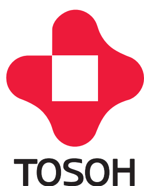

INCREASED PRODUCTIVITY FOR DOWNSTREAM PROCESSING OF BIOTHERAPEUTICS

Polymer-based chromatographic resins featuring high mechanical and chemical stability and high protein binding capacities can help to increase the throughput and robustness of biopharmaceutical manufacturing processes.

By Regina Römling and Egbert Müller, Tosoh Bioscience

Advances in genetic engineering and cell culture technology have raised upstream productivity in the production of recombinant proteins. Increased titres of up to 10 g/l stress the need for highly efficient and robust downstream processes (DSP). Most industrial bioprocess development groups apply generic DSP platforms that are designed to purify various candidates of the same class of proteins applying the same process design (1). Besides various filtration and virus inactivation steps, they usually involve two or three chromatographic unit operations with orthogonal separation modes. The use of high capacity chromatographic resins can help to overcome the DSP bottleneck by increasing process throughput and robustness. High sample amounts can be loaded and higher velocities applied without changing existing columns and other hardware.

Finding the best conditions for each chromatographic unit operation is of critical importance to establish a robust and efficient biopharmaceutical manufacturing process at large scale. Today the Design of Experiments (DoE) methodology and high-throughput screening (HTS) tools support efficient resin screening and method development. Applying these technologies also contributes to a better process understanding by increasing 'prior knowledge'. This is mandatory when following the Quality by Design (QbD) approach to establish robust and validated platforms.

PURIFICATION OF MONOCLONAL ANTIBODIES

Monoclonal antibodies (mAbs) represent the fastest growing segment of the biopharmaceutical industry. A variety of preparative modes of chromatography have been employed for the large-scale purification of mAbs. Most schemes involve the use of protein A affinity chromatography in the capture step, exploiting the specific interactions that take place between the Fc regions of the monoclonals and immobilised protein A, a cell wall component of *Staphylococcus aureus*. Today, recombinant protein A ligands are used for protein A affinity resin production. Protein A affinity chromatography is well established and highly specific to mAbs. More than 95 per cent purity can be achieved in a protein A capture step. In a typical three-step mAb process, protein A chromatography is usually combined with other modes of chromatography – such as cation exchange, anion exchange or hydrophobic interaction (HIC) – to achieve pharmaceutically acceptable purity levels.

Other strategies are focusing either on reducing the number of chromatographic unit operations to a two-step process, or on developing non-protein A platforms. Eliminating a protein A step would overcome the known drawbacks of protein A chromatography such as high resin costs, protein A leaching and the formation of aggregates due to the acidic conditions needed for elution. Recently Lain et al evaluated the use of cation exchange chromatography as a mAb capture step to replace the protein A step (2). They developed a high capacity capture step for mAbs based on Toyopearl GigaCap S-650M.

In 2009, the CMC Biotech Working Group published the A-MAb Case Study, summarising the joint efforts of several large biopharm manufacturers in exemplifying a QbD approach to monoclonal antibody product development (3). A-Mab – a humanised IgG1 monoclonal antibody – was used to follow typical groups or sequences of activities that occur in the development of a monoclonal antibody including upstream and downstream processing.

CHROMATOGRAPHIC PROCESS RESINS

A broad range of commercial chromatographic resins can be applied in large-scale mAb purification. Base particles range from soft agarose based matrices to porous glass and more rigid polymer and silica-based matrices. Common processes often combine resins of various suppliers in order to achieve maximum performance for each single step. Besides binding capacity, purity, robustness towards sanitisation and cleaning-in-place, the mechanical properties of the resin also have to be taken into account when selecting the chromatographic resin for a specific step. The rigidity of the resin influences both the ease of column packing and the bed stability in large-scale industrial columns at high velocities.

Toyopearl® resins are based on a rigid, cross-linked polymethacrylate particle. The high mechanical stability

pays off in excellent pressure flow characteristics and straightforward column packing. Now, that the first Toyopearl based protein A process resin – Toyopearl AF-rProtein A-650F – is being introduced, a complete mAb purification platform based on Toyopearl can be established.

EVALUATION OF PURIFICATION STEPS

A generic mAb process assumes that a pre-defined purification template works for all monoclonals. However, physicochemical differences among mAbs require a flexible platform approach. The overall scheme of the downstream process and the limits of operating conditions can be pre-defined, but individual conditions have to be adapted to the specific target mAb. HTS and DoE are powerful tools when defining the design space of downstream processes and considerably speed up parameter scouting.

The results of the CMC study can be used to set up a mAb purification platform based on polymeric high capacity resins. HTS techniques and the DoE methodology were applied to evaluate chromatographic steps for mAb purification. Various resins and method parameters for the protein A affinity step and subsequent ion exchange steps were evaluated. Robotic platforms for parallel microscale chromatography substantially facilitate the screening of chromatographic resins and support a fast optimisation of method parameters (4); they process small screening columns in 96-well plate format in a few hours. Today, high-throughput systems complement or even replace the tedious and sample consuming column-based screening in many industrial process development groups.

DYNAMIC BINDING CAPACITY

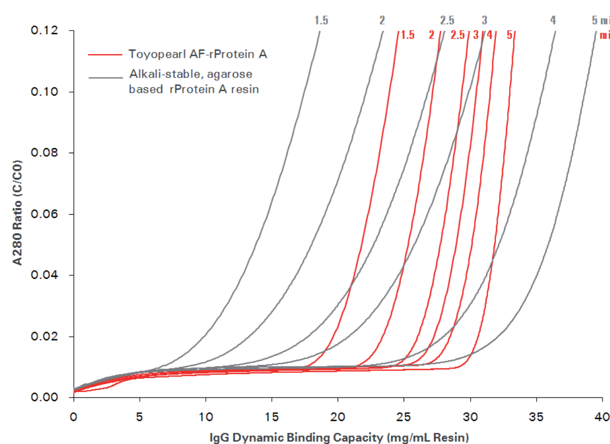

Figure 1

Breakthrough curves for hIgG loading (polyclonal, 10 mg/ml)
Typical DBC at 10% breakthrough:
33 mg/mL @ 60 cm/hr (5 min residence time) - 24 mg/mL @ 200 cm/hr (1.5 min residence time)
Column: 5 mm ID x 5 cm L
Mobile phase: 20 mM sodium phosphate buffer pH 7.2 containing 150 mM NaCl
Sample conc.: 10 mg/mL
Residence time: 1.5, 2.0, 2.5, 3.0, 4.0, 5.0 min

PROTEIN A AFFINITY

Toyopearl AF-rProtein A-650F is an attractive candidate to improve the efficiency of a protein A based mAb capture step because it retains its high dynamic IgG binding capacity at a broad range of velocities and also when loaded with high titre feedstocks (see Figure 1). Its recombinant protein A derived ligand is attached to the rigid base matrix by multiple stable bonds resulting in low ligand leaching and high stability towards alkaline clean-in-place (CIP) conditions. Different protein A affinity resins, including the Toyopearl AF-rProtein A-650F, packed in MediaScout® MiniColumns (Atoll GmbH, Weingarten, Germany) were tested by varying the binding buffer pH, the sample load and the residence time.

HCP REMOVAL

	Bed volume (µl)	Protein load (mg/ml gel)	pH	Flow rate (cm/h)	HCP (ppm)
Toyopearl AF-rProtein A	200	5	3.9	100	19.2
Competitive rProtein A resin	200	5	3.9	100	59.7
Toyopearl AF-rProtein A	200	5	3.9	250	9.8
Competitive rProtein A resin	200	5	3.9	250	30.5
Toyopearl AF-rProtein A	200	25	3.9	250	47.3
Competitive rProtein A resin	200	25	3.9	250	629.6

Table 1

Effect of residence time on the CHO host cell protein (HCP) removal for two alkali-stable rProtein A resins: Toyopearl AF-rProtein A-650F and a competitive agarose-based resin. HCP content detected by ELISA (Cygnus Technologies). *Data kindly provided by U. Breuninger, University of Applied Science Esslingen.*

In order to simulate a real feedstock sample, a pure monoclonal antibody (IgG1) was spiked into a Chinese hamster ovary (CHO) cell lysate. Purity and recovery of the antibody in the eluate were monitored. The purity of the antibody fraction was tested by measuring the amount of CHO host cell proteins (HCP), and the amount of leached protein A by immunoassays. Table 1 shows the HCP removal when varying sample load and/or flow rate (residence time) at fixed bed volume and pH. Fractions purified by the Toyopearl AF-rProtein A-650F showed a lower amount of remaining host cell proteins under all conditions tested when compared to those purified by a widely used competitive rProtein A resin providing a similar IgG binding capacity.

EVALUATION OF CATION EXCHANGE STEP

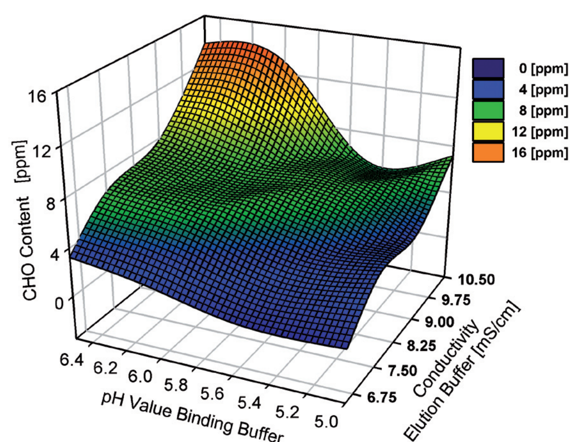

Figure 2

Influence of binding buffer pH and elution buffer ionic strength on the CHO protein content of the mAb fraction purified by cation exchange chromatography on Toyopearl GigaCap CM-650M. *Data kindly provided by U Breuninger at the University of Applied Science, Esslingen, Germany*

PRESSURE/FLOW CHARACTERISTICS

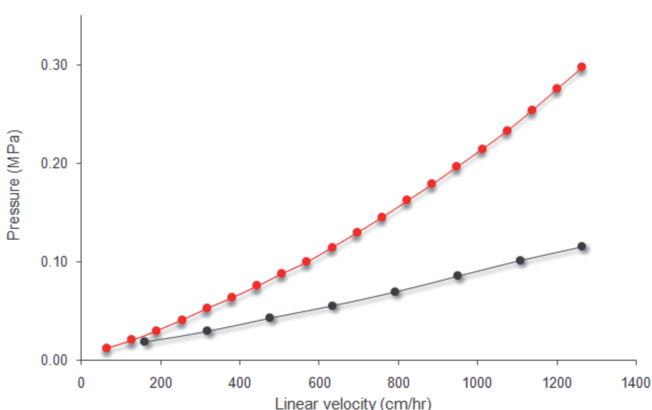

Figure 3

Pressure flow characteristics of polymethacrylate-based process resins. Rigid, polymeric process resins can be used at high flow rates. Toyopearl GigaCap S-650M cation exchange resin with a mean particle size of 75 µm (grey), as well as Toyopearl AF-rProtein A-650F (red) with a mean particle size of 45 µm can be used at linear velocities of more than 1,000 cm/hr.

ION EXCHANGE CHROMATOGRAPHY

The reduction of CHO proteins (CHOP) was also evaluated for a cation exchange step by varying the parameters protein load and pH, as well as ionic strength of binding and/or elution buffer in a DoE approach. At low pH of the binding buffer, the ionic strength of the elution buffer (shown as conductivity) has no significant influence on the amount of residual HCP eluting from the cation exchange resin (Toyopearl GigaCap CM-650M, see Figure 2). With increasing pH of the binding buffer up to neutral pH values, an increased influence of the ionic strength on the HCP removal can be observed. Similar high throughput screening experiments were performed for anion exchange and hydrophobic interaction chromatography (data not shown). By applying the ideal conditions for each unit operation, various downstream process platform strategies can be simulated easily at lab scale.

INCREASING DSP THROUGHPUT

Future challenges of mAb purification will be provided by the further increase in cell culture titres. Chromatographic operations thus become limited in terms of the throughput they can provide. The ability to run a chromatographic purification step at a broad range of velocities and sample loads expands the design space provided by a validated purification platform. The use of resins providing a high mechanical stability and maintaining high protein binding capacities at high velocities supports these requests.

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4. Bensch et al, Chemical Engineering & Techn 28(11): pp1,274-1,284, 2005

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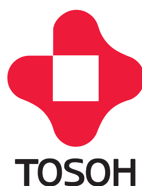

DEVELOPMENT OF A HIGH CAPACITY MIXED MODE RESIN FOR HIGH CONDUCTIVITY MAB FEEDSTOCKS

INTRODUCTION

Cation exchange resins are used in many protein purifications. The high capacity of these resins is very important to process throughput particularly as protein expression levels reach titers greater than 10 g/L.

