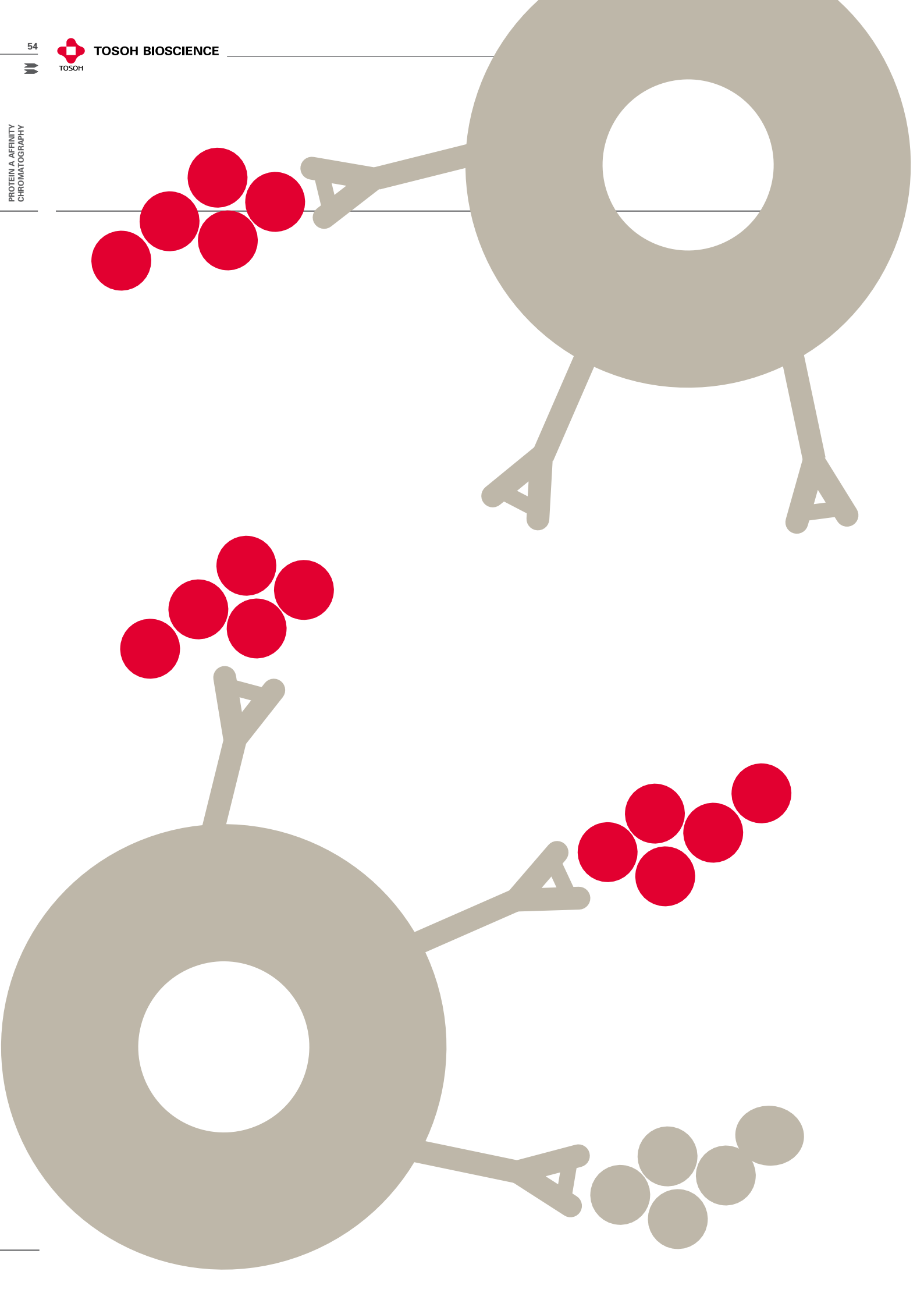


AFC
PROTEIN A AFFINITY CHROMATOGRAPHY

PROTEIN A AFFINITY PRODUCTS

- PROTEIN A AFFINITY
  TOYOPEARL AF-rProtein A HC-650F
  TOYOPEARL AF-rProtein A-650F

TOSOH FACT

TOYOPEARL AF-rProtein A HC-650F is an ultra-high capacity affinity resin designed for high throughput processing. It is the third generation of TOYOPEARL Protein A media. It is a rigid, alkaline resistant protein A affinity resin that offers the largest binding capacity for IgG of all base stable protein A media currently available on the market.

