# BIOPROCESSING APPLICATION NOTEBOOK

## PURIFICATION OF BIOMOLECULES

NO. #01 2019

# TOSOH BIOSCIENCE

# EDITORIAL
## DEAR READER

The purification and recovery of a biological target molecule out of a tissue, cell or fermentation broth - the so called downstream processing (DSP) - usually requires a combination of separation technologies. Bioprocess chromatography is the most important technique for this purpose.

Today, biopharmaceuticals are the fastest growing product segment of the pharmaceutical industry. An effective and affordable purification of therapeutic biomolecules is key for the successful development and commercialization of new drugs, no matter whether biologic, biosimilar, or biobetter. With new biopharmaceutical formats, such as bispecific mAbs, antibody fragments and antibody-drug-conjugates (ADCs) in the pipeline, rapid development of DSP steps will become even more important.

TOYOPEARL®, TSKgel®, and Ca++Pure-HA® process media are renowned for their quality, reliability, and productivity, and cover all modes of bioprocess chromatography, including affinity (AFC), ion exchange (IEX), mixed-mode (MXC), hydroxyapatite, hydrophobic interaction (HIC), and size exclusion (SEC). They are popular in the biotech and biopharmaceutical industry and are used in R&D, method development, and production from μL to 1000-L scales.

This application notebook compiles over twenty application notes covering important aspects of biopharmaceutical purification such as capturing of antibody constructs such as Fab, scFv, or mAB, mAb aggregate removal using salt-tolerant IEC or hydroxyapatite, intermediate purification of bi-specific antibodies with a non-affinity platform using MXC and HIC, antibody-drug conjugates (ADC) purification on HIC resin, or separation of oligonucleotides on highly-selective anion exchanger.

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BIOPROCESSING
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# 1. mAb FRAGMENT CAPTURING

# PURIFICATION OF Fab USING HIGH BINDING CAPACITY TOYOPEARL® AF-rProtein L-650F

## A High Capacity Protein L Resin for the Purification of Monoclonal Antibody Fragments
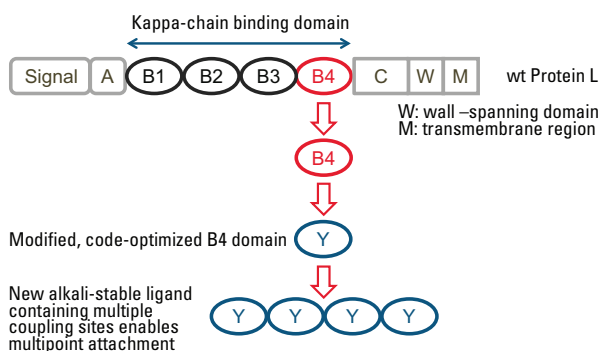
### INTRODUCTION

Protein L based affinity chromatography is used for the capture of antibodies and antibody fragments that do not bind to protein A. Unlike protein A and G, which bind to the Fc region of immunoglobulins (IgGs), protein L binds through interactions with the variable region of an antibody's kappa light chain. Figure 1 shows several possible binding sites of protein L to fragments (Fabs), single-chain variable fragments (scFvs) and domain antibodies (dAbs).



Fab (55 kDa)    scFv (25 kDa)    dAb (~15 kDa)

Antibody fragments
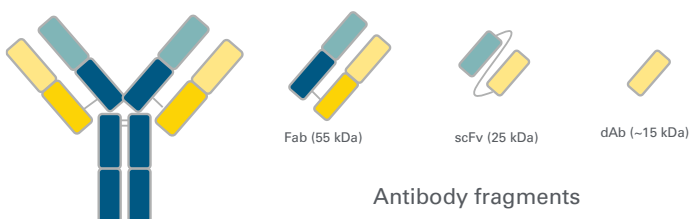
➤ Figure 1 ······································

TOYOPEARL AF-rProtein L-650F is an affinity chromatography resin that combines a rigid polymer matrix with a recombinant ligand, which is derived from the B4 domain of native protein L from Peptostreptococcus magnus and is expressed in E.coli (Figure 2). Code optimization of the domain results in high binding capacity and an improved alkaline stability of the ligand compared to the native molecule.