In many cases the feedstock may need to be diluted prior to loading onto a cation exchange resin to maintain the high protein dynamic binding capacity (DBC) reported by the manufacturer. The additional water, buffer volumes and process time, adds cost to the loading step.

The mixed mode resin TOYOPEARL® MX-Trp-650M shows both high DBC and tolerance to feedstocks with conductivities up to 17 mS/cm. It has been engineered to have excellent elution kinetics.

The data shows that TOYOPEARL MX-Trp-650M resin:

- Tolerates high conductivity feedstocks with minimal dilution required during loading.
- Exhibits mAb DBCs as high as 90-100 g/L.
- Results in improved resolution and peak shape compared to agarose materials.
- Minimizes process pool volumes with both fast binding and fast elution kinetics.

TOYOPEARL MX-Trp-650M is the optimal resin for protein capture steps where the selectivity and protein clearance needed is lower than with affinity resins. This novel resin an excellent choice for intermediate purifications.

RESIN STRUCTURE

TOYOPEARL MX-Trp-650M uses tryptophan as the active ligand (Figure 1). This amino acid has both weak carboxyl cation exchange and indole hydrophobic functional groups.

TOYOPEARL MX-Trp-650M STRUCTURE

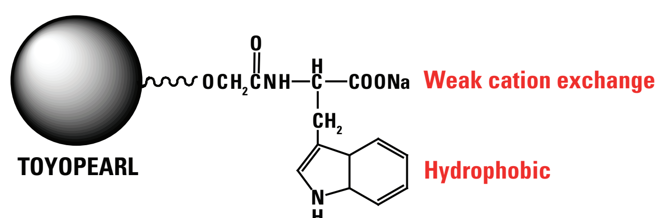

Figure 1

The 50-100 µm particle size of this resin is very useful for capture or intermediate purification steps.

DBC AT HIGH FEEDSTOCK CONDUCTIVITIES

Table 1 shows the DBC of TOYOPEARL MX-Trp-650M at two feedstock conductivities: 12 mS/cm and 17 mS/cm. For comparison purposes, data for a Brand M agarose resin is also shown.

DYNAMIC BINDING CAPACITIES AT HIGH CONDUCTIVITIES

RESIN	PARTICLE SIZE (µm)	ION EXCHANGE CAPACITY (meq)	DBC (g/L)	RECOVERY %
TOYOPEARL MX-Trp-650M (12mS/cm)	50-100	0.12	95	97
TOYOPEARL MX-Trp-650M (17mS/cm)	50-100	0.12	48	96
Brand M (Agarose 12mS/cm)	75 (median)	0.24	14	86
Brand M (Agarose 17mS/cm)	75 (median)	0.24	11	85

Table 1

Resins: TOYOPEARL MX-Trp-650M, Brand M; Column size: 6 mm ID × 4 cm L; Mobile phase: Buffer (12 mS/cm): 0.05 mol/L acetate buffer (pH 4.3, 4.7, 5.0) + 0.10 mol/L NaCl; Buffer (17 mS/cm): 0.05 mol/L acetate buffer (pH 4.3, 4.7, 5.0) + 0.15 mol/L NaCl; Flow rate: 1.0 mL/min (212 cm/hr); Detection: UV @ 280nm; Sample: human polyclonal IgG (1mg/mL); DBC calculated from 10% height of breakthrough curve.

DBC AT VARYING pH

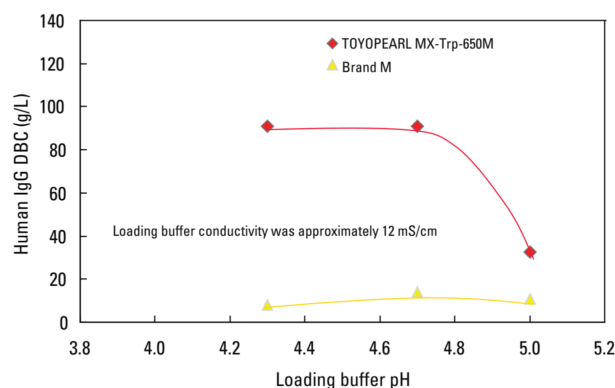

Figure 2

Resins: TOYOPEARL MX-Trp-650M, Brand M; Column size: 6 mm ID × 4 cm L; Mobile phase: Buffer A: 0.05 mol/L acetate buffer (pH 4.3, 4.7, 5.0) + 0.10 mol/L NaCl (12 mS/cm); Buffer B: 0.1 mol/L Tris-HCl buffer (pH 8.5) + 0.3 mol/L NaCl; Flow rate: 1.0 mL/min (212 cm/hr); Detection: UV @ 280nm; Sample: human polyclonal IgG (1mg/mL) DBC calculated from 10% height of breakthrough curve.

For the 12 mS/cm and 17 mS/cm measurements, the TOYOPEARL MX-Trp-650M resin shows almost 7× higher and 4× higher DBC, respectively, than Brand M.

DBC is maintained in 12 mS/cm loading buffers up to pH 4.8 (Figure 2).

MASS TRANSFER PARAMETERS

The mass transfer properties of a resin influence the economics of the loading and elution stages of a capture step, and the degree of resolution for intermediate purification. In keeping with the exceptional target binding and eluting properties of Tosoh's newer TOYOPEARL GigaCap® resins, the TOYOPEARL MX-Trp-650M also shows a narrow elution peak width to complement its higher capacity (Figure 3).

The mass transfer properties also contribute to minimal peak broadening when doing chromatography. Figure 4 shows the excellent peak shape for the new TOYOPEARL MX-Trp-650M and the much broader tailing associated with the Brand M agarose material.

RESIN CLEAN IN PLACE (CIP)

The new TOYOPEARL MX-Trp-650M resin has excellent stability to 0.5 mol/L NaOH and can be run for many CIP cycles without losing its high capacity (Figure 5).

CONCLUSION

TOYOPEARL MX-Trp-650M resin is a high capacity and high performance mixed mode resin for intermediate purification. The new product has 3-5 × the DBC of typical mixed mode resins so reductions in process buffer and pool volumes can be expected. The ability of this novel resin to bind protein from high conductivity feedstock minimizes the amount of water needed for dilution before loading the column.

NARROW ELUTION PEAK WIDTHS

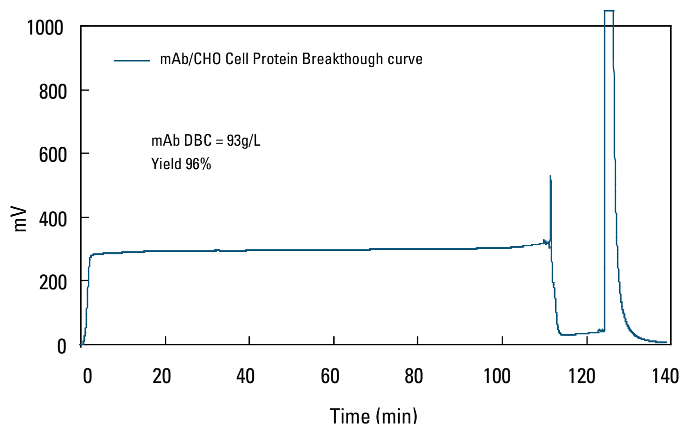

Figure 3

Resin: TOYOPEARL MX-Trp-650M; Column size: 6 mm ID × 4 cm L; Mobile phase: Buffer A: 0.05 mol/L acetate buffer (pH 4.7) + 0.1 mol/L NaCl (12 mS/cm); Buffer B: 0.1 mol/L Tris-HCl buffer (pH 8.5) + 0.3 mol/L NaCl; Flow rate: A: 1.0 mL/min (212 cm/h); B: 2.0 mL/min (started at 124 min); Detection: UV @ 280nm; Sample: CHO cell culture media, monoclonal antibody (1 mg/mL) diluted with Buffer A

GOOD RESOLUTION FOR INTERMEDIATE PURIFICATION

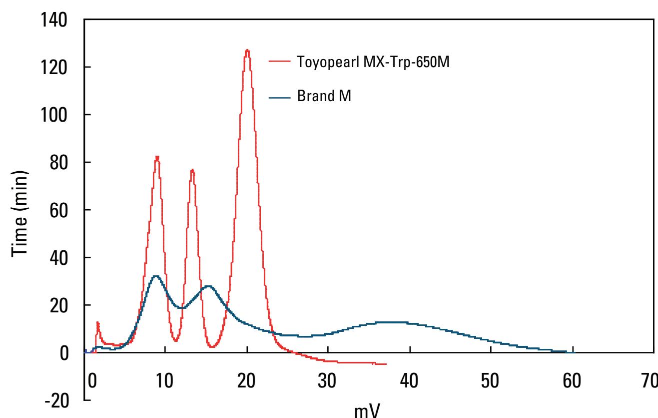

Figure 4

Resins: TOYOPEARL MX-Trp-650M, Brand M; Column size: 7.5 mm ID × 7.5 cm L; Mobile phase: Buffer A: 20 mmol/L phosphate (pH 7.0); Buffer B: 20 mmol/L phosphate + 1.0 mol/L NaCl (pH 7.0); Gradient: 30 min. linear gradient from buffer A to buffer B; Flow rate: 1.0 mL/min; Detection: UV @ 280nm; Sample: trypsinogen (6.6 mg/mL) cytochrome C (3.6 mg/mL) lysozyme (6.6 mg/mL); Sample vol.: 25 µL

STABILITY IN 0.5 mol/L NaOH

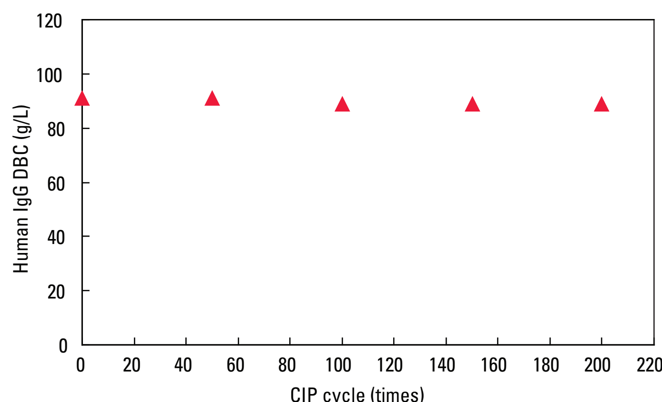

Figure 5

Alkaline cleaning (CIP) conditions
3CV: 0.5 mol/L NaOH,
5CV: 0.1 mol/L Tris-HCl pH 8.5 + 0.3 mol/L NaCl
Flow rate: 1 mL/min
DBC Measurement
Column Size: 6 mm ID × 4 cm L; Binding buffer: 0.05 mol/L acetate buffer (pH 4.7) + 0.1 mol/L NaCl; Flow rate: 1 mL/min.; Detection: UV @ 280 nm; Sample: polyclonal human IgG; Sample Load: 1 mg/mL

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Application Note

SEPARATION OF MONOCLONAL IMMUNOGLOBULIN G AND ITS AGGREGATES USING TOYOPEARL MX-TRP-650M

The importance of proper aggregate removal during polishing of a monoclonal antibody (mAb) for therapeutic use is beyond controversy. Severe anaphylactic reactions have been described in the literature for the application of aggregated proteins as a drug byproduct. Traditionally, ion exchange chromatography or hydrophobic interaction chromatography are utilized to purify a structurally homogeneous product. In case these platforms do not satisfy the requirements for mAb polishing, advanced chromatography resins need to be considered. For instance, mixed mode stationary phases like TOYOPEARL MX-Trp-650M may pave the way for more challenging polishing applications. This application note intends to give you some insights into how you could start handling mAb polishing with the tryptophan immobilized ligand.

SCREENING FOR THE APPROPRIATE CONDITIONS FOR MAB AGGREGATE REMOVAL

Mixed mode chromatography is one approach to combine the advantages of hydrophobic interaction chromatography (HIC) and ion exchange chromatography (IEX). The number of potential ligands for mixed mode chromatography is huge, as potential candidates can be found in various molecular classes. However, the preferred environment for an antibody restricts the ligand choice. Binding and elution with moderate salt and pH conditions, as well as capacities comparable to IEX are in focus of ligand selection. Moreover, the need for an appropriate selectivity sets tight bounds.

To fulfill these expectations, more complex structures are used, if compared to traditional IEX or HIC ligands. In accordance with this, method development becomes more complex, as well. In case no robotic system is at hand, a straight-forward approach how to handle the increased number of parameters affecting the process of binding and elution is described in our first example, the polishing of a humanized, monoclonal IgG. The major factors influencing binding and separation of proteins on TOYOPEARL MX-Trp-650M are the pH and the salt concentration.

