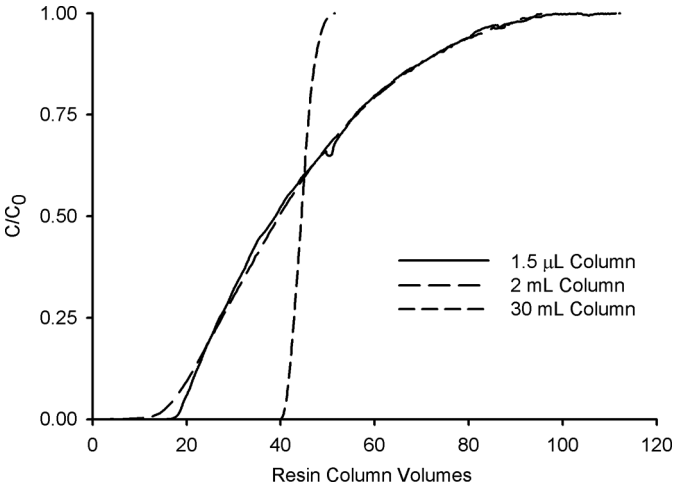

FIG. 5. Comparison of lyoszyme breakthrough curves determined on the 1.5 μL (1 cm height) and 2 μL (1 cm height) microfluidic chromatography columns compared to the laboratory scale 30 mL (15 cm height) column. Experiments performed at a linear velocity of 270 cm h^{-1}. Note the ~1300 fold difference in scale.

Laboratory Scale Column Elution Profiles

Figure 6 displays the egg white separation data generated using the two laboratory scale columns. Three peaks are clearly shown in each profile, the first being lysozyme, which is unretained, and two further peaks which are not fully resolved. In Fig. 6(a) for the 30 mL column conalbumin and ovalbumin represent the second and third peak respectively. There is some evidence that resolution using smaller injection volumes produced better resolution, as the column has sufficient time to remove the proteins over the linear gradient. In Fig. 6(b) when using 1.33 mL as the injection volume for the 2 mL column, no resolution was obtained between the second two peaks. This is almost certainly due to the reduction in the residence time in this much shorter column. The lack of resolution on both columns could be due to the fact that the isoelectric points for both proteins are comparatively close to each other. Conalbumin has a pI of 6.5, while ovalbumin has a pI of 4.7 (30) which could lead to some competitive binding. A longer bed height or a longer linear gradient would most likely improve resolution of the two proteins when using the same flowrates. The fact that there was incomplete resolution between peaks, however, does not detract from the ability to obtain useful insights from these columns on how the conalbumin-ovalbumin peaks alter with changing bed heights and potentially different mobile phase conditions i.e., pH, ionic strength etc.

Microfluidic Scale Elution Profiles

In assessing the utility of the microfluidic column in early stage bioprocess development it is important to not only assess binding capacity (Table 2) but also the degree