STRUCTURE OF TOYOPEARL AF-RPROTEIN L-650F



Kappa-chain binding domain

| Signal | A | B1 | B2 | B3 | B4 | C | W | M |    wt Protein L

W: wall –spanning domain
M: transmembrane region

B4

Modified, code-optimized B4 domain    Y

New alkali-stable ligand containing multiple coupling sites enables multipoint attachment    Y  Y  Y  Y

➤ Figure 2 ······································

The selected recombinant protein L ligand also has an affinity for a broad range antibody subclasses as demonstrated in Table 1. This application note demonstrates the ability of TOYOPEARL AF-rProtein L-650F resin to capture and purify Fabs with a high yield and high purity.

### TOYOPEARL AF-rProtein L-650F LIGAND WITH A BROAD AFFINITY RANGE FOR mAb SUBCLASSES

| Species | Class | Affinity |
|---|---|---|
| General | Kappa light chain | ++ |
| | Lambda light chain | - |
| | Heavy chain | - |
| | Fab | ++ |
| | ScFv | ++ |
| | Dab | ++ |
| Human | IgG(1-4) | + |
| | IgA | + |
| | IgD | + |
| | IgE | + |
| | IgM | + |
| Mouse | $IgG_1$ | + |
| | $IgG_2a$ | + |
| | $IgG_2b$ | + |
| | IgA | + |
| | IgM | + |
| Rat | $IgG_1$ | + |
| | IgG2a,b,c | + |
| | IgA | + |
| Hen | IgY | + |

➤ Table 1 ······································

### MATERIALS AND METHODS

**Digestion**
Humanized IgG1, 3 mg/mL, was digested using papain enzymatic protocol as described in the Pierce™ Fab Preparation Kit (catalog number: 0044985).

**Chromatography**
Purification of Fab: 100 µL of the papain digested IgG1 was loaded onto a TOYOPEARL AF-rProtein L-650F column, 5 mm ID × 2.5 cm (0.5 mL total volume). See chromatogram for operating conditions.

Size analysis of fractions: the collected peaks, 10 µL each, were injected onto a TSKgel UP-SW3000, 4.6 mm ID × 15 cm size exclusion chromatography (SEC) column. See chromatogram for operating conditions.
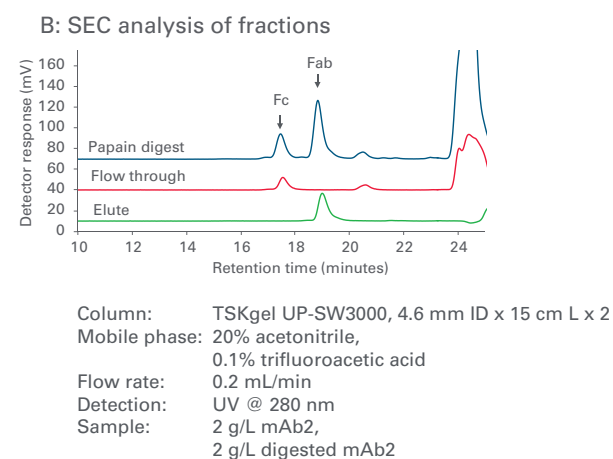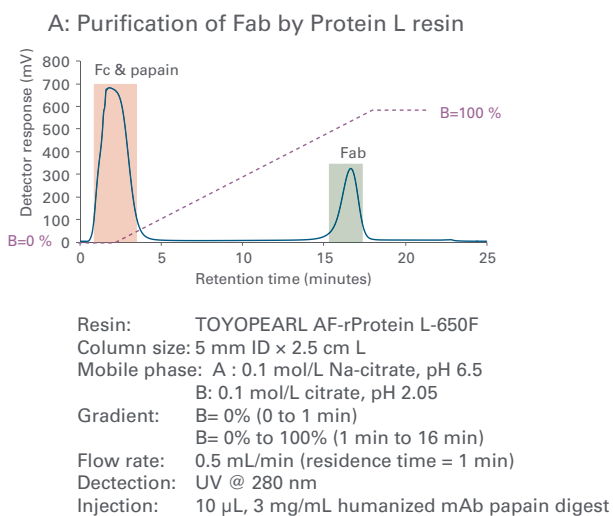
## RESULTS AND DISCUSSIONS

### Purification of Fab

Figure 3, panel A, shows the purification result of Fab from the IgG1 papain digested sample using the TOYOPEARL AF-rProtein L-650F column. Only two peaks are observed in the chromatographic profile - the flow through peak and the eluted peak. The eluted peak is eluted with 0.1 mol/L citrate buffer, pH 2.05 at ~90% gradient. The data suggests that Fab is bound strongly onto the TOYOPEARL AF-rProtein L-650F resin. Both peaks were collected for further analysis using a TSKgel UP-SW3000, 4.6 mm ID × 15 cm SEC column. The eluted peak is expected to contain only the Fab fragment.