ELUTION BY pH GRADIENT

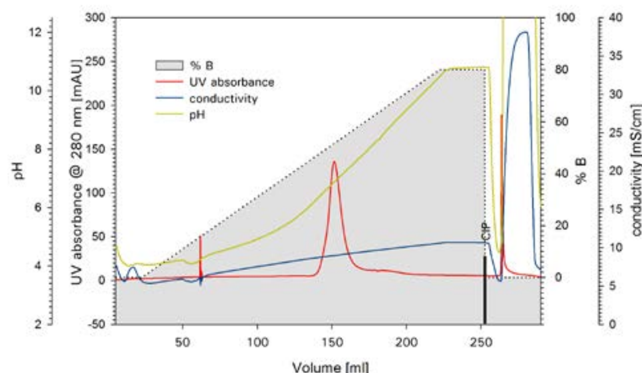


Figure 1b

Column: 6.6 mm ID x 2 cm L; Mobile phase A: buffer pH 4.0 + 0.2 mol/L NaCl; Mobile phase B: buffer pH 12.0 + 0.2 mol/L NaCL; Linear flow: 150 cm/h; Sample: 10 mg mAb + mAb aggregates (conc. 1 g/L)

ELUTION BY SALT GRADIENT

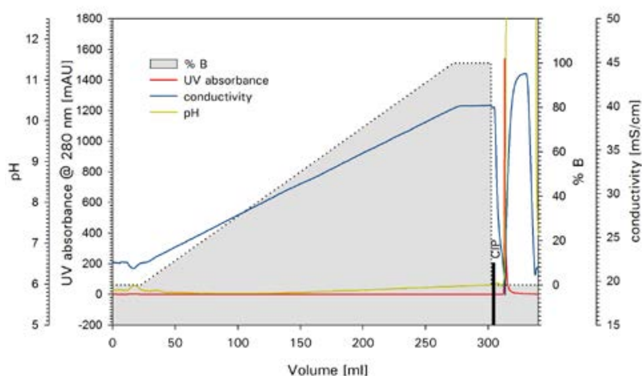


Figure 1a

Column: 6.6 mm ID x 2 cm L; Mobile phase A: buffer pH 4.0 + 0.2 mol/L NaCl; Mobile phase B: buffer pH 4.0 + 0.5 mol/L NaCl; Linear flow: 150 cm/h; Sample: 10 mg mAb + mAb aggregates (conc. 1 g/L)

ELUTION BY COMBINED pH AND SALT GRADIENT

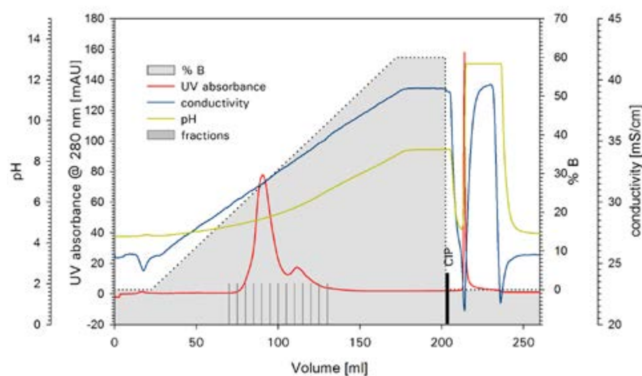


Figure 1c

Column: 6.6 mm ID x 2 cm L Mobile phase A: buffer pH 4.0 + 0.2 mol/L NaCl; Mobile phase B: buffer pH 12 + 0.4 mol/L NaCl; Linear flow: 150 cm/h; Sample: 10 mg mAb + mAb aggregates (conc. 1 g/L)

For a start, three linear gradient runs will provide hints on the actual working frame for a certain molecule. Figure 1a, b & c show three chromatograms of the mAb sample containing approximately 17 % aggregates. The three runs illustrate a salt gradient (constant pH), a pH gradient (constant salt concentration) and a combined salt and pH gradient. The pH span of the applied chromatofocusing buffersystem depends of course on the stability of the sample. These buffer systems are either commercially available as ready to use buffer systems or can be prepared by arranging various (zwitter-) ionic buffer salts with pKs values covering the desired pH span.

While the salt gradient does not allow protein recovery, the pH gradient leads to the elution of one protein peak. In contrast, the combined pH and salt gradient recovers the protein in two peaks, a monomer peak in the front, followed by the aggregates. Quantitative and qualitative analysis of the collected protein peaks was performed by size exclusion chromatography (SEC) using TSKgel G3000SWXL. The corresponding results are presented in figure 2.

OPTIMIZED pH & SALT GRADIENT ON TOYOPEARL MX-TRP-650M FOR THE SEPARATION OF MAB MONOMERS AND AGGREGATES

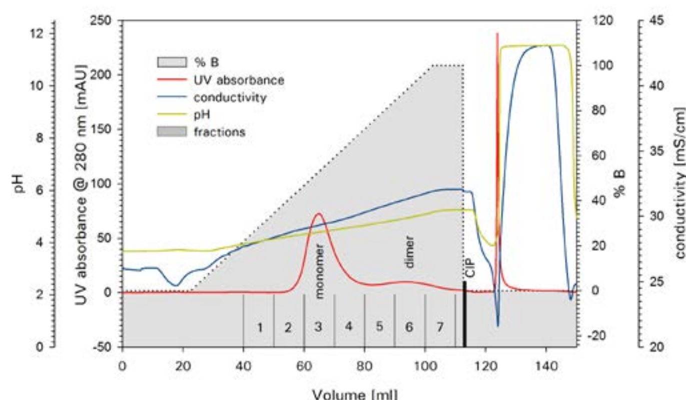


Figure 3

Column: 6.6 mm ID x 2 cm L; Mobile phase A: 0.1 mol/L acetate + 0.2 mol/L NaCl, pH 4.3; Mobile phase B: 0.1 mol/L acetate + 0.4 mol/L NaCl, pH 5.6; Linear flow: 150 cm/h; Sample: 10 mg mAb + mAb aggregates (conc. 1 g/L)

MAB AGGREGATE REMOVAL APPLYING AN UP-SCALABLE GRADIENT

The screening gradients are rather time consuming and inefficient in process scale, due to the covered range of pH 4.0 to 12.0. Nevertheless, the retrieved results allow narrowing the pH and salt concentration range to pH 4.0 to 6.0 and 0.2 M NaCl to 0.4 M NaCl, respectively. This frame can be covered by a sodium acetate buffer (Fig. 3). The significantly shortened gradient can be applied for separation, while the aggregate content in the monomer pool is below 1 %. SEC chromatograms of the collected fractions are presented in Figure 4.

From these results, we conclude that TOYOPEARL MX-Trp-650M can be utilized as a highly efficient tool for aggregate removal of mAbs, as it offers capacities comparable to IEX, high recovery and proper selectivity. With the presented screening gradients, it is possible to take advantage of a straight-forward method development approach, which does not necessarily require a robotic system. As a result, method development for mixed mode chromatography with the tryptophan ligand is not more elaborate than for traditional HIC or IEX resin, while it offers outstanding selectivity for mAbs and mAb aggregates. For even more challenging separations, one might consider systematic screening for method development, to play on the modulation opportunities typically owned by mixed mode resins. Enabling both procedures with adequate outcome, traditional method development and advanced systematic robotic screening, characterizes TOYOPEARL MX-Trp-650M as a perfect tool for the polishing of mAbs.

SEC CHROMATOGRAMS OF THE COLLECTED FRACTIONS OF COMBINED SALT & pH GRADIENT ON TOYOPEARL MX-TRP-650M (SEE FIG. 1C).

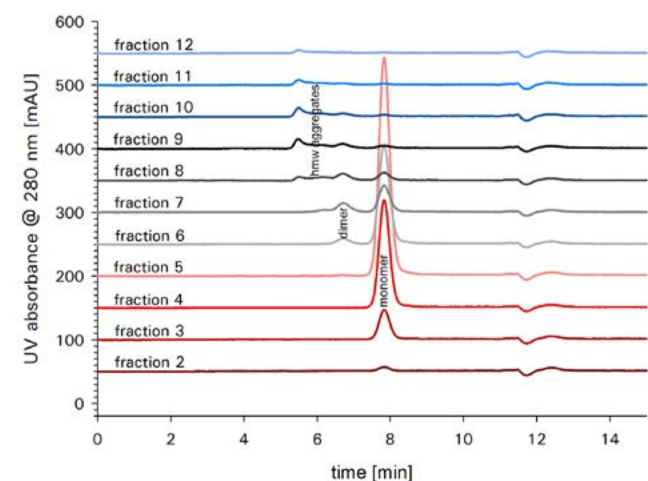


Figure 2

Column: 6.6 mm ID x 2 cm L; Mobile phase A: 0.1 mol/L acetate + 0.2 mol/L NaCl, pH 4.3; Mobile phase B: 0.1 mol/L acetate + 0.4 mol/L NaCl, pH 5.6; Linear flow: 150 cm/h; Sample: 10 mg mAb + mAb aggregates (conc. 1 g/L)

SEC CHROMATOGRAMS OF THE COLLECTED FRACTIONS OF OPTIMIZED GRADIENT ON TOYOPEARL MX-TRP-650M (SEE FIG. 3).

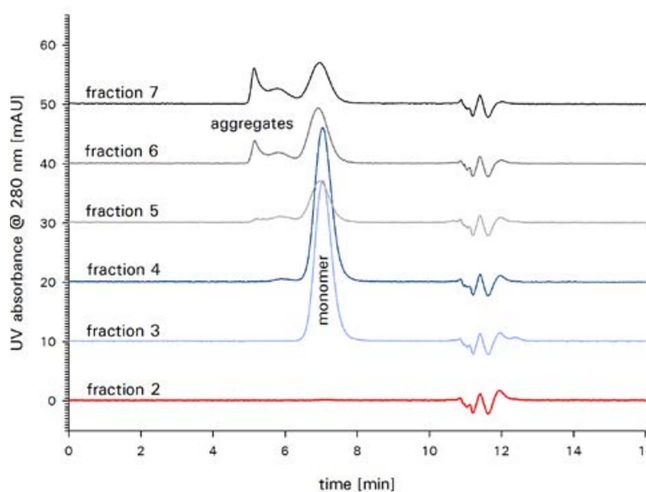


Figure 4

Column: TSKgel G3000SWXL 7.8 mm ID x 30 cm L; Mobile phase: 0.1 mol/L sodium phosphate + 0.1 mol/L sodium sulfate, pH 6.7; Flow rate: 1 ml/min; Detection: UV @ 280 nm; Sample: 100 µl of each fraction

TOSOH

TOYOPEARL® MX-Trp-650M
SALT SELECTIVITY AND TOLERANCE

TOYOPEARL MX-Trp-650M, a new, mixed-mode chromatography resin combining a weak cation exchange and a hydrophobic interaction ligand for process scale chromatography applications is the latest addition to the TOYOPEARL product line. This resin, capable of being run much the same as a standard cation exchanger or in a more traditional HIC mode, truly lives up to the mixed-mode moniker.

INTRODUCTION

Chromatographic resins with high capacities, selectivities, and salt tolerances differing from those seen with traditional ion exchange media are now in demand. Mixed-mode chromatography media offers an alternative to traditional single-mode media. The polymethacrylic base bead (TOYOPEARL HW-65) is chemically modified with the amino acid tryptophan, which combines a weak cationic group with a hydrophobic functional group. The resulting resin exhibits dynamic binding capacities of approximately 90 mg/mL for human IgG. TOYOPEARL MX-Trp-650M offers chromatographers selectivity and salt tolerance combined with binding capacities that are similar to traditional cation exchange resins.

EXPERIMENTAL CONDITIONS

For selectivity and salt tolerance comparisons of TOYOPEARL MX-Trp-650M and a traditional strong cation exchange (TOYOPEARL GigaCap® S-650M) resin, various buffering salts at a set pH value were used. For the selectivity comparisons between different buffering salts at a single pH value, pH 6.0 was selected. Since there are multiple buffering salts with an effective range that includes this pH point; a total of four buffering salts were selected for these experiments: sodium acetate, MES, Bis-Tris Propane and sodium citrate. 6.6 mm ID × 15.5 ± 1.0 cm columns were packed with new resin. A three protein mixture (trypsinogen, cytochrome C, and lysozyme) was loaded onto the column and eluted with a linear salt gradient (Figures 1-2). Resolution between the peaks was measured and recorded for comparison (Tables 1-2).