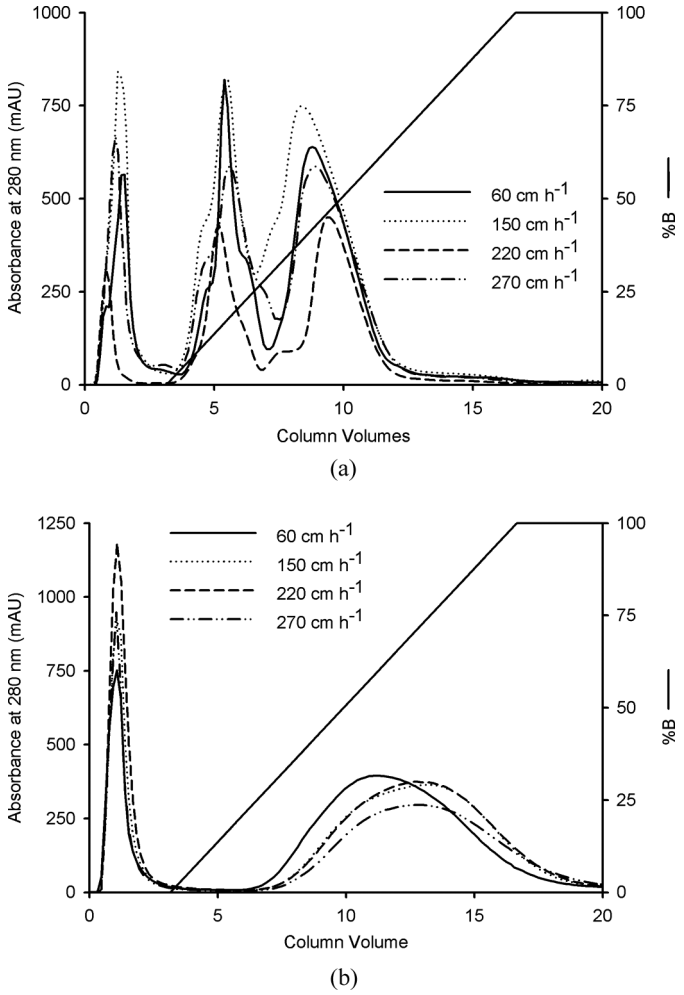

FIG. 6. Influence of mobile phase velocity on laboratory scale separation of a ternary protein mixture. (a) a 20 mL injection of 30 mg mL^{-1} egg white proteins (3.6% (w/w) lysozyme, 12% (w/w) conalbumin and 54% (w/w) ovalbumin) in 0.05 M Tris/HCl buffer pH 8 using a 30 mL column; (b) 1.33 mL injection of egg white protein using a 2 mL column. The first peak is lysozyme, then conalbumin and finally ovalbumin. Proteins were eluted using 0.05 M Tris/HCl/0.3 M NaCl pH 8 and the resin used was Q Sepharose Fast Flow.

of resolution that could potentially be obtained on the ultimate, large scale manufacturing column. One of the main technical challenges of achieving linear gradient separations at this scale is the production of the gradient. This could be due to local flow instabilities at nano flowrate changes explained below.

To visualize the linear gradient required for separation, fluorescently labelled lysozyme was used as buffer B instead of a salt gradient. Results were then overlaid over the separation data. Separation of egg white proteins was then instituted. Column volumes in this case were not quoted in terms of the resin column volume because the comparison required referred to the linear gradient of the separation which was not present when using breakthrough curves.

To institute a linear gradient at the microfluidic flowrates used here, nano flowrate changes are required. Very low flowrate changes are difficult to maintain as they are affected by factors such as temperature, variation in the piston seal quality over time, and there may be solvent compressibility problems (31). Extremely small air bubbles in the liquid that compress in a piston based pump could also cause instabilities within local velocities. Velocities then even out as the pressure reaches atmospheric pressure at the exit (32). The small scale of the microfluidic column therefore ensures that accurate gradients are difficult to obtain. The chip version developed by Brennen et al. (31) have developed gradients over minutes at nano-liter flow rates.

Irrespective of these technical challenges however, a separation of three proteins using a linear gradient was achieved on the microfluidic column as shown in Figs. 7 and 8. Figure 7 shows elution of the non-biding lysozyme peak first followed by a broader second peak being a mixture of conalbumin and ovalbumin. There is a shoulder evident on this second peak (at around 100 column volumes), which is most likely to be from conalbumin while the main body of the peak is ovalbumin.

Figure 8(a) provides a close-up view of this shoulder and peak. In order to verify if the degree of separation achieved is likely to be representative of large scale separations, it was necessary to generate comparative 2 mL column data (i.e., 1 cm length at the laboratory scale) using a similar 150 column volume gradient as shown in Fig. 8(b). Although the height of the conductivity when the curve returns to the baseline differs between the scales, the length of the gradient is the same.

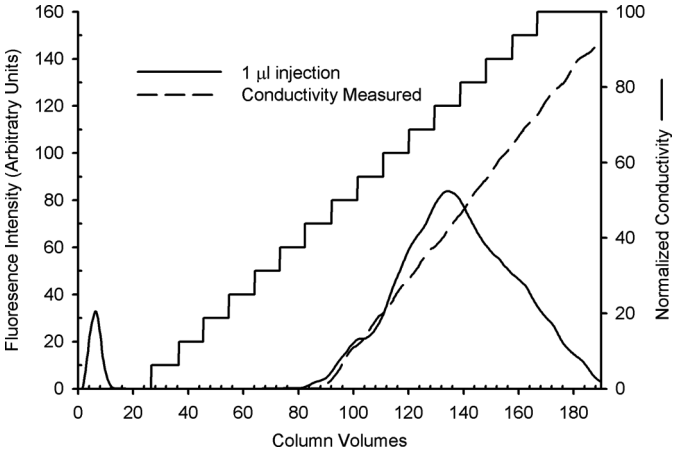

FIG. 7. Influence of mobile phase linear velocity on separation of a ternary protein mixture. A microfluidic column egg white protein separation using a series of 16 steps to produce a gradient over 150 column volumes. The same buffer conditions were used as in the standard scale chromatography column system. An injection of 1 µL volume was used containing 1 mg mL^{-1} lysozyme, 3.6 mg mL^{-1} conalbumin and 16.2 mg mL^{-1} ovalbumin in a fluorescently labelled solution with the standard buffer.