PROTEIN A AFFINITY CHROMATOGRAPHY

TOYOPEARL RESINS FOR PROTEIN A AFFINITY CHROMATOGRAPHY

Protein A affinity chromatography is the most commonly used capture step in antibody purification processes. Its high specificity for the binding of human immunoglobulin allows highly selective capturing of the target protein out of cell culture supernatant. The protein A capture step is most often followed by ion exchange, HIC or mixed-mode polishing steps in order to remove nucleic acids, aggregates and leached protein A.

The first protein A affinity resins were introduced in the 1970s based on native protein A ligands derived from the bacterium *Staphylococcus aureus*. These media suffered from insufficient alkaline stability, which limited the cleaning in place options for process use. State-of-the-art protein A resins carry recombinant protein A variants genetically engineered to provide maximum IgG affinity and base stability.

Tosoh Bioscience offers two protein A affinity resins, both based on alkaline stable, recombinant ligands coupled to the proven TOYOPEARL polymethacrylate matrix. The new ultra-high capacity TOYOPEARL AF-rProtein A HC-650M excels all other commercially available protein A media with regard to its IgG binding capacity.

PROTEIN A CHROMATOGRAPHY – HOW DOES IT WORK

Protein A is a 40-60 kDa surface protein originally found in the cell wall of the bacteria *Staphylococcus aureus*. Protein A and its recombinant derivatives bind the Fc region of immunoglobulins through interaction with the heavy chain. The binding strength of protein A for IgG depends on the source species of the immunoglobulin as well as the subclass of IgG. The standard protocol for antibody purification by protein A chromatography involves loading of the feedstock at physiological pH and ionic strength, washing unbound substances of the column with loading buffer and elution of the bound immunoglobulins by lowering the pH. The change in pH alters the degree of ionization of charged groups on the ligand and the bound antibody thus reducing the affinity. The fractions can be collected into neutralization buffer to return to a neutral pH.

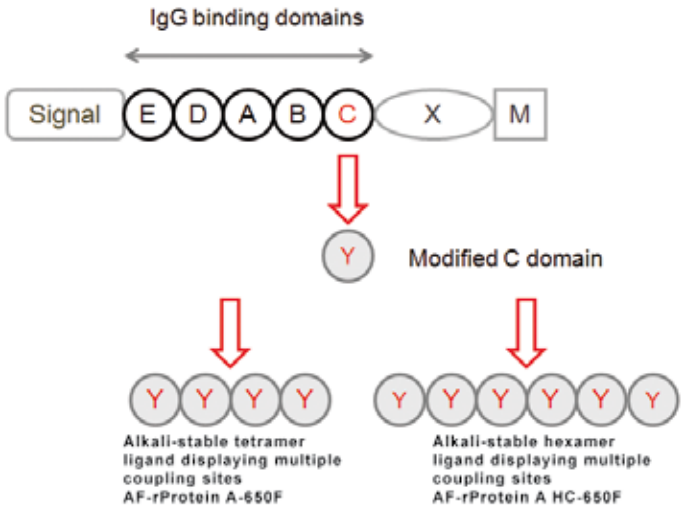
TOYOPEARL PROTEIN A RESINS

The ligands of all TOYOPEARL protein A resins are recombinant protein A variants expressed in *E. coli*. They are derived from one of the IgG binding domains of protein A. The amino acid sequence is optimized in order to increase the protein's stability towards alkaline solutions and to introduce additional lysine residues that can be utilized for multipoint attachment of the ligand to the TOYOPEARL matrix. The ligand of TOYOPEARL AF-rProtein A-650F consists of a tetramer of these modified protein A C domains. For the ultra-high capacity TOYOPEARL AF-rProtein A HC-650F this domain was further optimized and expressed as a hexamer in order to further increase IgG binding capacity (Figure 1).