TOYOPEARL MX-Trp-650M MULTIPLE BUFFERING SALTS AT PH 6.0

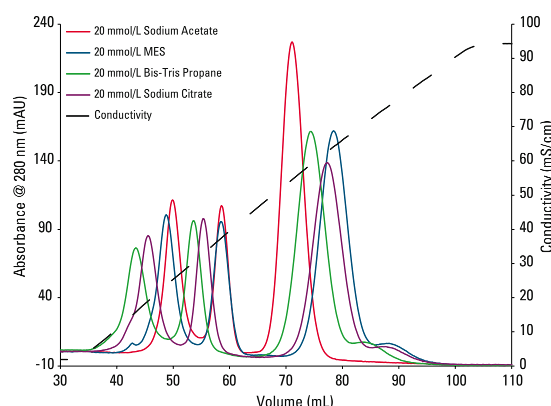


Figure 1

Resin: TOYOPEARL MX-Trp-650M
Column size: 6.6 mm ID × 15.5 cm (5.30 mL)
Buffer A (1): 20 mmol/L sodium acetate, Buffer A (2): 20 mmol/L MES, Buffer A (3): 20 mmol/L Bis-Tris Propane, Buffer A (4): 20 mmol/L sodium citrate, Buffer B: Buffer A + 1.0 mol/L NaCl
Gradient: 60 minutes 0% B – 100% B Flow rate: 1.14 mL/min (200 cm/hr); Detection: UV @ 280 nm, Temperature: ambient
Sample: 1. trypsinogen (6.6 mg/mL), 2. cytochrome C (3.6 mg/mL), 3. lysozyme (6.6 mg/mL)
Sample Load: 5% CV (4.45 mg total protein)

TOYOPEARL MX-Trp-650M pH 6.0 MULTI BUFFER RETENTION AND RESOLUTION

	Trypsinogen		Cytochrome C			Lysozyme		
	Retention (mL)	Cond. (mS/cm)	Retention (mL)	Cond. (mS/cm)	Trypsinogen/ Cytochrome C Resolution (Rs)	Retention (mL)	Cond. (mS/cm)	Cytochrome C/ Lysozyme Resolution (Rs)
Sodium Acetate	50.01	25.37	58.45	37.40	0.89	69.87	52.98	1.04
MES	48.77	22.74	58.46	36.40	0.98	78.43	62.84	1.58
Bis-Tris Propane	43.43	16.38	53.64	30.58	0.78	74.36	59.08	1.41
Sodium Citrate	45.60	21.48	55.36	34.86	0.76	77.26	63.84	1.59

Table 1

TOYOPEARL GigaCap S-650M MULTIPLE BUFFERING SALTS AT pH 6.0

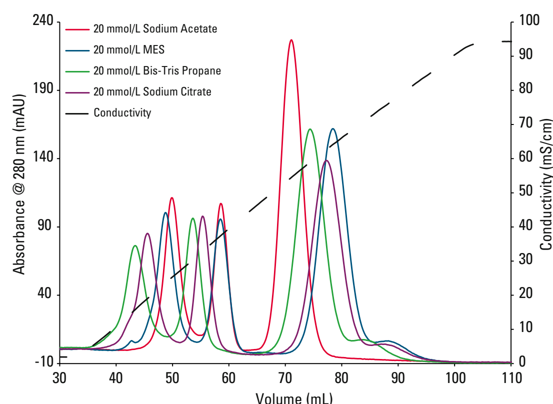


Figure 2

Resin: TOYOPEARL GigaCap S-650M
Column size: 6.6 mm ID × 15.5 cm (5.30 mL)
Buffer A (1): 20 mmol/L sodium acetate; Buffer A (2): 20 mmol/L MES; Buffer A (3): 20 mmol/L Bis-Tris Propane; Buffer A (4): 20 mmol/L sodium citrate; Buffer B: Buffer A + 1.0 mol/L NaCl
Gradient: 60 minutes 0% B – 100% B; Flow rate: 1.14 mL/min (200 cm/hr); Detection: UV @ 280 nm; Temperature: ambient
Sample: 1. trypsinogen (6.6 mg/mL), 2. cytochrome C (3.6 mg/mL); 3. lysozyme (6.6 mg/mL)
Sample Load: 5% CV (4.31 mg total protein)

The relative salt tolerance of the two resins tested in these experiments can be determined in part by peak conductivity for each of the proteins. Comparison of the conductivity at peak maximum (Table 3) as a function of the salt concentration required to desorb the proteins is indicative of the relative salt tolerance of the resins.

RESULTS

The order of elution for each of the chromatograms is as follows: trypsinogen, cytochrome C, and lysozyme. While the order of elution remained unchanged for all buffering salts used with TOYOPEARL MX-Trp-650M and TOYOPEARL GigaCap S-650M (Figure 1-2), the choice of buffer did have an effect on the resolution and the amount of NaCl needed to desorb each protein from the resin. The lysozyme was the most affected of the three proteins by the change in buffering salt (Table 1-2). Comparison of peak conductivities indicate that the TOYOPEARL MX-Trp-650M is more salt tolerant than TOYOPEARL GigaCap S-650M for all proteins with all of the buffers tested at pH 6.0 (Table 3).

TOYOPEARL GigaCap S-650M pH 6.0 MULTI BUFFER RETENTION AND RESOLUTION

	Trypsinogen		Cytochrome C			Lysozyme		
	Retention (mL)	Cond. (mS/cm)	Retention (mL)	Cond. (mS/cm)	Trypsinogen/ Cytochrome C Resolution (Rs)	Retention (mL)	Cond. (mS/cm)	Cytochrome C/ Lysozyme Resolution (Rs)
Sodium Acetate	43.44	17.17	52.46	30.04	1.16	57.20	36.92	0.75
MES	43.76	17.04	52.78	30.10	1.01	61.77	43.06	0.88
Bis-Tris Propane	37.16	8.00	48.70	25.46	1.31	59.09	40.51	0.95
Sodium Citrate	39.56	13.77	49.52	27.99	0.98	59.61	41.97	0.90

Table 2

TOYOPEARL MX-Trp-650M AND TOYOPEARL GigaCap S-650M SALT TOLERANCE

TOYOPEARL MX-Trp-650M			
	Trypsinogen Peak Conductivity (mS/cm)	Cytochrome C Peak Conductivity (mS/cm)	Lysozyme Peak Conductivity (mS/cm)
Sodium Acetate	25.37	37.40	52.98
MES	22.74	36.40	62.84
Bis-Tris Propane	16.38	30.58	59.08
Sodium Citrate	21.48	34.86	63.84
TOYOPEARL GigaCap S-650M			
	Trypsinogen Peak Conductivity (mS/cm)	Cytochrome C Peak Conductivity (mS/cm)	Lysozyme Peak Conductivity (mS/cm)
Sodium Acetate	17.17	30.04	36.92
MES	17.04	30.10	43.06
Bis-Tris Propane	8.00	25.46	40.51
Sodium Citrate	13.77	27.99	41.97

Table 3

CONCLUSIONS

TOYOPEARL MX-Trp-650M was able to separate all three test proteins in all four buffers tested; resin performance in different buffers did vary (Figure 1). MES, pH 6.0, produced the best results for the MX-Trp-650M, with the poorest results being the sodium acetate with respect to overall retentive properties and resolution (Table 1). TOYOPEARL GigaCap S-650M was also able to separate all three test proteins in the four buffers tested. Like the TOYOPEARL MX-Trp-650M, its performance in different buffers varied as well (Figure 2). Bis-Tris Propane, pH 6.0, produced the best results for the GigaCap S-650M with the poorest results being the sodium citrate with respects to overall retentive properties and resolution (Table 2). These results indicate that the TOYOPEARL MX-Trp-650M and TOYOPEARL GigaCap S-650M selectivities can vary depending on the buffer being used.

TOYOPEARL MX-Trp-650M is more salt tolerant than the traditional cation exchange resin tested in this experiment. For all four buffers tested at pH 6.0, TOYOPEARL MX-Trp-650M required higher concentrations of salt to desorb the trypsinogen, cytochrome C, and lysozyme than TOYOPEARL GigaCap S-650M (Table 3).

MIXED ELECTROLYTES IN HYDROPHOBIC INTERACTION CHROMATOGRAPHY

INTRODUCTION

While process productivity of ion exchange chromatography (IEX) has been improved due to advanced surface modifications of newly developed resins (such as the TOYOPEARL GigaCap series), hydrophobic interaction chromatography (HIC) with proteins has experienced comparably small performance increases. For HIC, additional parameters besides the resin surface modification need to be tuned. Especially capacities and recoveries of HIC applications cannot compete with those of modern IEX. The general method complexity allows various strategies to approach this goal, as for many applications, selectivity of HIC is striking. This application note addresses the electrolyte composition of the liquid phase as one parameter responsible for protein adsorption and desorption. One standard salt used for HIC is ammonium sulfate. Its salting-out potential is well-known and also applicable for non-chromatography based purification methods, such as protein precipitation. Apart from sodium citrate and sodium sulfate, which also show great salting-out potentials for many proteins, salts like sodium acetate and sodium chloride belong to the manufacturing scale relevant (buffer-) salts. The latter are not typically related to HIC as their salting-out potential is weaker. The herein presented results illustrate the benefit of often neglected salts in HIC and mixtures of them, regarding capacity and selectivity.

DYNAMIC BINDING CAPACITY

The impact of the electrolyte composition of the liquid phase in HIC on the dynamic binding capacity for lysozyme and a monoclonal antibody (mAb) was investigated. The solubility limiting concentration of ammonium sulfate was optically determined to be 2.2 M for lysozyme and 1.1 M for the mAb. Hence, concentrations of 2 M and 1 M ammonium sulfate were chosen, respectively, as a reference value for the capacity comparison. Table 1a & b list exemplary dynamic binding capacities that could be achieved using ammonium sulfate and mixtures of ammonium sulfate, sodium citrate, sodium sulfate, sodium acetate and sodium chloride for protein adsorption onto TOYOPEARL PPG-600M, TOYOPEARL Phenyl-600M and TOYOPEARL Butyl-600M. Capacities were measured at 10 % breakthrough, pH 7.0 and a protein concentration of 1 g/L. The feed stream was set to 150 cm/h. The corresponding resins were packed to a total column volume of 1 mL with an inner diameter of 6.6 mm.

The dynamic binding capacities for the two presented proteins can be increased for certain salt mixtures and resins. Improvements up to roughly 50 % are possible.

SELECTIVITY

The point of elution of a certain protein does not only depend on the applied salt molarity. Factors like pH and temperature are well-known to influence protein desorption. Further, the standard protein separations presented in figure 1 – 3 illustrate that the protein elution does not correlate linearly with the decreasing salt concentration in the liquid phase for ammonium sulfate and the exemplary presented salt mixtures. The different figures refer each to one commonly used TOYOPEARL HIC ligand: PPG, Phenyl and Butyl. For selectivity testing, cytochrome C (1), ribonuclease A (2) and lysozyme (3) were separated. 1 mL columns with an inner diameter of 6.6 mm were used. 10 mg/mLresin of each protein were loaded. A constant flow of 150 cm/h was applied.

Figure 1 illustrates the chromatograms using TOYOPEARL PPG-600M. Lysozyme, which is represented by peak 3 elutes the latest for the mixture of sodium sulfate and sodium chloride. For the same mixture, ribonuclease A (2) elutes right after the gradient starts. For ammonium sulfate as a single salt, the very same proteins elute in between. This means, that this mixture provides increased resolution for the proteins, compared to ammonium sulfate. The same behavior is observed for TOYOPEARL Phenyl-600M (Figure 2) and TOYOPEARL Butyl-600M (Figure 3).

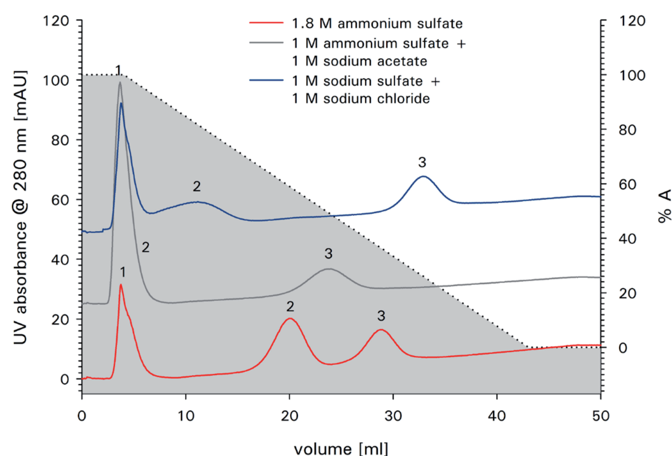


Figure 1

Separation of cytochrome C (1), ribonuclease A (2) and lysozyme (3) on TOYOPEARL PPG-600M. Ribonuclease A is hardly retained for the mixtures. Lysozyme is further retained for the sodium sulfate + sodium chloride mixture than for ammonium sulfate as a single salt.

