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Cation exchange chromatography in antibody purification: pH screening for optimised binding and HCP removal☆

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Abstract

The production of pharmaceutical antibodies requires reliable and rapid processes with high purity and yield. Although protein A gels selectively and efficiently bind antibodies in the capture step, intense research is going on to find alternatives that can abolish the drawbacks of protein A chromatography. Ion exchangers e.g. are more robust, considerably cheaper and can eliminate ligand leaching. For the strong cation exchangers Fractogel® EMD SO_3^- (M) and Fractogel® EMD SE Hicap (M) we have evaluated the influence of pH for optimised binding and removal of host cell protein (HCP). In a fast initial screening we measured batch binding capacities. Subsequent scale-down to 96-well plate format proved that assay miniaturisation still provided reliable data. We demonstrated with the principle of residence time that scout columns are suitable for dynamic studies. The optimum pH range from batch binding was transferred to scout columns which were then used to screen for maximum dynamic capacities. In addition IEF titration curve analysis was employed to define a final operational pH. With this pH we ran labscale columns to purify monoclonal antibody. The cation exchangers showed high step yields and host cell proteins in the pools from gradient elution were reduced very effectively.
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Keywords: Cation exchange chromatography; Residence time; Resin screening; Comparative study; Monoclonal antibodies; Purification; Host cell protein removal

1. Introduction

Cation exchange chromatography (CEC) is a well-established unit operation in the downstream processing of monoclonal antibodies. In many processes this type of chromatography is part of a multi-step sequence which starts with an affinity step for capture and then adds further chromatography steps (most commonly cation exchange chromatography, anion exchange chromatography (AEC) or hydrophobic interaction chromatography (HIC)) in different combinations [1,2]. The order of the sequence and therefore also the position of the CEC step in the overall process can vary as it depends on several factors, e.g. the composition of the cell culture supernatant (concentration and character of impurities), the stability profile of the antibody, or a manufacturer's preferred approach. When affinity chromatography, most often represented by a resin with immobilised protein A as ligand, is applied for capture, the CEC step will be part of intermediate purification or polishing. In these later stages of a downstream process, step productivity (defined by dynamic binding capacity of a resin and operational flow rate amongst others) remains an issue for the drug manufacturer for the reason of process economy. In a comparative study on different cation exchangers the methacrylate based resins Fractogel® EMD SO_3^- (M) and Fractogel® EMD SE Hicap (M) were found to have the highest overall binding capacity for the antibody tested [3]. Besides productivity, however, the potential of a step for removal of impurities may have to be rated even higher, as it is an absolute requirement to meet the purity specifications, set by the regulatory authorities, at the end of the process.

Resins from different vendors are likely to show differences in their separation behaviour, even if they belong to the same type of chromatography resin [4] or even possess the same ligand [5–8]. Referring to the latter case this can be due to dissimilarities in resin properties like ligand density and accessibility or secondary effects of the base matrix.

At least two different strategies have been followed in the literature to find the operational conditions for optimum resin performance: modelling and prediction on the one hand

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[9,10] and empirical screening on the other hand. Screening of chromatographic resins (or phases) has in some cases been based on small columns (termed scout(ing)/screening columns or scout(ing)/screening kits). The selectivity of HIC resins was compared [6], or a separation on a cation exchanger optimised [11]. Even if some automation is implemented [12] the column strategy limits the number of experiments because runs are usually carried out in series. A large number of experiments, though, can easily be created in the initial screening procedure when a variety of resins and multiple parameters are tested in order to develop a well-defined and robust purification process. The throughput in resin screening can be increased considerably when experiments are performed in batch mode. Batch mode operation can be parallelised and thus more experiments conducted per time unit. A further leap in throughput is gained by miniaturisation to microtitre plate formats. Typical for this format is besides parallel operation low material consumption (resin and sample) and a high degree of automation when robots are employed. Bensch et al. [13] in their recent review about high-throughput screening of chromatography gels covered some applications for the automated multi-well format and also some for column screening. Thiemann et al. [14] used an automated parallel system based on 96-well plates to optimise the selectivity in the separation of a protein mixture in batch mode altering pH and solution conductivity. Optimum parameters were determined, transferred to a small column (filled with 2 ml of resin) and the protein separation then run on this column.

The present study investigates the influence of pH for optimised binding and removal of host cell protein (HCP) employing the strong cation exchange resins Fractogel® EMD SO_3^- (M) and Fractogel® EMD SE Hicap (M) for the purification of a monoclonal antibody (mAb). In the initial screening we utilised a parallel "normal"-scale batch mode assay to measure the impact of pH on the static binding capacity (SBC) of the resins for the mAb. The SBC assay was then adapted to the 96-well format. Having established the optimum pH we narrowed down the pH window for subsequent column binding experiments. Before defining a column size for the dynamic experiments, the influence of residence time, achieved with different bed heights and flow rates, on dynamic binding capacity (DBC) was examined. The pH for maximum DBC was identified, compared with the IEF titration curve analysis of the mAb containing cell culture supernatant, and a final pH defined. With this pH a preparative chromatographic run was carried out in which the mAb was purified from mammalian cell culture.