Multipoint attachment of the ligand to the TOYOPEARL matrix enhances the chemical and thermal stability of the resin. In practice this pays off for a low level of protein A leaching and also for a high resistance to alkaline solutions. Both resins are based on the TOYOPEARL HW-65F base bead with a particle size of 45 µm.

FIGURE 1

RECOMBINANT PROTEIN A DERIVED LIGANDS



FEATURES

- High IgG binding capacity
- Recombinant protein A ligand
- TOYOPEARL polymer matrix

BENEFITS

- Increased productivity of antibody purification
- Lower buffer consumption
- Alkaline stable
- Low protein A leakage
- High mechanical stability
- High chemical stability

PROTEIN A AFFINITY CHROMATOGRAPHY

ULTRA-HIGH CAPACITY TOYOPEARL AF-rPROTEIN A HC-650F

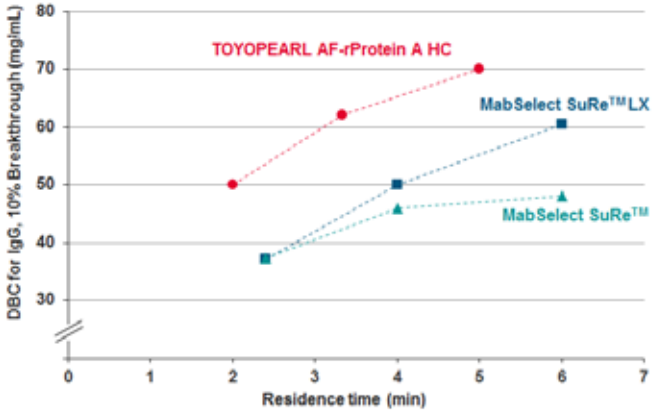
TOYOPEARL AF-rProtein A HC-650F is the newest affinity resin introduced by Tosoh Bioscience. It exhibits dynamic binding capacities of greater than 65 g/L at residence times of 5 minutes and greater than 50 g/L at 2 minutes residence time with feed stock concentrations from 1.0 g/L to 10.0 g/L (Figure 2).

Improved mass transfer characteristics allow it to maintain a larger percent of its capacity at lower residence times (Figure 3) relative to agarose based, caustic stable resins.

The multipoint attachment of the enhanced recombinant protein A ligand to the TOYOPEARL matrix is resulting in excellent base stability for up to 200 CIP cycles with 0.1 mol/L NaOH at 15 min contact time (Figure 4). It maintains 80% of initial dynamic binding capacity after 40 CIP cycles with 0.5 mol/L NaOH (Figure 5).

FIGURE 3

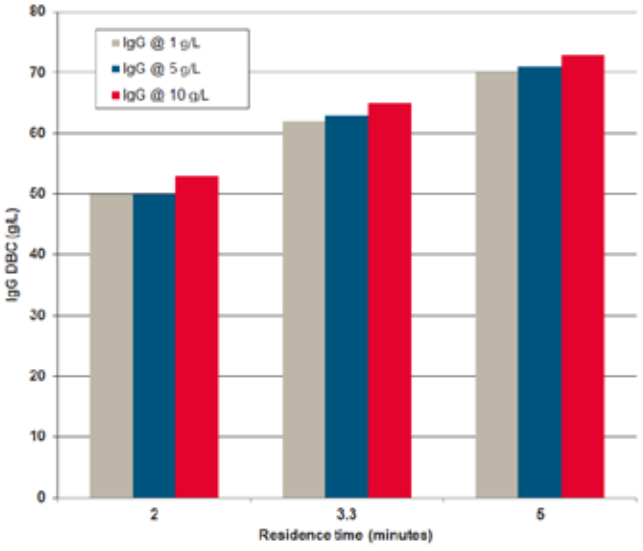
DBC OF HIGH CAPACITY PROTEIN A MEDIA



Column: TOYOPEARL AF-rProtein A HC-650F (5 mm ID x 5 cm L)
Mobile phase: 20 mmol/L sodium phosphate, 150 mmol/L NaCl pH 7.4;
Residence time: 2, 3.3, 5 min; Detection: UV @ 280 nm;
Sample: polyclonal human IgG @ 1 g/L in mobile phase;
DBC measured at 10 % breakthrough. MabSelect SuRe™ and MabSelect SuRe™ LX DBC data from GE brochure. MabSelect SuRe™ and MabSelect SuRe™ LX are registered trademarks of GE Healthcare Bio-Sciences AB, Uppsala, Sweden.