TOYOPEARL Butyl-600M, as the most hydrophobic resin among the tested ones, allows binding of cytochrome C (1) for some salts or salt mixtures. Only the mixture of ammonium sulfate and sodium acetate does not cause cytochrome C binding, while lysozyme is almost as much retained as for ammonium sulfate as a single salt. Ribonuclease A is only weakly bound to the resin using this mixture. If lysozyme would represent the target molecule, the almost exclusive binding of lysozyme would allow higher binding capacities as for the other two liquid phase compositions, where parts of the resin capacity are occupied by the other sample components.

It can be concluded that every target protein and its related impurities require distinct salts or salt mixtures to achieve highest resolution, purity and capacity. On the one hand, this enlarges the set of parameters in HIC, but on the other hand, this allows selectivity modulation. Higher resolution of target substances and impurities can be achieved. Capacities can also benefit from salt mixtures. Besides a more sophisticated binding of the target component, general capacities up to 150 % of the ammonium sulfate capacity can be reached. Using mixed electrolytes in HIC opens up an additional opportunity to improve HIC separations as an alternative to traditional HIC applications using ammonium sulfate.

Salt	Capacity [mg/mL] PPG-600M	Capacity [mg/mL] Phenyl-600M	Capacity [mg/mL] Butyl-600M
2 M ammonium sulfate	30	46	15
1 M sodium sulfate + 1 M sodium acetate	39	63	18
1 M ammonium sulfate + 1 M sodium chloride	31	54	10
0.9 M trisodium citrate + 0.9 M sodium chloride	38	43	20

Table 1a

Dynamic binding capacities for lysozyme and salt mixtures on TOYOPEARL PPG-600M, TOYOPEARL Phenyl-600M and TOYOPEARL-Butyl-600M. The capacity determined applying ammonium sulfate serves as a reference standard.

Salt	Capacity [mg/mL] PPG-600M	Capacity [mg/mL] Phenyl-600M	Capacity [mg/mL] Butyl-600M
1 M ammonium sulfate	18	20	18
1.7 M sodium chloride + 0.7 M ammonium sulfate	30	25	15
1.3 M sodium chloride + 0.6 M ammonium sulfate	25	20	12

Table 1b

Capacities at 10 % breakthrough for mAb and representative mixtures on TOYOPEARL PPG-600M, TOYOPEARL Phenyl-600M and TOYOPEARL-Butyl-600M. The capacity for ammonium sulfate may be regarded as a reference standard.

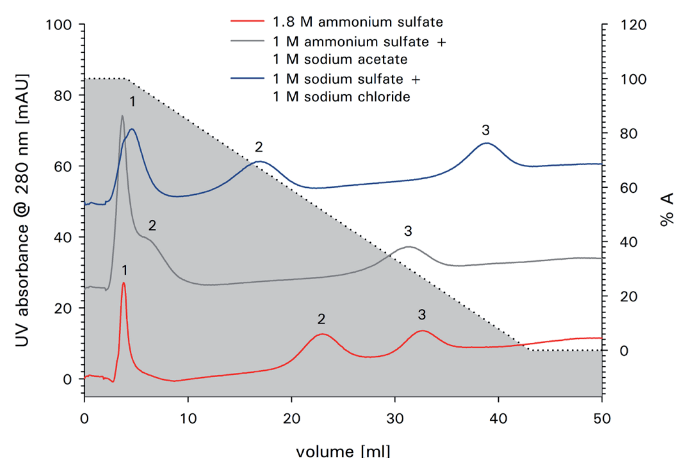


Figure 2

The three standard molecules cytochrome C (1), ribonuclease A (2) and lysozyme (3) are separated using TOYOPEARL Phenyl-600M. The retention of and lysozyme for the mixtures is at least as strong as for ammonium sulfate. For ribonuclease A and the two mixtures, a significant decrease in retention, compared to ammonium sulfate, can be observed.

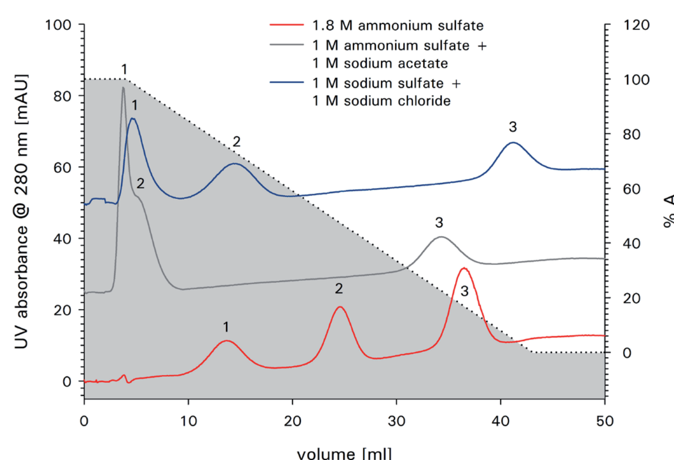


Figure 3

Separation of cytochrome C (1), ribonuclease A (2) and lysozyme (3) on TOYOPEARL Butyl-600M. Ammonium sulfate is compared to mixtures of ammonium sulfate + sodium acetate and sodium sulfate + sodium chloride. Resolution of ribonuclease A and lysozyme is increased for the mixtures. Binding of cytochrome C cannot be achieved for the mixtures, although in total, higher molarities are applied.

Application Note

PURIFICATION OF OLIGONUCLEOTIDES ON TOYOPEARL GigaCap® Q-650S

TOYOPEARL GigaCap Q-650S, a high capacity/high resolution anion exchange resin for process scale applications, was recently introduced by Tosoh Corporation. This resin, with dynamic binding capacities approaching 190 g/L for bovine serum albumin (BSA), is the newest member of the TOYOPEARL® product line.

TOYOPEARL GigaCap Q-650S maintains the high capacity of our popular TOYOPEARL GigaCap Q-650M and the 35 µm particle size provides high resolution for improved separation of process impurities and aggregates.

INTRODUCTION

The purification of oligonucleotides using anion exchange chromatography has traditionally fallen to resins such as TSKgel® SuperQ-5PW (20) that offer high resolution and selectivity in conjunction with excellent mechanical stability at very high column pressures. TOYOPEARL GigaCap Q-650S resin offers a low pressure alternative to oligonucleotide purification while preserving the selectivity, resolution and yields of those higher pressure processes.

TOYOPEARL and TSKgel products are hydroxylated methacrylic polymer resins and are made commercially in many different pore sizes and particle diameters. TOYOPEARL resins vary from TSKgel resins by having a lower degree of crosslinking. Lower crosslinking makes available a larger number of resin sites for ligand immobilization when producing TOYOPEARL resins. This lower degree of crosslinking also makes for a less rigid bead. Therefore a functionalized TOYOPEARL resin will have a lower pressure rating than the corresponding TSKgel material.

Because similarly functionalized TSKgel and TOYOPEARL resin types have the same backbone polymer chemistry, the selectivity for proteins, oligonucleotides and their attendant impurities remains the same. TOYOPEARL resin products can be used at high linear velocities and withstand operating pressures up to 0.3 MPa while TSKgel resins can withstand operating pressures of up to 2.0 MPa.

Table 1 shows the comparative properties of TOYOPEARL GigaCap Q-650S and TSKgel SuperQ-5PW (20) resins and dynamic binding capacities for the oligonucleotide used in these experiments. The following experiments detail the purification of an oligonucleotide using TOYOPEARL GigaCap Q-650S and TSKgel SuperQ-5PW (20) resins.

Oligonucleotides are short, linear sequences of deoxyribonucleic acid or ribonucleic acid that are generally manufactured by chemical synthesis. Because of the unique structure of these molecules and the way they are synthesized, oligonucleotides require special considerations during chromatographic purification.

During the synthesis of the oligonucleotide, there are a small percentage of sequences where a segment may either be deleted or have more than one segment attached (N-1 and N+1 respectively are the common nomenclature).

	TSKgel SuperQ-5PW (20)	TOYOPEARL GigaCap Q-650S
Particle size (µm)	20	35
Pore Diameter (nm)	100	100
Ion exchange capacity (eq/L resin)	0.14	0.17
DBC oligo (g/L resin)	46.4	36.8
Max pressure	2.0 MPa	0.3 MPa

Table 1

Properties of TSKgel SuperQ-5PW and TOYOPEARL GigaCap Q-650S

TSKgel SuperQ-5PW (20), 1.0 mg LOAD

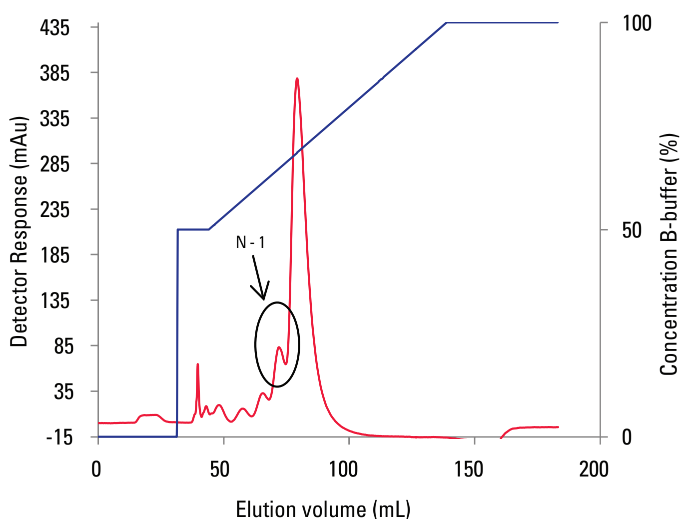


Figure 1

Resin: TSKgel SuperQ-5PW (20); Column size: 6.6 mm ID × 18.5 cm (6.3 mL); Mobile phase: A: 20 mmol/L NaOH; B: 20 mmol/L NaOH, 3.0 mol/L NaCl; Gradient: 50% B (2 CV); 50-100% B (15 CV); 100% B (2 CV); Flow rate: 200 cm/hr (1.14 mL/min); Detection: UV @ 254 nm; Sample load: 1.0 mg; Sample: crude phosphorothioate deoxyoligonucleotide

TOYOPEARL GigaCap-Q-650S, 1.0 mg LOAD

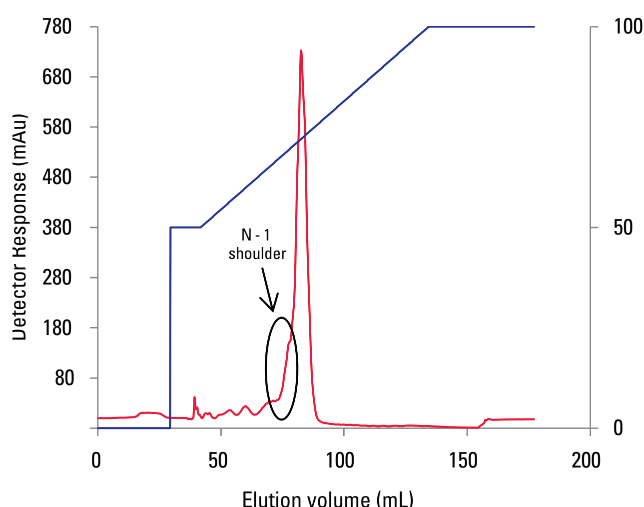


Figure 2

Resin: TOYOPEARL GigaCap Q-650S; Column size: 6.6 mm ID × 18.5 cm (6.3 mL); Mobile phase: A: 20 mmol/L NaOH; B: 20 mmol/L NaOH, 3.0 mol/L NaCl; Gradient: 50% B (2 CV); 50-100% B (15 CV); 100% B (2 CV); Flow rate: 200 cm/hr (1.14 mL/min); Detection: UV @ 254 nm; Sample load: 1.0 mg; Sample:crude phosphorothioate deoxyoligonucleotide

PURIFICATION OF OLIGONUCLEOTIDE AT 80% DBC ON TSKgel SuperQ-5PW (20) RESIN

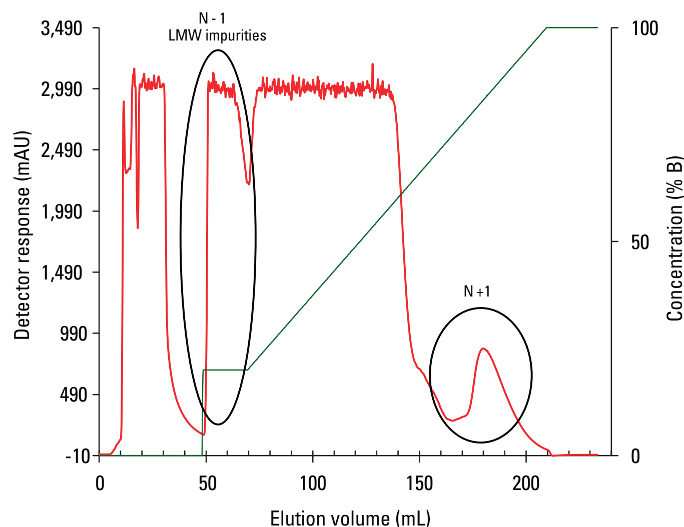


Figure 3

Resin: TSKgel SuperQ-5PW (20); Column size: 6.6 mm ID × 18.5 cm (6.3 mL); Mobile phase: A: 20 mmol/L NaOH; B: 20 mmol/L NaOH, 3.0 mol/L NaCl; Gradient: 20% B (2 CV), 20-100% B (20 CV); 100% B (2 CV); Flow rate: 200 cm/hr (1.14 mL/min); Detection: UV @ 254 nm; Sample load: 235 mg; Sample: crude phosphorothioate deoxyoligonucleotide

Taken collectively, these synthesis errors may produce measurable amounts of impurities. The similarity in the impurities to the target molecule requires a high resolution technique to adequately isolate the target molecule.