2. Experimental

2.1. Materials

Acetic acid (Cat#1.00063), di-sodium hydrogenphosphate di-hydrate (Cat#1.06580), 5 M hydrochloric acid (Cat#1.09911), sodium acetate (Cat#1.06268), sodium azide (NaN_3, Cat#1.06688), sodium dihydrogenphosphate ($NaH_2PO_4xH_2O$, Cat#1.06346), 5 M sodium hydroxide solution (Cat#1.09913), sodium sulfate (Na_2SO_4, Cat#1.06649) were purchased from Merck KGaA (Darmstadt, Germany). The chemicals described above were of a reagent grade or higher. Purified human polyclonal immunoglobulin G (IgG), Gammanorm 165 mg/ml, was purchased from Octapharma, AB (Stockholm, Sweden). NS/0 cell culture supernatant with mAb was supplied in-house (pH was 7.2 and conductivity was 17 mS/cm at 25 °C). Chromatographic resins: UNOsphere™ S was bought from Bio-Rad Labs (Hercules, CA, USA), SP Sepharose® XL (SP Seph XL) and CM Sepharose™ Fast Flow were from GE Healthcare Europe GmbH (Freiburg, Germany), Fractogel® EMD SO_3^- (M), Fractogel® EMD SE Hicap (M), Fractogel® EMD COO^- (M), and Fractoprep® SO_3 were obtained from Merck KGaA (Darmstadt, Germany).

2.1.1. IEF

Precast PhastGel pH range 3–9 (Cat#17-0543-01) and PhastGel™ Silver Kit (Cat#17-0617-01) were obtained from GE Healthcare Europe GmbH (Freiburg, Germany).

2.1.2. HPLC

TSK G3000SWXL size-exclusion column (5 μm, 300 mm × 7.8 mm inner diameter (i.d.), Cat#08541) was from Tosoh Bioscience GmbH (Stuttgart, Germany), guard column cartridge GFC-3000 (4 mm × 3 mm i.d., Cat#AJO-4488) was from Phenomenex (Torrance, USA).

2.1.3. ELISA

NS/0 ELISA kit for measurement of NS/0 host cell proteins (Cat#F220) and sample diluent (Cat#F223 A) were purchased from Cygnus Technologies (Southport, NC, USA)

2.1.4. SDS-PAGE

Novex 10% Bis-Tris Gel NuPAGE® (Cat#NP0301), MES SDS running buffer (Cat#NP0002), SeeBlue® plus2 molecular weight marker (MWM) protein mix (Cat#LC5925) were ordered from Invitrogen (Groningen, The Netherlands).

2.2. Methods

2.2.1. pH screening for optimised batch binding

The pH screening for optimum static binding capacities of the cation exchangers was determined in a 100 ml scale batch assay using a monoclonal antibody from NS/0 cell culture supernatant. Batch binding buffers of defined pH values in the range from pH 5.0 to 7.0 were prepared from a buffered solution containing 10 mM $NaH_2PO_4 \cdot H_2O$ + 27 mM NaCl (pH 6.3). The pH was adjusted with 5 M HCl or 5 M NaOH, respectively.

Resin treatment was the same for all batch binding experiments regardless of scale. The resins were equilibrated to the respective binding buffer (10 mM $NaH_2PO_4 \cdot H_2O$ + 27 mM NaCl, pH varying from 7.0 to 5.0) on a sintered glass frit (porosity 2) by washing three times with 6 bed volumes of de-ionised water followed by three times washing with 6 bed volumes of binding buffer. Afterwards equilibrated resin cakes were resuspended in binding buffer to prepare 50% (v/v) stock slurries (resin sediment volume/total slurry volume). After settling overnight sedimented bed volumes were determined and slurry concentrations adjusted by adding or removing buffer.

The mAb containing sample solution was prepared from a NS/0 hybridoma cell culture supernatant (CCS). The sample was concentrated seventeen-fold and diafiltered with 8 dia-volumes of 10 mM $NaH_2PO_4 \cdot H_2O$ + 27 mM NaCl buffered solution (pH 6.0, conductivity ≈4 mS/cm) using a 30 kDa polyethersulfone tangential flow cartridge (PTTK 30k PES, Millipore Corp., Bedford, USA). Protein solutions of the same defined pH values were prepared by diluting ultra-/diafiltered CCS concentrate with appropriate batch binding buffer to yield a final concentration of 2 mg/ml of the intact monoclonal antibody Afterwards the pH had to be readjusted using 1 M HCl or 1 M NaOH.

Binding assays were performed by mixing 2 ml of a 50% (v/v) resin slurry (corresponds to 1 ml of settled resin) with 90 ml of the mAb solution (using 250 to 500 ml size beakers). The assays were incubated on a rotary shaker for 20 min at 200 rpm and at room temperature. Afterwards samples were withdrawn and filtered through 0.22 μm syringe filters. Residual IgG concentrations in the permeates were determined by analytical size-exclusion chromatography (SEC HPLC). Static binding capacities (the amount of IgG bound per ml resin settled) were calculated from the decrease of IgG concentration in solution and the volume of settled resin used.

2.2.2. Batch binding on different scales

In these experiments batch binding was compared on different scales. Resins were incubated with purified polyclonal IgG. Resin treatment was as described before, however using a pH 6.0 binding buffer only.

The batch binding assay on the 100 ml scale was conducted by mixing 2 ml of stock resin slurry (corresponding to 1 ml of resin settled) with 100 ml polyclonal IgG solution. The assays were incubated on a rotary shaker at 200 rpm and at room temperature.