FIGURE 2

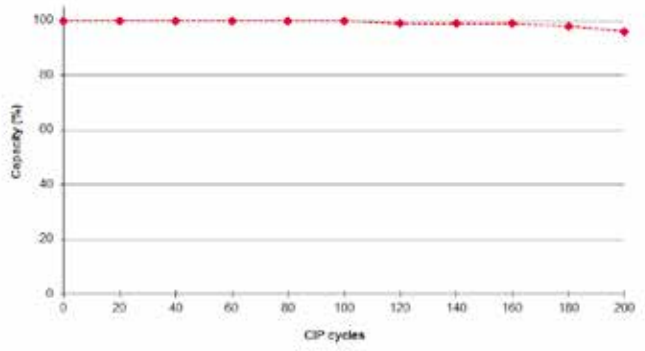
DBC AT VARIOUS LOADS AND RESIDENCE TIMES



Column: TOYOPEARL AF-rProtein A HC-650F (5 mm ID x 5 cm L)
Mobile phase: 20 mmol/L sodium phosphate, 150 mmol/L NaCl pH 7.4;
Residence time: 2, 3.3, 5 min; Detection: UV @ 280 nm
Sample: polyclonal human IgG @ 1, 5, 10 g/L in mobile phase
DBC measured at 10 % breakthrough

FIGURE 4

CIP STUDY WITH 0.1 M NaOH



Column size: 5 mm ID x 5 cm L; Wash procedure: A: 20 mmol/L Na_2HPO_4 0.15 mol/L NaCl, pH 7.4 (10 CV)
B: 0.1 mol/L citrate, pH 3.0 (5 CV)
C: 20 mmol/L Na_2HPO_4, 0.15 mol/L NaCl, pH 7.4 (7 CV)
D: 0.1 mol/L NaOH (3 CV – 15 min contact time)
E: 20 mmol/L Na_2HPO_4, 0.15 mol/L NaCl, pH 7.4 (5 CV)

PROTEIN A AFFINITY CHROMATOGRAPHY

The binding of the enhanced rProtein A ligand to the TOYOPEARL base bead via multipoint attachment is not only resulting in high alkaline stability but also the reason for low ligand leakage (Table 1).

Achievement of high linear velocities at relatively low pressure enables high throughput at production scale using equipment with moderate pressure limitations (Figure 6).

TABLE I

PROTEIN A LIGAND LEAKAGE

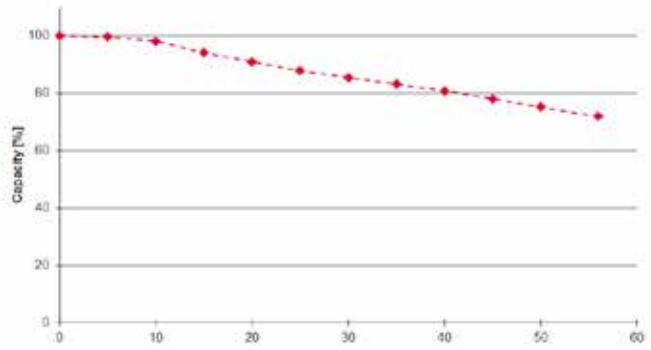
Amount of ligand leakage	Before CIP		After 200 CIP cycles	
	Elution Buffer		Elution Buffer	
	citrate (pH 3.0)	glycine-HCl (pH 3.0)	citrate (pH 3.0)	glycine-HCl (pH 3.0)
(ppm)	1.7	1.6	0.6	0.5