EXPERIMENTAL CONDITIONS / RESULTS

The data presented here demonstrate the similar capabilities of TOYOPEARL GigaCap Q-650S and TSKgel SuperQ-5PW (20) resins to purify a phosphorothioate deoxyribonucleotide (24-mer).

Experiments were carried out on 6.6 mm ID × 18.0 ± 0.5 cm columns packed with TOYOPEARL GigaCap Q-650S and TSKgel SuperQ-5PW (20) resins. The columns were first under-loaded with a 1.0 mg sample of crude oligonucleotide to better visualize resin performance, Figures 1-2. As can be seen from these chromatograms, the N-1 peak was slightly better resolved with the TSKgel SuperQ-5PW (20) than with the TOYOPEARL GigaCap Q-650S, perhaps due to the smaller particle size of the TSKgel resin. HPLC analysis of fractions taken across the peaks (data not shown) revealed that both resins were able to adequately resolve the full length oligonucleotide.

After optimizing the elution gradient, the performance of the resins was then compared at 80% of each resin's respective dynamic binding capacity for this oligonucleotide, Figures 3-4. As can be seen in the chromatograms, there was a visible N+1 peak that was resolved from the largest oligonucleotide peak in addition to the N-1 peak. Many of the low molecular weight impurities are visually resolved as well.

PURIFICATION OF OLIGONUCLEOTIDE AT 80% DBC ON TOYOPEARL GigaCap Q-650S RESIN

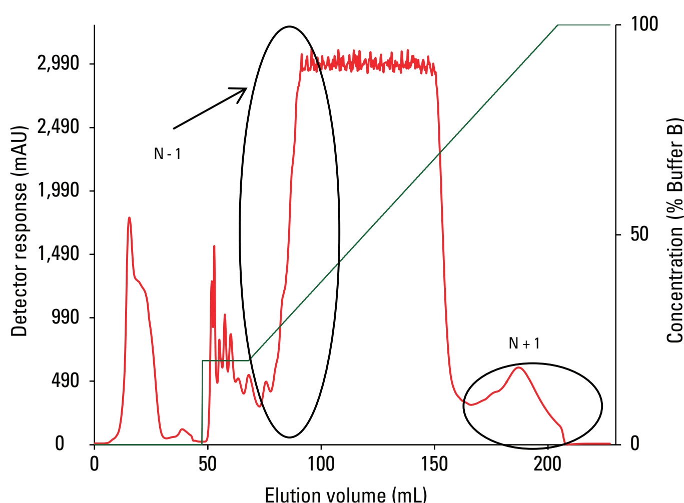


Figure 4

Resin: TOYOPEARL GigaCap Q-650S; Column size: 6.6 mm ID × 18 cm (6.16 mL); Mobile phase: A: 20 mmol/L NaOH; B: mobile phase A + 3.0 mol/L NaCl; Gradient: step to 20% B (2 CV); 20% - 100% B (20 CV); 100% B (2 CV); Flow rate: 200 cm/hr (1.14 mL/min); Detection: UV @ 254 nm; Injection vol.: 181.4 mg; Sample: crude phosphorothioate deoxyribonucleotide

Though the chromatograms in Figures 3 and 4 went off scale for UV, the general shape of the chromatograms is unchanged from that of the corresponding chromatogram when only 1.0 mg was loaded. HPLC analysis of fraction purity (data not shown) indicates that selectivity and resolution are maintained even at 80% DBC loading conditions.

TSKgel SuperQ-5PW (20) RESIN: 80% DBC ELUTION PEAK WITH FRACTION PURITY HISTOGRAM

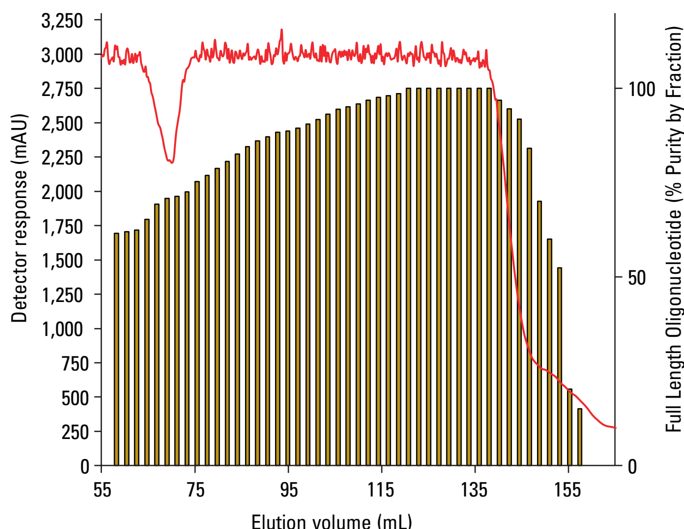


Figure 5

Resin: TOYOPEARL GigaCap Q-650S; Column size: 6.6 mm ID × 18.5 cm (6.3 mL); Mobile phase: A: 20 mmol/L NaOH; B: 20 mmol/L NaOH, 3.0 mol/L NaCl; Gradient: 50% B (2 CV); 50-100% B (15 CV); 100% B (2 CV); Flow rate: 200 cm/hr (1.14 mL/min); Detection: UV @ 254 nm; Sample load: 1.0 mg; Sample:crude phosphorothioate deoxyoligonucleotide

TOYOPEARL GigaCap Q-650S RESIN: 80% DBC ELUTION PEAK WITH FRACTION PURITY HISTOGRAM

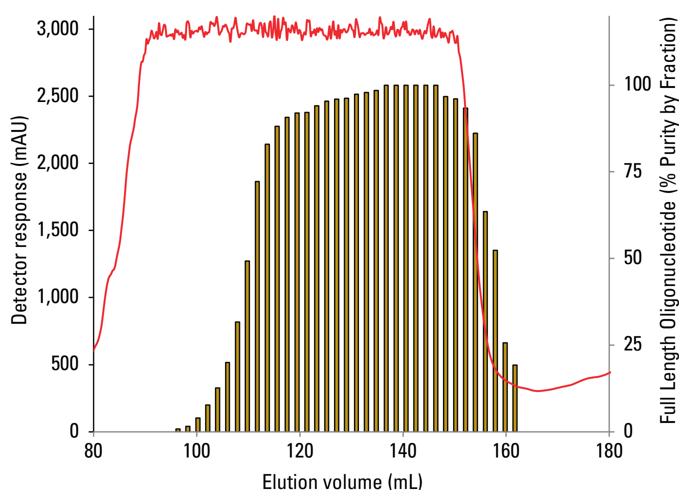


Figure 6

Resin: TOYOPEARL GigaCap Q-650S; Column size: 6.6 mm ID × 18.5 cm (6.3 mL); Mobile phase: A: 20 mmol/L NaOH; B: 20 mmol/L NaOH, 3.0 mol/L NaCl; Gradient: 50% B (2 CV); 50-100% B (15 CV); 100% B (2 CV); Flow rate: 200 cm/hr (1.14 mL/min); Detection: UV @ 254 nm; Sample load: 1.0 mg; Sample: crude phosphorothioate deoxyoligonucleotide

Resin	Crude Oligo Purity	Final Oligo Purity	% Yield
TSKgel SuperQ-5PW (20)	66.5%	96.4%	72.5%
TOYOPEARL Giga-Cap Q-650S	66.5%	96.9%	81.3%

Table 1

Oligonucleotide purity and yield from 80% DBC purifications

An enlarged image of the main oligonucleotide peak, overlaid with a histogram showing HPLC results for fraction purity, highlights the chromatographic separation of the full length oligonucleotide, Figures 5-6. At 80% DBC, the TSKgel SuperQ-5PW (20) resin had some breakthrough of the full length product from the main peak into the N-1 peak while the TOYOPEARL Q-650S did not. This indicates that the TOYOPEARL Q-650S was better able to maintain resolution at 80% DBC loading conditions.

After pooling fractions of purified oligonucleotide, the yield and purity of the final product was determined for each resin, Table 2. The TSKgel SuperQ-5PW (20) and TOYOPEARL GigaCap Q-650S generated very high purity full length oligonucleotide (96.4% and 96.9% respectively) from crude synthesis material. The yield of full length oligonucleotide was almost 9% greater on the TOYOPEARL GigaCap Q-650S than the yield from the TSKgel SuperQ-5PW (20).

Product yield is affected by the amount of crude material loaded onto the column. In general, as column loading approaches saturating conditions, yield will decrease. This phenomenon appears to be more pronounced with the TSKgel SuperQ-5PW (20) resin than with the TOYOPEARL GigaCap Q-650S resin.

Recovery was determined by comparing the amount of full length oligonucleotide present in the crude sample loaded onto the column with the amount of full length oligonucleotide present in the fraction pool.

CONCLUSION

TOYOPEARL GigaCap Q-650S is capable of delivering oligonucleotides of comparable purity to that seen with the TSKgel SuperQ-5PW (20) resin and at slightly higher process yields under the same loading conditions but at lower pressures. This capability allows chromatographers to purify oligonucleotides without the added expense of purchasing high pressure manufacturing equipment.

Fast Semi-Preparative Analysis and Scaling-up with TSK-GEL PW Resins

TSK-GEL PW Series

Introduction

Biopharmaceutical researchers are often challenged with the need to rapidly develop preparative quantities of a product for pre-clinical tests or phase I trials. The direct scale-up of an analytical method is often the most expedient answer to this challenge. As an easy solution Tosoh Bioscience offers 20 µm and 30 µm ion exchange bulk media (TSK-GEL DEAE-5PW, SuperQ-5PW and SP-5PW) and hydrophobic interaction bulk media (TSK-GEL Ether-5PW and Phenyl-5PW) with the same characteristics as the analytical columns.

Characteristics

- Highly crosslinked polymethacrylate
- Particle size 20 µm and 30 µm
- Mechanically stable (up to 20 bar)
- Chemically stable (pH 2-12)
- Very suitable for scaling-up
- High capacity
- Fast semi-preparative analysis
- Identical selectivity as with analytical columns

Scaling-up

TSK-GEL 5PW preparative liquid chromatography resins are made from the same polymer (G5000PW), as in the pre-packed TSK-GEL 5PW analytical columns, and provide identical selectivity, (illustrated in Figure 1). Therefore, methods can easily be transformed from analytical to preparative scale chemistry.

Analytical to preparative scale purification of superoxide dismutase with TSK-GEL DEAE-5PW columns.