Assays in 200 μl format were performed in a 96-well filter plate with a 0.22 μm hydrophilic low protein binding Durapore® membrane (Multiscreen HTS™, GV, Cat#MSGVN2210, Millipore Corp.–Billerica, MA, USA). Solutions were transferred manually with a 12-channel Multipette (Eppendorf AG, Hamburg, Deutschland). Fifty percent (v/v) stock slurries were diluted with binding buffer to obtain 3% (v/v) working resin slurries. Working slurry were transferred (100 μl) into each well and the liquid filtered off with the help of a MultiScreen vacuum manifold (Cat MAVM0960R Millipore Corp., Billerica, MA, USA) leaving 3 μl of resin sediment in each well. To each well 200 μl of IgG solution (2 mg/ml) were added. The covered filter plate was incubated for 20 min at room temperature on a rotary shaker (VARIOMAG Monoshake, H + P Labortechnik, Germany) at high frequency. Afterwards the IgG solution was separated from the resin by filtration with help of a MultiScreen vacuum manifold. IgG solution was collected as permeate in a 96-well UV microplate (Cat#3635, Corning, USA).

IgG concentrations in the permeates were determined by measuring the absorption at 280 nm with a UV spectrophotometer (UV-2000, Hitachi, Japan respectively BIO-TEK Instruments Inc., USA), Static binding capacities (the amount of IgG bound per ml resin settled) were calculated from the decrease of IgG concentration in solution and the volume of settled resin used according to the following Eq. (1):

$$\mathrm{SBC}\left(\frac{\mathrm{mg}}{\mathrm{ml}}\right) = \frac{V_S\,(\mathrm{ml}) \times [C_0 - C_e]\,(\mathrm{mg/ml})}{V_R\,(\mathrm{ml})} \quad (1)$$

V_S, volume of protein solution; V_R, volume of settled resin; C_0, initial IgG concentration in solution (start); C_e, final IgG concentration in solution (end); SBC, static binding capacity.

2.2.3. pH screening for maximised dynamic binding

Dynamic binding was performed with an ÄKTAexplorer 100 chromatographic system (GE Healthcare, Uppsala, Sweden). Before the column size was specified for the experiments evaluating the influence of pH on DBC, the influence of residence time on DBC was analysed. Specific residence times were set by different combinations of bed height and flow rates. Three different columns packed with the strong cation exchange resin Fractoprep® SO_3 were used: 200 mm × 10 mm i.d., packed to 18% compression; 127 mm × 10 mm i.d., packed to 20% compression; 19.4 mm × 8.1 mm i.d., packed to 20% compression (the latter column being called scout column). Columns were equilibrated with running buffer (25 mM Na-acetate, pH 5.0, 4 mS/cm). Protein solution (2 mg/ml human polyclonal IgG in running buffer) was loaded at different flow rates (respectively residence times). Loading was stopped when the concentration of mAb in the column effluent (detected at UV 280 nm) was 10% relative to the mAb concentration of the feed (this loading is defined as 10% breakthrough). Unbound protein was washed out with running buffer. Elution was effected by changing to 100% elution buffer (25 mM Na-acetate +1 M NaCl, pH 5.0) and DBC calculated from protein collected in the eluate fractions according to the following Eq. (2):

$$\mathrm{DBC}\left(\frac{\mathrm{mg}}{\mathrm{ml}}\right) = \frac{V_E\,(\mathrm{ml}) \times C_e\,(\mathrm{mg/ml})}{V_R\,(\mathrm{ml})} \quad (2)$$

V_E, volume of eluate; V_R, volume of settled resin; C_e, IgG concentration in eluate fraction; DBC, dynamic binding capacity.

Scout columns were then chosen to screen the optimum pH for maximum DBC. All resins tested were packed into empty columns of 19.4 mm × 8.1 mm i.d. (from Merck KGaA) in-house and then used as scout columns. The residence time during sample loading and washout was maintained constant at 2 min, the linear flow rate for elution was set to 250 cm/h. Columns were equilibrated with running buffer (25 mM Na–acetate + 25 mM Na–phosphate) with varying pH in the range 4.5–6.5, adjusted with 5 M HCl or 5 M NaOH. A 5 mg/ml buffered solution of mAb in running buffer was loaded onto the column until 10% breakthrough. The washout was conducted with 10 column volumes of running buffer, elution was carried out with 18 column volumes of elution buffer. The DBC was calculated from the amount of protein recovered in the eluate fraction.

2.2.4. IEF titration curve analysis

Electrophoretic titration curve analysis is a two-dimensional technique to analyse protein charge characteristics. The titration curve analysis was performed using a PhastSystem Separation and Control Unit (GE Healthcare). The sample was electrophoresed on a homogeneous polyacrylamide precast IEF

PhastGel covering the pH range 3–9 applying an optimised method (Separation Technique file no. 100 PhastSystem™). In the first dimension the pH gradient was generated by running the gel for 150 Vh at 2000 V, 2.5 mA, 3.5 W, 15 °C. The gel was then rotated clockwise 90° and 3 μl sample were applied perpendicularly to the gradient across the middle of the gel (using the PhastGel titration curve sample applicator).