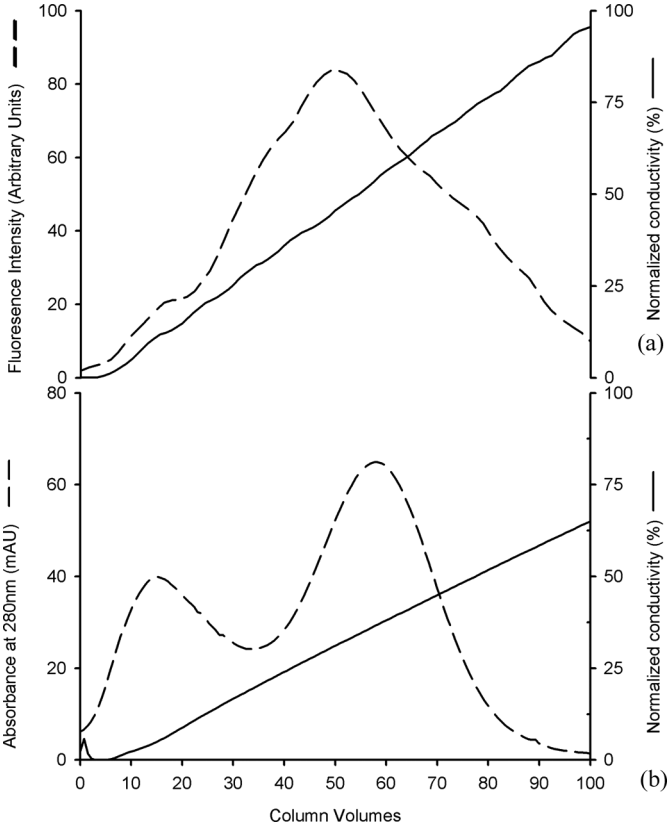

FIG. 8. Comparison of both scales of the ternary protein separation at the elution stage using 150 column volumes. Linear gradient separation of the three egg white proteins using (a) the microfluidic column and (b) the 2 mL column. Both columns were run at 270 cm h^{-1}, while the injection volume used for the 2 mL column was 1.33 mL.

Although full resolution was not observed between the conalbumin and ovalbumin peaks when using 150 column volumes with the 2 mL column, the data in Figs. 7 and 8 represents a first step in the generation of elution profiles of ion exchange chromatography using a microfluidic column.

Comparison with Other Microscale Chromatography Techniques

As shown in Fig. 1 the performance and potential industrial application of the microfluidic column established here needs to be considered against other similar technologies for high throughput chromatographic process screening. The other systems presented use complex liquid handling systems facilitating the use of many different chromatographic separations at once. The advantage of the approach presented within this paper is the use of extremely small volume (1.5 µL) chromatography columns. The microfluidic system is capable of developing into the use of highly parallel chromatography columns using true flowrate conditions. All other systems within the literature

operate using batch conditions or with the use of pseudo linear gradients. The advantage of the microfluidic system is that it is capable of producing separations using true linear gradients as well as parallel conditions.

CONCLUSIONS

The data presented here displays positive initial results on the performance of a 1.5 μL microfluidic chromatography column packed with process scale chromatography resin. Results in terms of packing quality and performance (HETP) and frontal and elution chromatography between the microfluidic column and laboratory scale 2 mL and 30 mL columns showed good quantitative agreement both in terms of maximum dynamic binding capacity and also separation of binding and non-binding proteins. Protein requirement has decreased 10,000-fold for breakthrough analysis and 3000-fold for elution between the microfluidic and the 30 mL column. Therefore the work described in this paper is representative of the proof of principle of a potentially powerful tool for the generation of microfluidic process bed data for the biopharmaceutical industry and is useful for early stage bioprocess development.

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