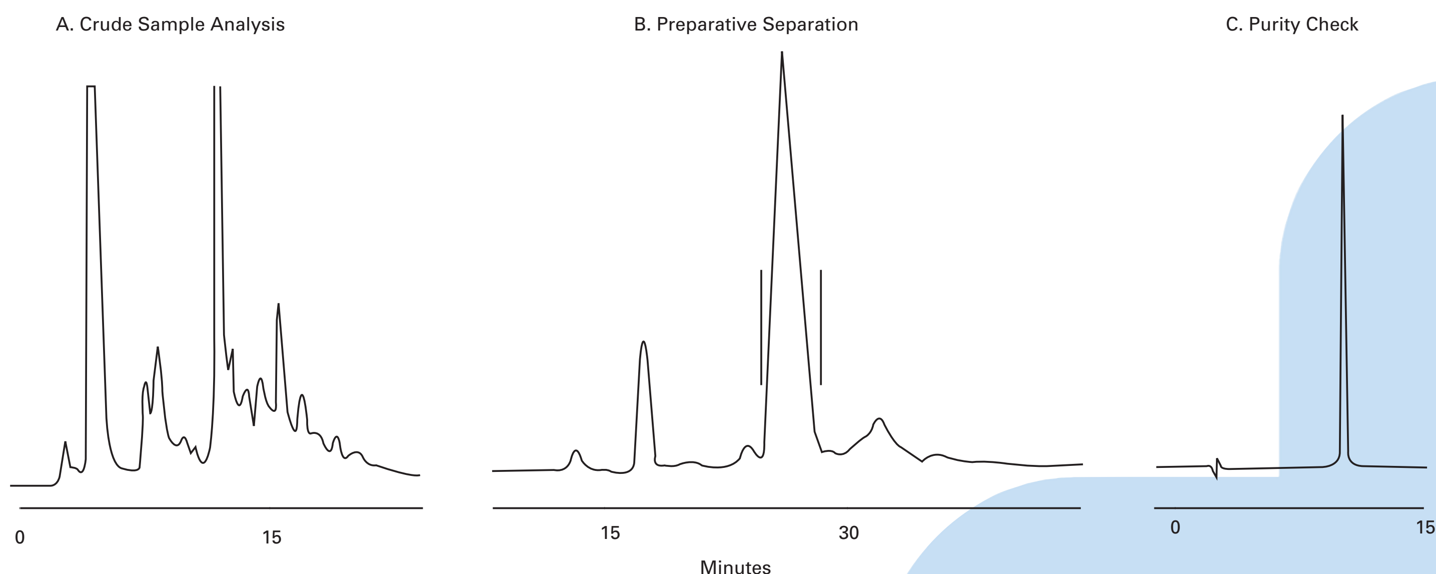


Figure 1

Column: TSKgel DEAE-5PW, A. 7.5 mm ID x 7.5 cm L, 10 µm; B. 55 mm ID x 20 cm L, 20 µm; C. 7.5 mm ID x 7.5 cm L, 10 µm
Sample: Commercially available superoxide dismutase: A. 1.0 mg, B. 300 mg, C. fraction analysis of B.
Elution: A. 120 min, B. 180 min, C. 30 min, linear gradient from 0.1 M to 0.3 M NaCl in 0.02 M Tris-HCl, pH 7.5
Flow rate: A. 1.0 ml/min., B. 30 ml/min., C. 1.0 ml/min.
Detection: UV @ 280 nm

Process scale

For scale up to process scale, Tosoh Bioscience offers the TOYOPEARL® line of resins in 35, 65 and 100 µm particle sizes. Toyopearl has the same backbone chemistry as TSK-GEL-5PW resins, so selectivity is maintained when scaling up from one resin to the other. This is demonstrated in Figure 2 showing an easy scale up to Toyopearl process resins for the separation of a mix of four standard proteins. Figure 3 demonstrates a separation on a DEAE resin. Only resolution decreases due to the larger particle size of the process resins. So researchers can be assured that chromatographic conditions developed on an anlytical or semi-preparative column will work for large productions as well.

TSK-GEL Phenyl-5PW resins for analytical to preparative separations

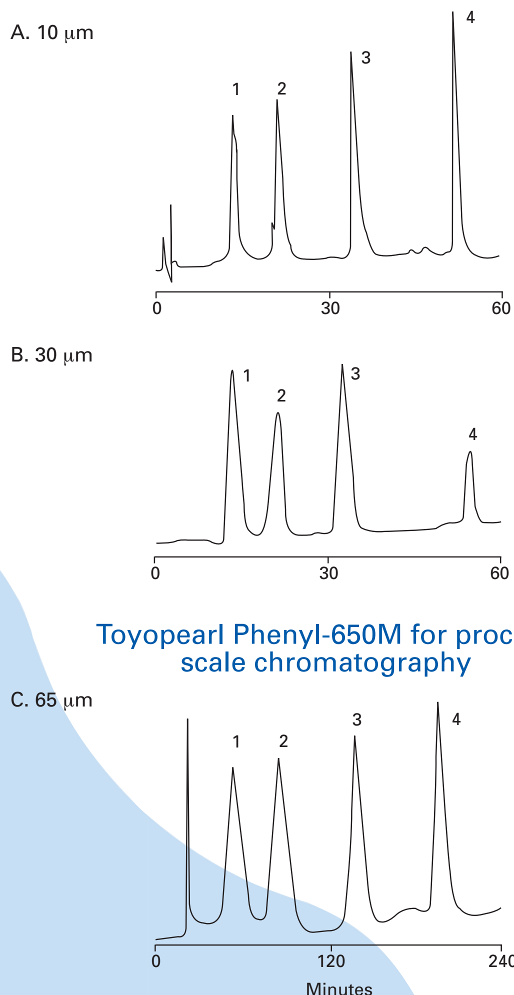


Figure 2

Column:	TSKgel Phenyl-5PW, 7.5 mm ID x 7.5 cm L
Sample:	1. myoglobin, 2. ribonuclease, 3. lysozyme, 4. alpha-chymotrypsinogen
Injection:	100 µl containing 1 mg/ml of each protein
Elution:	60 min. linear gradient for 10 and 30 µm TSK-GEL resins; 240 min. linear gradient for 65 µm Toyopearl resin from 1.8 M to 0 M $(NH_4)_2SO_4$ in 0.1 M phosphate buffer, pH 7.0
Flow rate:	0.5 ml/min for TSK-GEL resins (A&B), 2.0 ml/min for Toyopearl resin (C)
Detection:	UV @ 280 nm

Mechanical stability

TSK-GEL bulk resins are designed for preparative LC applications and have been used in columns up to 25 cm inernal diameter. The mechanical stability and excellent permeability of TSK-GEL bulk resins allow high linear flow rates (Figure 4).

Scale-up easily with TSK-GEL Columns

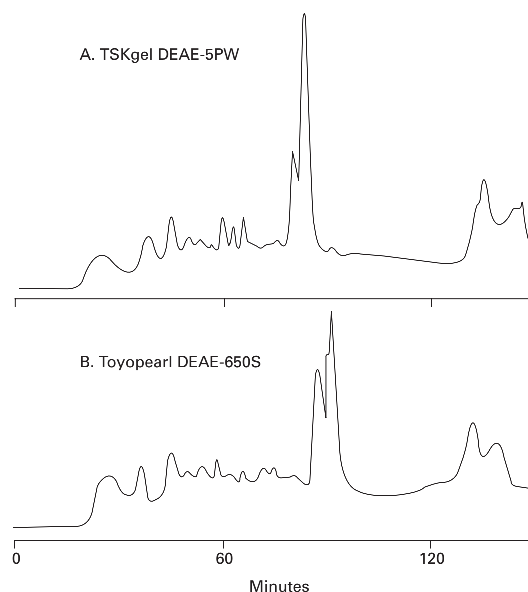


Figure 3

Column:	TSKgel DEAE-5PW, A. 7.5 mm ID x 7.5 cm L, 10 µm B. 55 mm ID x 20 cm L, 20 µm
Sample:	1. ovalbumin, 2. soybean trypsin inhibitor
Injection:	A. 0.2 mg each in 0.1 ml, B. 4.7 mg each in 4.7 ml
Elution:	A. 60 min, B. 120 min, linear gradient from 0 M to 0.5 M NaCl in 0.02 M Tris-HCl, pH 8.0
Flow rate:	A. 0.1 ml/min, B. 20 ml/min.
Detection:	UV @ 280 nm

Pressure/flow relationship for 30 µm TSK-GEL Phenyl-5PW

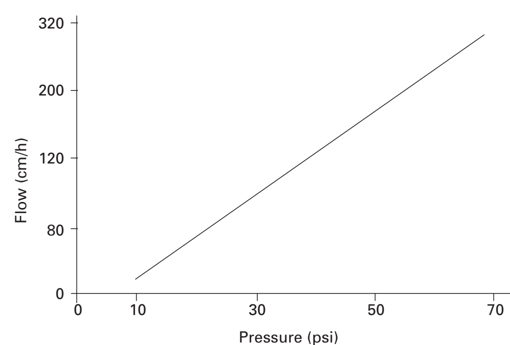


Figure 4

Column:	TSK-GEL Phenyl-5PW, 55 mm ID x 20 cm L, 20 µm
Eluent:	distilled water

Ordering Information

TSK-GEL anion exchange resins

Part #	Product description**	Container size (mL)	Particle size (µm)	Ion exchange capacity (meq/mL resin)	Adsorption capacity (mg/mL resin) (BSA)
43383	SuperQ-5PW (20)	25	15-25	0.12-0.18	52-88
18535		250			
18546		1,000			
18547		5,000			
43283	SuperQ-5PW (30)	25	20-40	0.12-0.18	52-88
18536		250			
18548		1,000			
18549		5,000			
43381	DEAE-5PW (20)	25	15-25	0.05-0.11	25-45
14710		250			
14711		1,000			
18436		5,000			
43281	DEAE-5PW (30)	25	20-40	0.05-0.11	20-40
14712		250			
14713		1,000			
18370		5,000			

TSK-GEL cation exchange resins

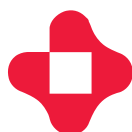
Part #	Product description**	Container size (mL)	Particle size (µm)	Ion exchange capacity (meq/mL resin)	Adsorption capacity (mg/mL resin) (lysozyme)
43382	SP-5PW (20)	25	15-25	0.06-0.12	20-40
14714		250			
14715		1,000			
18435		5,000			
43282	SP-5PW (30)	25	20-40	0.06-0.12	20-40
14716		250			
14717		1,000			
18384		5,000			

TSK-GEL 5PW HIC resins

Part #	Product description**	Container size (mL)	Particle size (µm)	Adsorption capacity (mg/mL resin) (lysozyme)
43276	Ether-5PW (20)	25	10-30	10-30
16052		250		
16053		1,000		
43176	Ether-5PW (30)	25	20-40	10-30
16050		250		
16051		1,000		
43277	Phenyl-5PW (20)	25	10-30	10-30
14718		250		
14719		1,000		
43177	Phenyl-5PW (30)	25	20-40	10-30
14720		250		
14721		1000		

TSK-GEL LABPAK

Part #	Product description	Container size (mL)	Particle size (µm)
43278	HICPAK PW (20) (Ether-5PW, Phenyl-5PW)	2 x 25 mL	10-30
43175	HICPAK PW (30) (Ether-5PW, Phenyl-5PW)	2 x 25 mL	20-40
43380	IEXPAK PW (20) (SP-5PW, DEAE-5PW, SuperQ-5PW)	3 x 25mL	15-25
43280	IEXPAK PW (30) (SP-5PW, DEAE-5PW, SuperQ-5PW)	3 x 25mL	20-40



TOSOH BIOSCIENCE GMBH

Use of Hydrophobic Interaction Chromatography With a Non-Salt Buffer System for Improving Process Economics in Purification of Monoclonal Antibodies

Originally presented at Waterside Conference on Monoclonal and Recombinant Antibodies
Miami, Florida USA, April 30–May 3, 2000
Pete Gagnon, Validated Biosystems Inc., San Miguel de Allende, Mexico
E-mail: <pete@validated.com>

Abstract

One of the barriers to more prevalent application of hydrophobic interaction chromatography (HIC) for purification of monoclonal antibodies is that the sample often elutes at salt concentrations that require buffer exchange before the next purification method can be performed. The selectivity provided by HIC is often so powerful that the technique is used despite these limitations, but residual salts constrain process design nevertheless. This report describes a non-salt-based (NSB) binding buffer system that mimics the selectivity of salt-based HIC buffers, but does so at conductivities directly compatible with ion exchange chromatography (IEC). This makes it possible to apply HIC fractions directly to IEC without intermediate buffer exchange, greatly extending the flexibility of HIC with respect to sequential placement in multi-method purification schemes. Results are shown illustrating practical application of the NSB system to multi-step purification of monoclonal antibodies.

Introduction

Hydrophobic interaction chromatography has been traditionally classified as a “high-salt” technique, however this classification is not explanatory in terms of the mechanism by which salts promote binding. Several investigators have noted that precipitating salts are excluded from protein surfaces. (1-3) This leaves a pure water hydration sheath surrounding the protein. The thermodynamic discontinuity between the pure water sheath and the high-salt bulk solution creates an exclusionary pressure so that when pure-water-hydrated proteins encounter other such proteins, their association is stabilized. Given the presence of a preferential binding substrate in the system, such as a hydrophobic stationary phase, proteins associate instead with the column.(3) The higher the salt concentration, the more strongly the proteins are excluded from the mobile phase, and the stronger their association with the stationary phase.