In the second dimension the gel was run for 50 Vh at 1000 V, 2.5 mA, 0.2 W, 15 °C. Proteins become negatively or positively charged depending on their p*I* and start to migrate from the center (or remain at the center) to the cathode or anode. The rate of their migration will depend on the magnitude of their charge.

Protein bands were visualised by silver staining using the Phast silver staining kit according to the manufacturer's instructions. The gel was fixed with 20% trichloroacetic acid (TCA) for 5 min at 20 °C. Afterwards the gel was washed twice with an aqueous solution containing 10% ethanol and 5% acetic acid at 50 °C for 2 and 4 min, respectively. Washing was followed by 6 min incubation with 5% glutaraldehyde at 50 °C. Again the gel was washed twice with the 10% ethanol and 5% acetic acid containing solution at 50 °C for 3 and 5 min, respectively. The gel was then washed twice with reagent grade water for 2 min at 50 °C. The gel was incubated for 10 min at 40 °C in 0.4% silver nitrate solution. After staining the gel was washed twice with reagent grade water for 0.5 min at 30 °C. The gel was developed in 2.5% sodium carbonate solution with 0.013% glutaraldehyde for approx. 3.5 min and then incubated in background reduction solution (0.1 M sodium thiosulfate solution) for 1.5 min at 30 °C. The development was stopped by washing the gel for 5 min at 50 °C with reagent grade water.

2.2.5. Preparative chromatographic runs

Fractogel® EMD SO_3^- (M) and Fractogel® EMD SE Hicap (M) were packed into chromatographic columns of 200 mm × 16 mm i.d. to 25% compression. Capture runs with mAb were performed on an ÄKTAexplorer 100 chromatographic system. Equilibration was carried out with running buffer (20 mM Na-phosphate + 24 mM NaCl, pH 6.0, 4.4 mS/cm). Sample material for column loading was prepared from a NS/0 cell culture supernatant which was concentrated 16-fold by use of a tangential flow ultrafiltration system with a 30 kDa polyethersulphone (PES) membrane (Millipore Corp., USA) and diluted with sample dilution buffer (10 mM $NaH_2PO_4 \cdot 2H_2O$, pH 5.4) to yield a conductivity of 4 mS/cm. pH was adjusted to 6.0 with 1 M HCl, final conductivity was ≈4.4 mS/cm. Elution buffer was 20 mM Na-phosphate +1 M NaCl, pH 6.0. Columns were loaded with 2125 ml of conditioned cell culture supernatant (mAb titre: 0.32 mg/ml yielding a load of ≈17 mg of mAb/ml of packed bed). Linear gradient elution was performed applying 0–50% elution buffer in 10 column volumes. The linear flow rate during equilibration, wash, and elution was 300 cm/h. After each separation a two-part cleaning-in-place (CIP) protocol was operated with 4 column volumes of elution buffer at a flow rate of 4 column volumes per hour followed by 4 column volumes of 1 M NaOH/1 M NaCl at a flow rate of 3 column volumes per hour.

2.2.6. SEC HPLC

Analytical size-exclusion chromatography (SEC) was conducted using a G3000SWXL column (Tosoh Bioscience GmbH) with a GFC-3000 guard cartridge (Phenomenex). The mobile phase was 25 mM $NaH_2PO_4xH_2O$ buffer solution (pH adjusted to 7.0 with 5 M NaOH) containing 150 mM $NaSO_4$ and 0.05% NaN_3. The column was operated isocratically at a constant flow rate (1 ml/min) using a LaChrom HPLC system (Merck–Hitachi, Darmstadt, Germany). The sample volume applied was 20 μl. The concentration of intact mAb in unknown samples was determined using a standard calibration curve generated with the purified antibody.

2.2.7. HCP ELISA

Host cell protein was detected using a commercial microtitre plate ELISA method specific for the hydridoma cell line NS/0 (Cygnus Technologies, NC, USA). Samples were diluted with sample dilution buffer (consisting of 2 mg/ml IgG in phosphate buffered saline (PBS), pH 7.0) employed with the kit and analysed according to the manufacturer's standard assay protocol. A plate spectrophotometer (Tecan Safire II, Ser. No. 501000005, Tecan AG, Männedorf, Switzerland) was set to dual wave length at 450 nm/630 nm (test/reference) to read the colorimetric reaction of standards and samples.

2.2.8. SDS-PAGE

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a homogeneous 10% Bis-TRIS NuPAGE® pre-cast polyacrylamide gel (Novex, Invitrogen) in combination with MES SDS running buffer. Pooled mAb eluates and purified mAb reference material were diluted to a final mAb concentration of 80 μg/ml. Flow-through fractions and starting material were diluted 1:4 with PBS, pH 7.0. Sixty microlitres of samples were mixed with 20 μl 4 × concentrated lithium dodecylsulfate (LDS) sample buffer, incubated at 70 °C for 10 min and centrifuged at 6000 × *g*. SeeBlue® Plus2 molecular weight marker protein mix was diluted 1:40 with 1 × LDS sample buffer and directly applied onto the gel. Fifteen microlitres of each sample and the MWM protein mix were loaded to the gel and electrophoresed at 200 V/125 mA for 35 min under non-reducing conditions. Protein bands were visualised by silver staining [15].