Amount of ligand leakage was determined with TOYOPEARL AF-rProtein A HC-650F ELISA

ppm=µg/g IgG

FIGURE 5

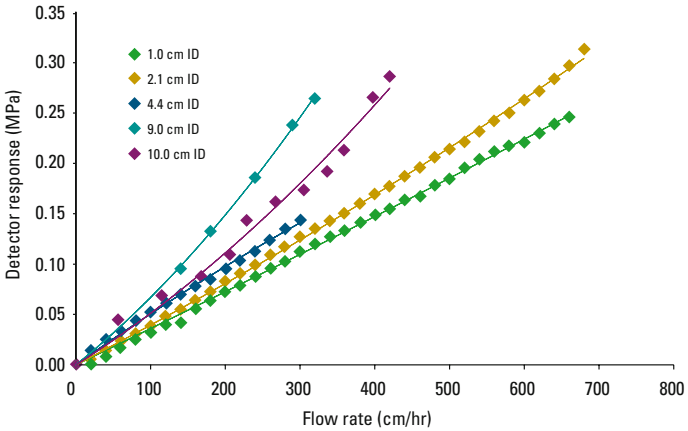
CIP STUDY WITH 0.5 M NaOH



Column size: 5 mm ID × 5 cm L; Wash procedure: A: 20 mmol/L Na_2HPO_4 0.15 mol/L NaCl, pH 7.4 (10 CV)
B: 0.1 mol/L citrate, pH 3.0 (5 CV)
C: 20 mmol/L Na_2HPO_4, 0.15 mol/L NaCl, pH 7.4 (7 CV)
D: 0.5 mol/L NaOH (3 CV – 15 min contact time)
E: 20 mmol/L Na_2HPO_4, 0.15 mol/L NaCl, pH 7.4 (5 CV)
Capacity: DBC was determined at 10 % breakthrough after every 5 cycles

FIGURE 6

PRESSURE/FLOW CURVE



Column size: 1.0 cm ID, 2.1 cm ID, 4.4 cm ID, 9.0 cm ID, 10.0 cm ID; 20 cm normalized bed height; Mobile phase: DI H_2O

PROTEIN A AFFINITY CHROMATOGRAPHY

TOYOPEARL AF-rPROTEIN A-650F

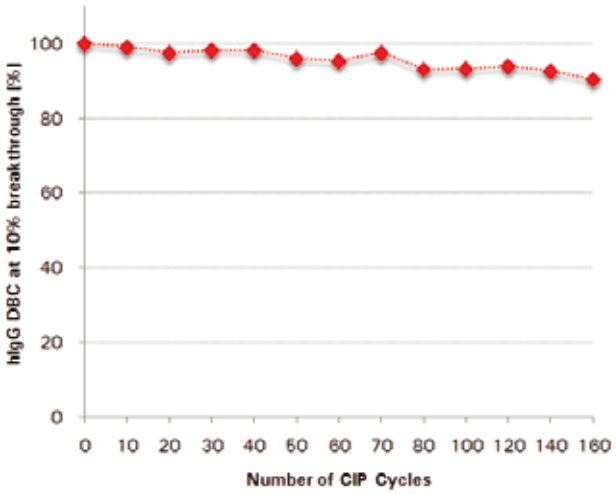
The standard TOYOPEARL AF-rProtein A-650F resin binds human and mouse immunoglobulin G, IgM, and Fab fragments. Typical static IgG binding capacity is > 45 mg/ml resin and typical dynamic IgG binding capacity at 10 % breakthrough is > 30 mg/mL resin at 2 minutes residence time (1 mg/mL protein load). Fast mass transfer kinetics support high binding capacities at high flow rates. IgG breakthrough curves (Figure 7) at various linear velocities demonstrate the superior kinetic performance of TOYOPEARL AF-rProtein A-650F.

The structure of the recombinant ligand and its multipoint attachment to the base matrix enhances the stability of TOYOPEARL AF-rProtein A-650F in 0.1 - 0.5 M NaOH. The dynamic binding capacity remains high after repeated CIP cycles. After more than 150 CIP cycles with 0.1 M NaOH at 16 min contact time per cycle more than 90 % of initial dynamic binding capacity was retained (Figure 8). When performing cleaning-in-place with 0.5 M NaOH the resin maintains about 80 % of IgG binding capacity after 50 cycles.