The amino acid glycine has been shown to be excluded from protein surfaces in a manner similar to precipitating salts.(1,2). Although not widely regarded as a protein precipitant, it has been used to efficiently precipitate fibrinogen.(4) Glycine has also been reported to enhance protein retention on HIC columns of weak-to-moderate hydrophobicity.(5) The degree of enhancement on these columns is not sufficient to promote retention independently, but more recent results show that glycine is able to support dynamic capacities greater than 25mg IgG /mL of gel on Phenyl columns.(6) Mole per mole, glycine is a far less effective binding promoter than the usual HIC salts such as ammonium sulfate, but it features electrostatic characteristics that make it much more compelling from a process perspective. Between the pKas of its carboxyl (2.35) and amino (9.76) groups, glycine is zwitterionic and contributes nothing to conductivity.(7) This allows HIC fractions to be applied directly to ion exchangers with little more than pH titration of the sample, thereby eliminating more costly intermediate sample equilibration steps such as buffer exchange chromatography or diafiltration.(6)

The only limitation of glycine in comparison to traditional HIC salts is its relative cost. In previous work, crude sample was equilibrated by addition of glycine to a concentration of 2.5M.(6) The column was equilibrated in the same buffer, and then washed with the same buffer after sample application. Still more glycine was consumed in the elution gradient. In the present study, the sample is equilibrated by direct addition of sodium chloride to a concentration of 2.0M. The column is equilibrated and washed with the 2.0 M sodium chloride buffer, then the system is switched to 2.5 M glycine, and the elution gradient is applied. This scheme conserves the advantages of low conductivity elution while substantially reducing scale-up process expense. To demonstrate the ability of NSB-HIC to improve purification process continuity, a three-step purification is illustrated, beginning with filtered ascites, first-step cation exchange, second-step NSB-HIC, and final-step anion exchange chromatography.

Results and Discussion

The results of this study demonstrate the ability of NSB-HIC to support effective multi-method IgG purification procedures, without requirement for intermediate sample preparation steps such as buffer exchange chromatography or diafiltration. Since compatibility of HIC fractions with downstream ion exchange steps was a primary objective of this study, the inclusion of 0.05M sodium chloride in the eluting buffer requires explanation. Simply put, IgG solubility is depressed in the complete absence of salts, and it elutes in a smear rather than a sharp peak. Chromatograms for the three purification steps are illustrated in **Figures 1–3**. A reduced SDS PAGE gel of selected fractions is illustrated in **Figure 4**.

The implications for purification process economics are substantial. **Table 1** illustrates some of the cost factors associated with intermediate processing steps. In addition to placing an unnecessary burden on process development and validation, these factors inflate the production cost of every lot of purified antibody that is subsequently manufactured. Even to the extent that glycine is more expensive than traditional HIC salts, the compensatory benefits of eliminating intermediate steps are overwhelming.

Table 1. Cost Factors Associated With Intermediate Sample Equilibration by Buffer Exchange Chromatography, Ultrafiltration and Diafiltration

- Direct expense of hardware
- Direct expense of media
- Process development expenses (method specifications, cleaning, cycle limits, storage)
- Validation expenses (method specifications, cleaning, cycle limits, storage)
- Documentation expense (SOPs, process records)
- Product losses (direct adsorption, internal hold-up volumes, other inefficiencies)
- Frequent formation of aggregates (diafiltration)
- Additional process time (reduces facility throughput/capacity)
- Increased process variability coincident with increased process complexity
- Additional opportunities for process/operator errors
- Equipment and media storage during non-use periods

The combination of NSB-HIC with ion exchange chromatography supports other immediate and long-term economic benefits as well. Protein A and protein G affinity chromatography are widely used for antibody purification despite their grossly disproportionate expense. This is largely due to the absence of more economical generic alternatives. This and other studies have shown that two-step NSB-HIC/ion exchange purifications can consistently produce antibody at a purity level sufficient for diagnostic applications or for animal studies.(6,8) This study and ongoing work indicate that three-step NSB-HIC/ion exchange purifications can likewise fulfill purification needs for later clinical studies and commercial manufacturing.

NSB-HIC/ion exchange puri-fication methods promise far greater benefits over affinity methods in other ways. The antibody industry is becoming increasingly aware that long overlooked features of affinity purification seriously com-plicate both process develop-ment and validation, and even threaten the long-term commercial success of affinity purified products. **Table 2** summarizes some of the nearly 200 publications that describe immunotoxic effects of both protein A and protein G.(9) The better known denaturative effects of these methods are also serious concerns. These factors risk both product efficacy and patient health. Combined with the direct cost burden of affinity purification, they highlight the need for the industry to evolve toward purification methods that avoid these critical liabilities.

Table 2. Immunological Side Effects of Protein A and Protein G*

Complement Activation Accelerated IgG catabolism Agglutination of granulocytes Leukocyte chemotaxis	Proliferation of B lymphocytes
	Proliferation of T lymphocytes
Histamine release from Leukocytes Induction of hypersensitivity Anaphylaxis	Altered ion transport across lymphocyte membranes
Migration of IgG receptors on cell surfaces Macrophage stimulation	Stimulation of DNA synthesis in activated B lymphocytes
Induction of rheumatoid factor Lymphokine secretion	Induction of immunoglobulin secretion by B lymphocytes
Potentiation of immune response	Proliferation of natural killer activity of human lymphocytes
Modulation of phagocyte function Platelet injury	Inhibition of aggregate binding by lymphocytes
**Refer to reference 9 for citations*	Inhibition of lymphocyte colony formation Inhibition of binding to Fc receptors

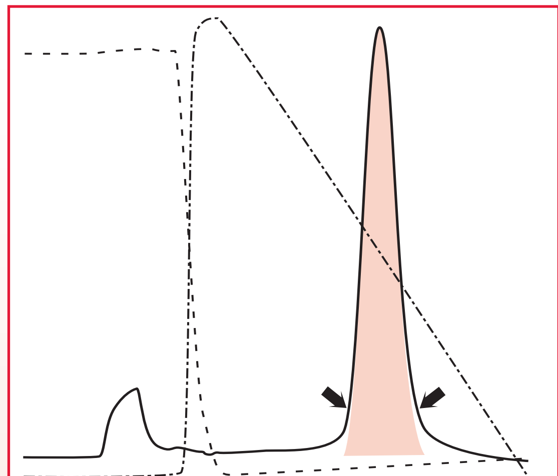


Figure 2. NSB-HIC of cation exchange pool on Phenyl-5PW-HR. Arrows indicate pool boundary. Shaded area indicates antibody distribution. Simple dashed line indicates conductivity. Double dashed line indicates glycine gradient. 1.5 AUFS. See methods section for conditions.

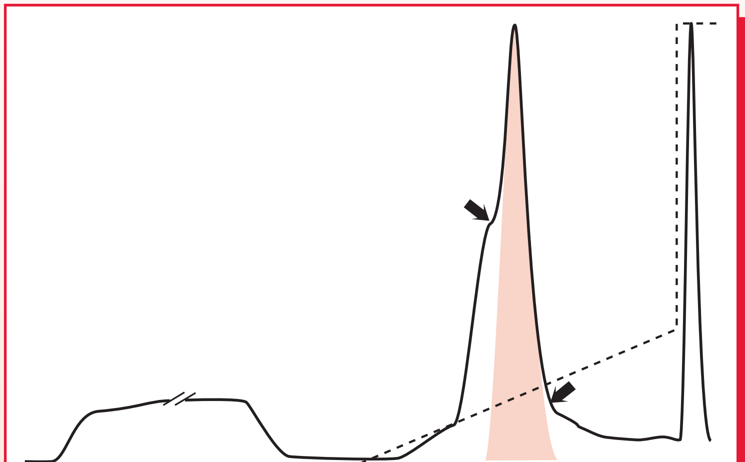


Figure 1. Cation exchange of ascites on SP-5PW-HR. Arrows indicate pool boundaries. Shaded area indicates antibody distribution. Dashed line indicates salt gradient. 3.0 AUFS. See methods section for conditions.

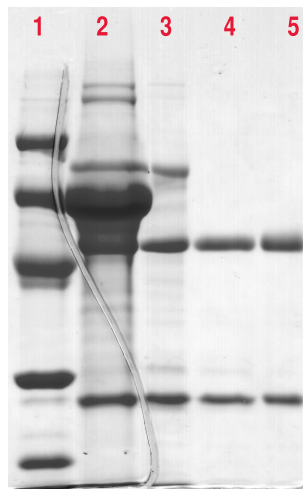


Figure 4. Reduced SDS-PAGE of purification steps.

1. Molecular weight stds.
2. Ascites
3. Cation exchange pool
4. NSB-HIC pool
5. Anion exchange pool

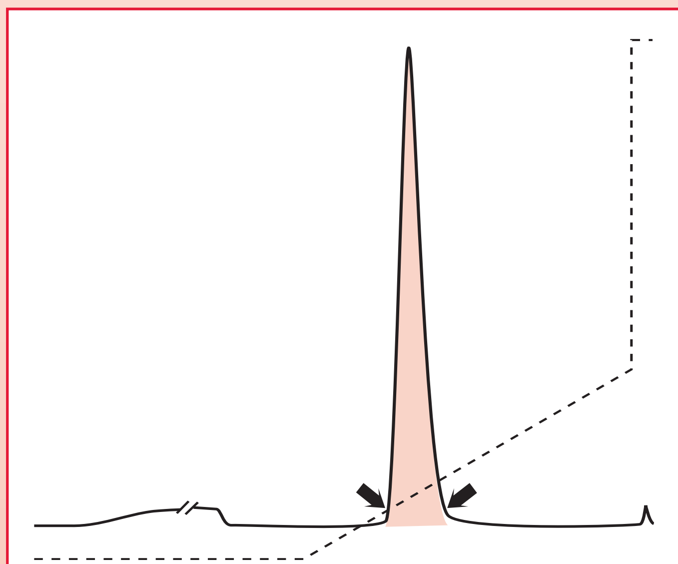


Figure 3. Anion exchange of NSB-HIC fraction on DEAE-5PW-HR. Arrows indicate pool boundaries. Shaded area indicates antibody distribution. Dashed line indicates salt gradient. 2.0 AUFS. See methods section for conditions.

Acknowledgements

The author would like to thank Anna Pujol at Becton Dickinson for her assistance with analyzing column fractions, and Tosoh Bioscience for providing materials and support to conduct this study.

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Materials and Methods

Prepacked TSK MD-G Phenyl-5PW-HR, SP-5PW-HR, and DEAE-5PW-HR columns (68mm x 10mm, 5.3mL) were obtained from Tosoh Bioscience (Montgomeryville, PA). Salts and buffers were obtained from Sigma Chemical Company (St. Louis, MO). Murine IgG1 ascites were obtained from Becton Dickinson BioSciences (San Jose, CA).

Buffers were prepared with reverse osmosis/deionized water and vacuum filtered to 0.22µm before use. For the cation exchange step, buffer A was 0.05M MES, pH 5.5. Buffer B was 0.05M MES, 1.0 M sodium chloride, pH 5.5. For NSB-HIC, buffer A was 0.05M sodium phosphate, 2.0M sodium chloride, 20mM EDTA, pH 7.5. Buffer B was 0.05M Tris , 2.5M glycine, pH 7.5. Buffer C was 0.05M Tris, 0.05M sodium chloride, 10% ethylene glycol, pH 7.5. For the anion exchange step, buffer A was 0.05M Tris, pH 8.5. Buffer B was 0.05M Tris, 1.0M sodium chloride, pH 8.5.

Chromatography conditions, cation exchange step: equilibrate column with 10 column volumes (CV) buffer A at a linear flow rate of 400cm/hr. Filter 5mL ascites to 0.22 µm. Equilibrate sample pH by addition of 0.5 mL 1.0M MES, pH 5.5. Load sample by on-line dilution, 20% sample, 80% buffer A. Wash, 5CV buffer A. Elute, 10CV linear gradient to 25% buffer B. Strip, 2CV 100% buffer B. Conditions for NSB-HIC: equilibrate column, 5CV buffer A at a linear flow rate of 400 cm/hr. Equilibrate IgG pool from previous step by addition of sodium chloride to 2.0M. Load without dilution. Wash 5CV buffer A, then 2CV buffer B. Elute, 10CV linear gradient to buffer C. Conditions for anion exchange: equilibrate column with 10CV buffer A at a linear flow rate of 400 cm/hr. Equilibrate pH of IgG pool from previous step by addition of 1.0M Tris pH 8.5, 10% (v:v). Load sample by on-line dilution 50% sample, 50% buffer A. Wash, 5CV buffer A. Elute, 10CV linear gradient to 30% buffer B. Strip, 2CV 100% buffer B.

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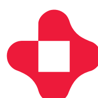

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