3. Results and discussion

3.1. pH optimum of static binding capacity

In ion exchange chromatography solution pH is important because it can influence the binding strength between functional groups on the resin and target molecules. With strong cation exchangers as applied in this study the sulfonic acid ligands are fully deprotonated throughout the pH range from 7.0 to 5.0 which was investigated. The mAb with an isoelectric point (p*I*) of ≈7.9–8.5, however, becomes increasingly protonated when the pH shifts towards the more acidic solution. Fig. 1 illustrates the influence of pH on the static binding capacity of two strong

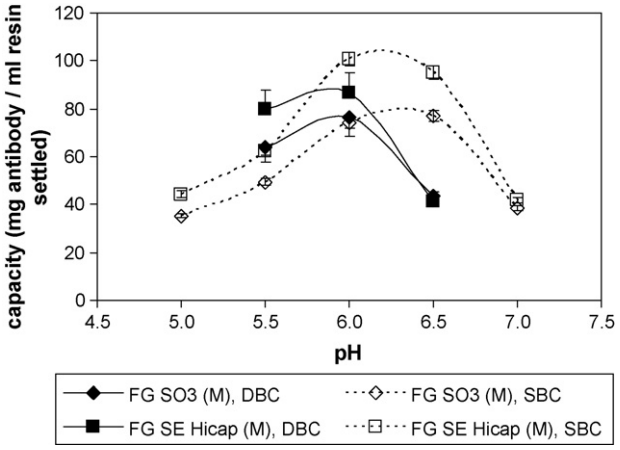


Fig. 1. Influence of pH on SBC and DBC of Fractogel® EMD cation exchangers. SBC: 10 mM Pi buffer + 26.5 mM NaCl (≈4 mS/cm). DBC: 10% breakthrough, 5 mg/ml mAb in 25 mM acetate/25 mM Pi buffer (≈4 mS/cm), residence time = 2 min. "FG": "Fractogel® EMD".

cation exchangers for a mAb. Both resins examined displayed a broad binding optimum around pH 6.3.

As pointed out earlier, for other resins although possessing the same type of ligand, optimum conditions may vary due to differences in resin nature. SP Sepharose XL e.g. was observed to show higher binding capacities at more acidic pH values of 4.5–5.0 [16].

3.2. Batch binding on different scales

Related to considerations to scale down screening assays and go the way towards miniaturisation and multi-well formats in future, a direct comparison was made of assay performance on different scales. Resins were incubated with antibody solution and the static binding capacities after a specified time interval calculated from residual antibody in the permeate. Fig. 2 compares the static binding capacities of two different cation exchangers for purified polyclonal IgG in the 100 ml scale (using beakers) and in the 96-well plate format.

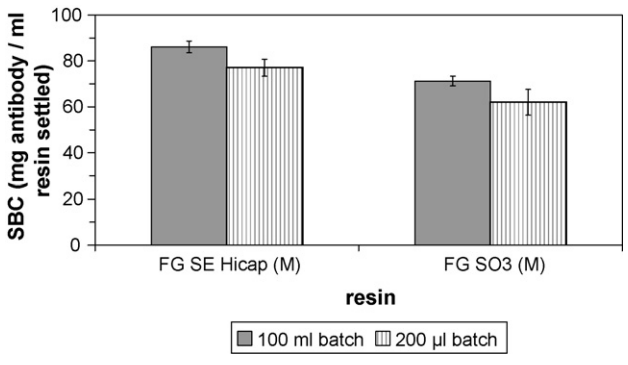


Fig. 2. Static binding capacities for polyclonal hu IgG on different scales. Resins were incubated on a rotary shaker with 2 mg/ml IgG in 20 mM phosphate buffer plus 26 mM NaCl (≈4 mS/cm), pH 6, for 20 min at room temperature. Hundred milliliters batch: 1 ml of settled resin plus 100 ml of IgG solution. Two hundred microlitres batch: 3 µl of settled resin plus 200 µl of IgG solution. "FG": "Fractogel® EMD".

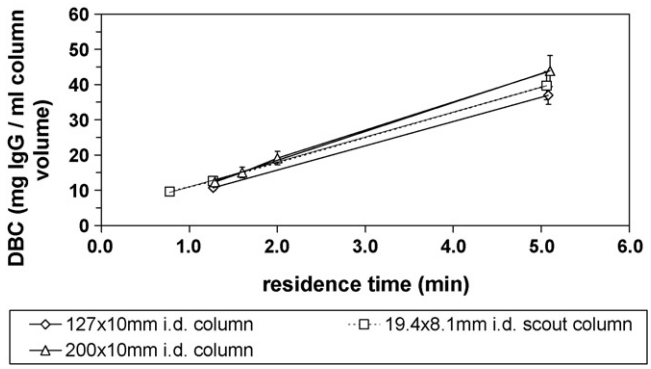


Fig. 3. Influence of residence time on DBC. A 2 mg/ml solution of polyclonal IgG in 25 mM Na-acetate, pH 5.0 was loaded to Fractoprep SO_3 up to 10% breakthrough using columns of different dimensions and different flow rates.

Both scales yielded similar data. In the 96-well plate format each resin was measured 24 times. The comparison shows that the assay format can be reduced and still provide accurate and reliable data.