TOYOPEARL AF-rProtein A-650F is also stable in ethanol, 6 M urea, 6 M guanidinium chloride, and 1 % phosphoric acid, respectively. Static binding capacity of the resin is not impaired when heated for 30 minutes to temperatures of up to 90 °C. Figure 9 shows the thermal stability of the resin. It can be stored at room temperature at production site. Recommended conditions for long term storage are a storage solution of 20 % ethanol and temperature of 4 - 8 °C.

FIGURE 8

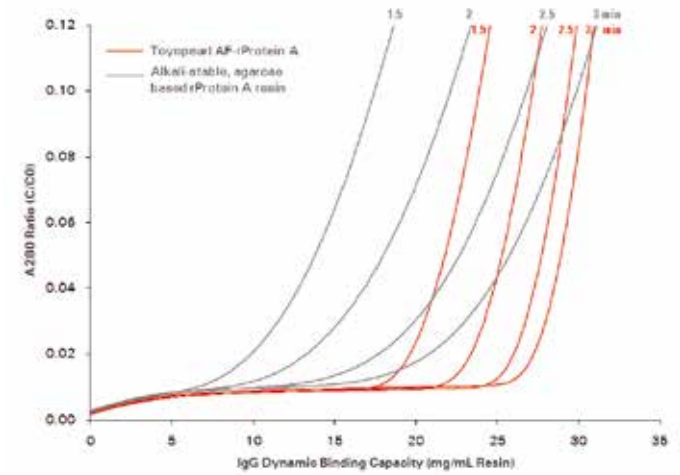
CLEANING-IN-PLACE STUDY WITH 0.1 M NaOH



Column: 5 mm ID x 5 cm L
10 column volumes binding buffer pH 7.4
5 column volumes elution buffer pH 3.0
3 column volumes binding buffer containing 0.1 mol/L NaOH, 16 min contact time
3 column volumes binding buffer pH 7.4

FIGURE 7

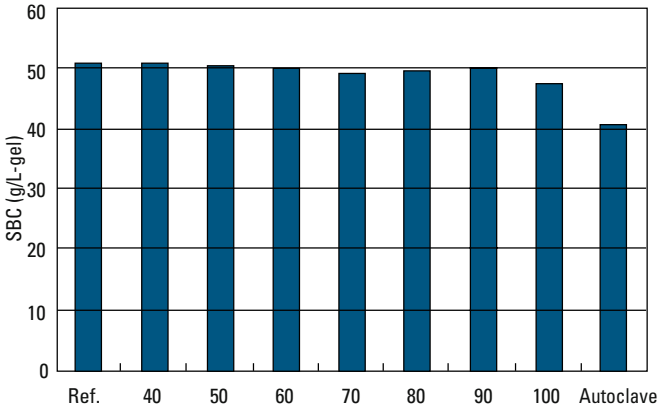
DYNAMIC BINDING CAPACITY



Breakthrough curves for h-IgG loading (polyclonal, 10 g/L) Typical DBC at 10 % breakthrough: 30,5 g/L @ 100 cm/h (3 min residence time) - 24 g/L @ 200 cm/h (1.5 min residence time); Column: 5 mm ID x 5 cm L; Mobile phase: 20 mmol/L sodium phosphate buffer pH 7.2 containing 150 mmol/L NaCl; Sample conc.: 10 g/L; Residence time: 1.5, 2.0, 2.5, 3.0 min

FIGURE 9

TEMPERATURE STABILITY



Resin: TOYOPEARL AF-rProtein A-650F; Mobile phase: deionized H_2O; Autoclave settings: 120 °C, 1.2 bar, 15 min; Heating time: 30 min;
TOYOPEARL AF-rProtein is stable at 35 °C for least 3 years (data not shown)

PROTEIN A AFFINITY CHROMATOGRAPHY

PURIFICATION OF MONOCLONAL ANTIBODIES

Typically antibodies are captured at near neutral pH and eluted using acidic conditions. The clarified feedstock is loaded onto the column at a neutral pH. After sufficient washing with the loading buffer, the antibody is eluted at low pH. However, the physicochemical properties of different mAbs are varying depending on the expression system and antibody subclass. Therefore a generic method needs to be optimized for each individual target in order to establish conditions that will bind the highest amount of the target molecule in the shortest time and elute it with the highest purity. For initial scouting of method parameters we recommend using pre-packed ToyoScreen columns or robotic high throughput screening devices with ToyoScreen RoboColumns.