3.3. The principle of residence time

In dynamic binding experiments column runs would preferentially be carried out with small columns in order to reduce the need for valuable sample. Before the decision was made for a particular column size, however, the influence of residence time on DBC was determined with columns of different length and at different flow rates. For protein A resins it has already been described that the dynamic binding of antibodies is governed by residence time [17]. Fig. 3 where DBC is plotted against residence time illustrates that this principle also holds for the ion exchange resin tested. It becomes obvious that binding capacity only depends on residence time, regardless by which combinations of bed height and flow rate a residence time was achieved. This means that scout columns can indeed be used for DBC measurements.

3.4. pH optimum of dynamic binding capacity

Once the "principle of residence time" was proved, the decision was made to use scout columns for the dynamic experiments. The results from batch screening allowed to narrow the pH window down for the following dynamic experiments. In this particular case with only one screening parameter, namely pH, batch screening has not a great benefit. In comprehensive screening, though, when various chromatography resins and a multitude of parameters such as mobile and stationary phase, column characteristics, and operating variables are covered, the saving of resources can be enormous. Fig. 4 summarises the results. For both resins the maximum dynamic capacity was identified at pH 6.0.

Fig. 1 shows the binding capacities from both the static experiment in the 100 ml scale batch assay (method described in Section 2.2.1) and the dynamic experiment on scout columns (method described in Section 2.2.3), both methods using the monoclonal antibody, in one diagram. Both capacity optima

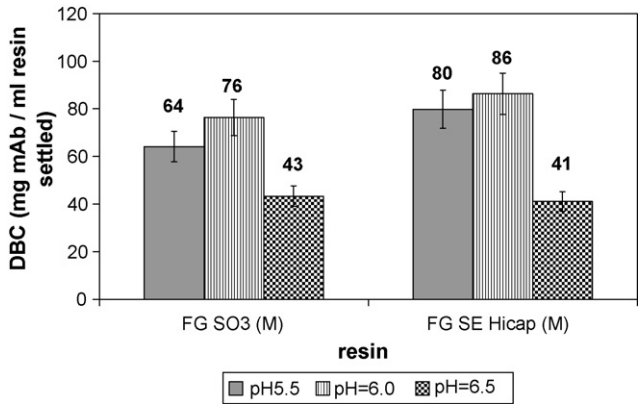


Fig. 4. Influence of pH on the DBC of cation exchangers for monoclonal antibody. Scout columns of 19.4 mm × 8.1 mm i.d., residence time 2 min, columns loaded with 5 mg/ml of mAb in a solution of 25 mM Na–acetate +25 mM Na–phosphate, 4 mS/cm, to 10% breakthrough.

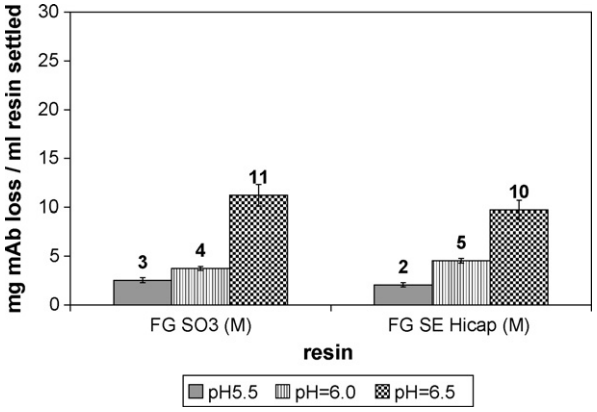


Fig. 5. Loss of monoclonal antibody during DBC determination at different pH values. Scout columns were loaded with mAb solution until 10% breakthrough. The cumulative amount of antibody in the breakthrough and in the subsequent wash is defined as mAb loss.

lie in the same area. The binding optimum under dynamic conditions is shifted by about 0.5 pH units towards more acidic solutions. Capacity levels remain very similar. Trying to explain this binding behaviour one might discuss the binding strength at pH ≥6.5 as relatively weak. When an additional force in the form of flow is applied the binding strength is no longer large enough and binding capacity decreases.

Fig. 5 gives supportive data that the binding strength is indeed weaker at pH 6.5 than at lower pH values. It shows the cumulative loss of mAb during loading (the mAb portion which is found in the flow-through) and subsequent wash. The loss of mAb is much larger at pH 6.5 than at pH values below reflecting weaker binding. mAb concentrations in the cumulative flow-through and wash volume were determined by measuring the absorption at 280 nm with a UV spectrophotometer.

Summarising the comparison of SBC and DBC it becomes clear that SBC can deliver reasonable start conditions for DBC measurements. Conditions must then be refined to locate the DBC optimum.

Besides the direct comparison of SBC and DBC for Fractogel® EMD SO_3^- (M) and Fractogel® EMD SE Hicap (M) we included four more resins in the pH screening for optimum DBC: SP Sepharose XL, UNOsphere S, CM Sepharose Fast Flow, and Fractogel EMD COO^- (M). The results are presented in Fig. 6.

Fig. 6 presents relative DBCs. For each resin its peak capacity was set to 100%. pH 5.5 appears to have a central position in the pH profile of all resins shown here. The pH optima of the two Fractogel resins are above this value, the optima for SP Sepharose XL and UNOsphere S are below (for UNOsphere S it is a bit unclear as pH 5.5 wasn't measured, the trend, however, points in that direction). Maximum DBC in the more acidic region for SP Sepharose XL is in line with the findings for SBC mentioned earlier in Section 3.1. Optimum DBC spans over roughly one pH unit for all resins (again some reservations with the UNOsphere resin). Being able to operate a resin at maximum capacity around pH 6 rather than further below would be an advantage. These resins could be used directly with cell-free mammalian cell culture supernatant. Cell culture supernatants with increased levels of DNA tend to form precipitates at pH values of ≤5.5. In such a situation the use of resins with lower pH optima would be prohibitive to operate at their optimum.

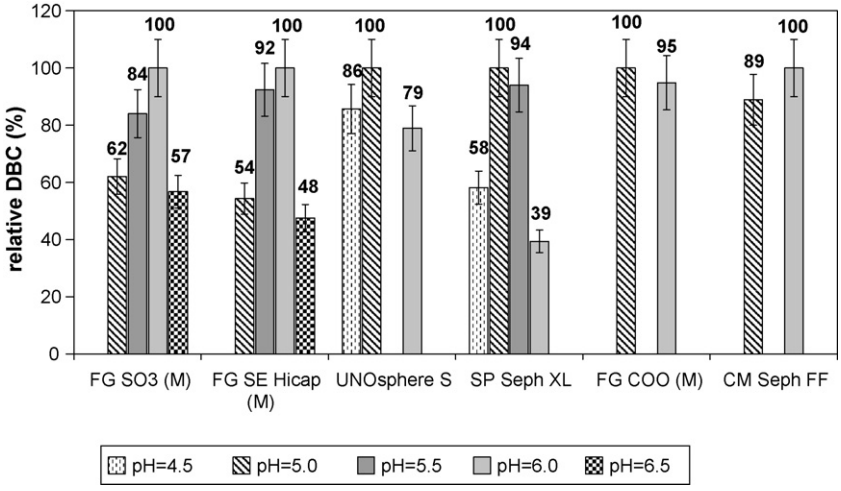


Fig. 6. DBC of cation exchange resins: influence of pH on mAb binding. Experimental conditions as before.

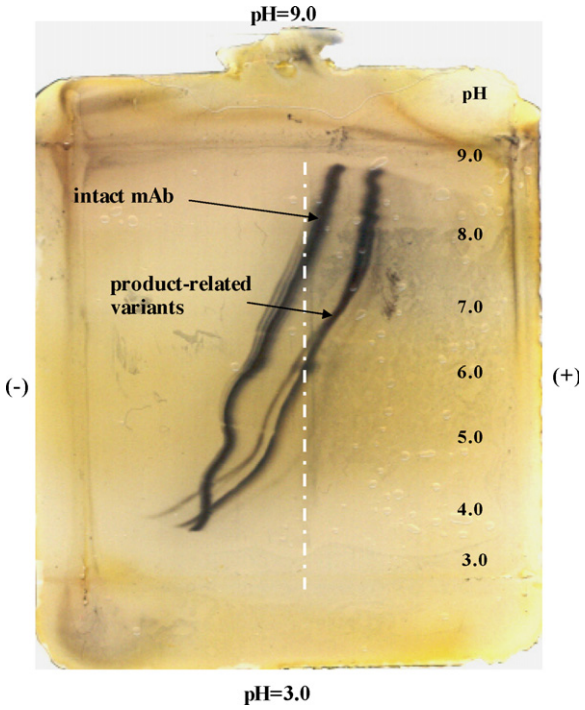


Fig. 7. IEF titration curve analysis of NS/0 cell culture supernatant. Electrophoretic titration curve analysis was performed using a PhastSystem. The sample was electrophoresed on a homogeneous polyacrylamide precast IEF PhastGel covering the pH range 3–9. Protein bands were visualised by silver staining.

The two weak cation exchange resins CM Sepharose Fast Flow and Fractogel EMD COO^- (M) were only measured at pH 5.0 and 6.0. DBCs did not show any impact of pH but were rather constant over the range measured.

3.5. IEF titration curve analysis

As the importance of throughput of a chromatographic step may even be outweighed by its potential for removal of impurities [1], the impact of pH was also looked at with regard to separating host cell proteins from the antibody. An IEF titration curve analysis was carried out with the cell culture supernatant. This analysis gives a clear picture of the p*I* values of different proteins in a mixture. The p*I* of a protein is worth to be used as an initial guidance trying to predict its binding behaviour to ion exchangers. Text books and other references often mention as a rule of thumb that a protein binds to an anion exchanger when pH > p*I*, or to a cation exchanger when pH < p*I*. But it is also stated, that protein retention on an ionic surface cannot be satisfactorily explained by the p*I* value of a protein, because this process is more complex and is influenced by factors such as intramolecular charge asymmetry in the protein, the nature of the support surfaces and mobile phase effects [10,18].