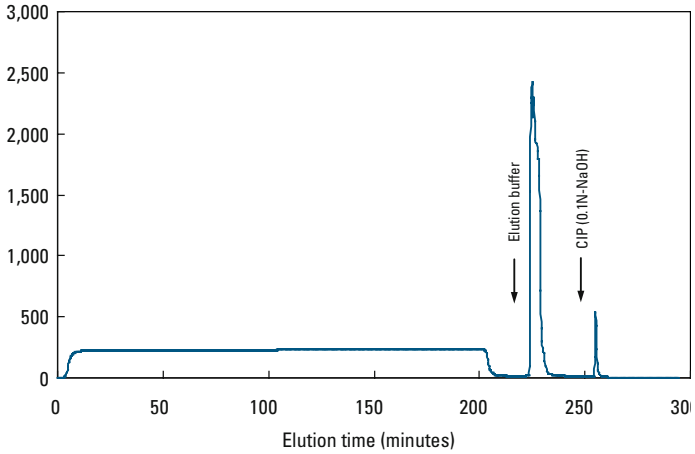
Suitable load/wash buffers are 20-100 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.2 - 7.5 or 100 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.2 - 7.5. Washing at reduced pH (e.g. pH 6) might further improve host cell protein reduction. Suitable elution buffers are 100 mmol/L citrate, 100 mmol/L acetate, or 100 mmol/L glycine-HCl. The pH shift required for mAb elution depends on the particular mAb and ranges from pH 3.0 to 4.5. For cleaning and sanitization the use of 0.1 to 0.5 molar NaOH is recommended. Depending on the origin and subclass of the antibody, contact time, concentration, and frequency of CIP cycles the conditions should be optimized.

TOYOPEARL AF-rProtein A HC-650F was used for the purification of a monoclonal antibody from CHO cell culture supernatant with a concentration of 1.0 g/L (Figure 10) at 5 minutes residence time in a 5 cm bed height column. As can be seen from the chromatogram, tailing is minimal on the elution peak and the eluted mAb is > 95% pure by SEC.

Figure 11 shows the binding capacities for the capturing of a therapeutic monoclonal IgG1 spiked at different concentrations into CHO cell culture fluid. The binding capacity of TOYOPEARL AF-rProtein A HC-650F for this specific antibody is increasing dramatically with increasing feed concentrations. Furthermore, when applying a feed concentration of 10 mg mAb/mL a capacity of more than 100 mg mAb/mL resin was even reached at 1 min. residence time.

FIGURE 10

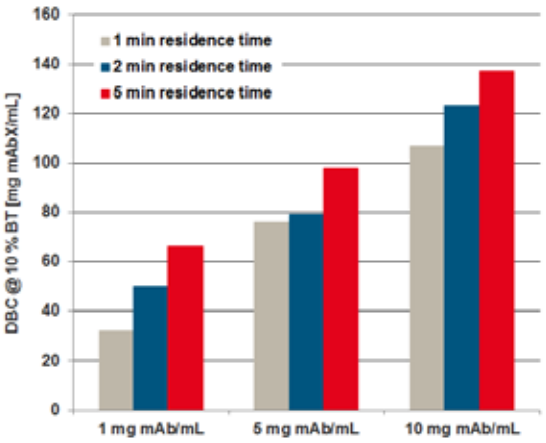
PURIFICATION OF MONOCLONAL ANTIBODY



Resin: TOYOPEARL Protein A; Column size: 5 mm ID × 5.0 cm L; Mobile phase: Buffer A: 20 mmol/L sodium phosphate containing 0.15 mol/L NaCl, pH 7.4, Buffer B: 0.1 mol/L citrate, pH 3.0; Flow rate: 61 cm/h (0.2 mL/min); Residence time: 5 min; Sample: 40 mL of CHO cell culture, containing 1.0 g/L humanized IgG_1