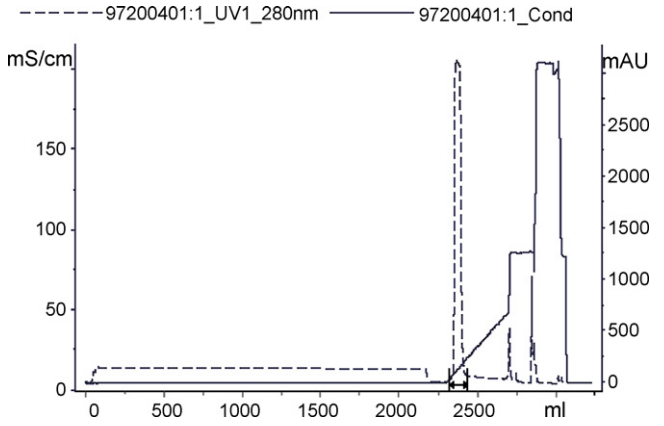


Fig. 8. Purification of a monoclonal antibody on a 200 mm × 16 mm i.d. column packed with Fractogel® EMD SO_3^- (M) (to 25% compression). Arrows indicate the eluate pool.

Fig. 7 shows the IEF titration curves. The intact antibody had a p*I* of ≈7.9–8.5 (upper curve). Two protein bands with p*I* ≈ 6 were identified as product-related variants (Western blot, data not shown). Below these bands can be seen some very faint curves, all with acidic p*I* values in the range 4.5–5.5. These curves belong to host cell proteins.

Based on these IEF results one may anticipate with some reservation that on a cation exchanger at pH 6 rather than at a more acidic pH host cell proteins may flow through the column while the antibody binds. Thus, both the DBC studies and the IEF titration curve analysis would support an operational pH around 6 rather than at more acidic pH.

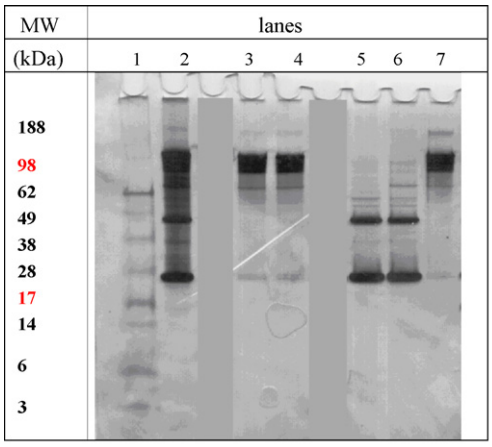
3.6. Preparative chromatographic runs

Fractogel® EMD SO_3^- (M) and Fractogel® EMD SE Hicap (M) were packed into 200 mm × 16 mm i.d. columns and a monoclonal antibody was purified applying a linear salt gradient. Fig. 8 shows the example chromatogram for the SO_3^- resin (the SE Hicap resin produced a very similar trace). Elution of the antibody occurred at 12–13 mS/cm. No antibody aggregates were formed during elution as analysed by SEC HPLC (data not shown).

Table 1 summarises yield and purification of the tentacle cation exchanger runs. Antibody capture on packed columns showed high yields of 95–98%. Host cell proteins were reduced efficiently by at least a factor of 700 to a final level of 80–220 ng/mg antibody (80–220 ppm).

Table 1
Purification of a monoclonal antibody from NS/0 cell culture supernatant on Fractogel® EMD cation exchangers

	mAb concentration (mg/ml)	mAb recovery (%)	HCP (ng/mg mAb)
Starting material	0.31	/	155,349
mAb pool from Fractogel® EMD SO_3^- (M)	6.57	98	218
mAb pool from Fractogel® EMD SE Hicap (M)	8.50	95	84



lane	sample
1	MW standard: See blue plus 2 prestained
2	conditioned CCS
3	mAb pool from Fractogel EMD SO3 (M)
4	mAb pool from Fractogel EMD SE Hicap (M)
5	flow-through Fractogel EMD SO3 (M)
6	flow-through Fractogel EMD SE Hicap (M)
7	mAb standard (after protein A column, CEC, AEC)

Fig. 9. SDS-PAGE analysis of mAb purification on strong Fractogel® EMD cation exchangers.

SDS-PAGE analysis (see Fig. 9) revealed a high purity of the mAb after the optimised CEC step, comparable to mAb reference material obtained from a three-step purification comprising a protein A column, cation and anion exchanger.

Follman and Fahrner [19] in their approach of investigating chromatographic purification processes for antibodies without protein A resins reported a sixteen-fold reduction of HCP to a level of 14,000 ppm when they subjected a Chinese hamster ovary (CHO) cell derived monoclonal IgG to chromatography on SP Sepharose Fast Flow as a first step. Typical HCP levels after protein A chromatography are 200–3000 ppm [1]. Compared with these literature data the preparative runs of the Fractogel® EMD cation exchangers produced very pure antibody with high yield.

4. Conclusion

The influence of pH on antibody binding to strong Fractogel® EMD cation exchangers was investigated, measuring binding as static binding capacities initially. Although in this case we used a macroscopic experimental set-up (1 ml of settled resin was incubated with 90 ml of antibody solution in small beakers), batch mode operation has the potential for high-throughput screening when the assay is miniaturised and automated (adaptation to multi-well plate formats). In order to assess the performance of the miniaturised assay it was directly compared to the macroscopic scale and was found to provide satisfactory reproducibility. We demonstrated that static binding capacities are suitable to predict and confine the conditions for dynamic binding experiments using columns. We confirmed the importance of residence time for studies on ion exchangers and showed that scout columns can be used to measure dynamic binding capacities. An optimised pH for preparative runs was defined based on results from dynamic binding capacities and IEF titration curve analysis. Cation exchangers turned out as highly efficient tools for antibody purification.

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