FIGURE 11

DBC FOR A SPECIFIC mAb AT VARIOUS LOADS AND VELOCITIES



Column: TOYOPEARL AF-rProtein A HC-650F (6.6 mm ID × 2 cm L)
Mobile phase: 100 mmol/L sodium phosphate pH 6.5;
Residence time: 1, 2, 5 min; Detection: UV @ 280 nm
Sample: monoclonal antibody mAbX @ 1, 5, 10 g/L in mobile phase
DBC measured at 10 % breakthrough

ToyoScreen PREPACKED COLUMNS FOR PROCESS DEVELOPMENT

ToyoScreen columns packed with the TOYOPEARL AF-rProtein A resins are available in 1 mL and 5 mL resin volumes. ToyoScreen columns provide a convenient way to perform early resin screening for both target retention and recovery. Multiple columns can be connected in series for additional capacity. ToyoScreen RoboColumns are miniaturized chromatographic columns for operation with a robotic liquid handling system, such as the Freedom EVO® from TECAN. This approach allows automated highthroughput, small-scale biochromatographic separations of protein samples by running up to eight individual columns simultaneously. ToyoScreen RoboColumns packed with TOYOPEARL Protein A resins are available with 200 µL and 600 µL resin volumes.

PROTEIN A AFFINITY CHROMATOGRAPHY

ORDERING INFORMATION

ToyoScreen PROCESS DEVELOPMENT COLUMNS FOR AFC

PART #	PRODUCT DESCRIPTION	PACKAGE
0023430	ToyoScreen AF-rProtein A HC-650F	1 mL x 5 each
0023431	ToyoScreen AF-rProtein A HC-650F	5 mL x 1 each
0023432	ToyoScreen AF-rProtein A HC-650F	5 mL x 5 each
0022809	ToyoScreen AF-rProtein A-650F	1 mL x 5 each
0022810	ToyoScreen AF-rProtein A-650F	5 mL x 1 each
0022811	ToyoScreen AF-rProtein A-650F	5 mL x 5 each
0045061	ToyoScreen RoboColumn AF-rProtein A-650F	200 µL x 8 (each)
0045062	ToyoScreen RoboColumn AF-rProtein A-650F	600 µL x 8 (each)
0045063	ToyoScreen RoboColumn AF-rProtein A HC-650F	200 µL x 8 (each)
0045064	ToyoScreen RoboColumn AF-rProtein A HC-650F	600 µL x 8 (each)

ToyoScreen COLUMN ACCESSORIES

PART #	PRODUCT DESCRIPTION
0021400	ToyoScreen Column Holder
0045099	RoboColumn Array Plate

TOYOPEARL AFFINITY CHROMATOGRAPHY RESIN

GROUP SPECIFIC RESINS

PART #	PRODUCT DESCRIPTION	CONTAINER SIZE (mL)	TYPICAL CAPACITY
0023425	TOYOPEARL AF-rProtein A HC-650F NEW	10	≥68 g/L (IgG)
0023426		25	
0023427		100	
0023428		1,000	
0023429		5,000	
0023434		50,000	
0022803	TOYOPEARL AF-rProtein A-650F	10	≥45 g/L (IgG)
0022804		25	
0022805		100	
0022806		1,000	
0022807		5,000	
0022808		50,000	

PROTEIN A IMMUNOASSAYS & STANDARDS

PART #	PRODUCT DESCRIPTION
0023433	Protein A-R40 ELISA Kit for TOYOPEARL AF-rProtein A HC-650F
0022815	Protein A-R28 ELISA Kit for TOYOPEARL AF-rProtein A-650F
0022836	Protein A-R28 STD 0.5 mL (10 mg/L) for TOYOPEARL AF-rProtein A-650F