Figure 3, panel B, shows results of the size exclusion analysis of the digested sample, the flow through and eluted peaks. The SEC profile showed that the digested sample contained two different molecular weight products based on the estimated retention times. As mentioned above, the papain digestion dissociates Fc and Fab regions of IgG and the protein L ligand only has an affinity for the Fab fragment. Therefore, there is no binding of the Fc fragment to the protein L resin and only Fab can be bound and eluted from the protein L column. Data from Figure 3, panel B, shows that the first/flow through peak from the papain digested sample has a retention time of 17 minutes and the second/eluted peak has a retention time of ~19 minutes.

PURIFICATION OF FAB USING TOYOPEARL AF-RPROTEIN L-650F



A: Purification of Fab by Protein L resin

Resin:       TOYOPEARL AF-rProtein L-650F
Column size: 5 mm ID × 2.5 cm L
Mobile phase: A : 0.1 mol/L Na-citrate, pH 6.5
             B: 0.1 mol/L citrate, pH 2.05
Gradient:    B= 0% (0 to 1 min)
             B= 0% to 100% (1 min to 16 min)
Flow rate:   0.5 mL/min (residence time = 1 min)
Dectection:  UV @ 280 nm
Injection:   10 µL, 3 mg/mL humanized mAb papain digest

B: SEC analysis of fractions

Column:      TSKgel UP-SW3000, 4.6 mm ID x 15 cm L x 2
Mobile phase: 20% acetonitrile,
             0.1% trifluoroacetic acid
Flow rate:   0.2 mL/min
Detection:   UV @ 280 nm
Sample:      2 g/L mAb2,
             2 g/L digested mAb2

➤ Figure 3 ·································································

The 17 minute retention time peak is suggested to be the Fc fragment because it has the same retention time with the flow through peak. And the 19 minute retention time peak is suggested to be the Fab fragment due to it having the same retention time as the eluted peak from the TOYOPEARL AF-rProtein L-650F column.

## DYNAMIC BINDING CAPACITY

A purified Fab sample was loaded onto the TOYOPEARL AF-rProtein L-650F resin, 0.83 mL column volume with 10% breakthrough at 3.4 minutes and 4.0 minutes residence times to determine the dynamic binding capacity (DBC) of a Fab fragment on the resin. Table 1 shows the comparison data between TOYOPEARL AF-rProtein L-650F resin to its competitor 85 µm agarose-based particle size protein L resin at various residence times. Due to the excellent mass transfer characteristics of TOYOPEARL AF-rProtein L-650F resin, dynamic binding capacities at 1 to 3 minutes residence time excel capacities obtained with the agarose-based resin (nearly double) as shown in Table 2. As the molecular weight of fragments is much smaller compared to full length IgGs, a dynamic binding capacity of about 50 mg/mL for a Fab with a typical molecular weight of 55 kDa equals a DBC of >130 mg/L for a ~150 kDa IgG when considering molar binding capacities.

DYNAMIC BINDING CAPACITY OF PROTEIN L RESINS FOR FAb

| | IgG SBC (g/L-resin) | Fab DBC (g/L-resin) | |
| --- | --- | --- | --- |
| | | Residence time | |
| | | 3.4 min | 4.0 min |
| rProtein L-650F | 72 | 33 | 50 |
| Competitor product | 42 | 19 | 26 |

Column:     TOYOPEARL AF-rProtein L-650F/competitor resin,
            4.6 mm ID × 50 mm (0.83 mL)
Detection:  UV @ 280 nm
Sample:     2 g/L human Fab in 0.1 mol/L Na-phosphate, pH 6.5
            DBC measured at 10% breakthrough

➤ Table 2 ··········································································

## CONCLUSIONS

The dynamic binding capacity of TOYOPEARL AF-rProtein L-650F resin exceeds the protein L agarose-based, 85 µm chromatography media currently available. Due to the high binding capacity, high yield and ease of use, TOYOPEARL AF-rProtein L-650F resin can considerably improve process economics of protein L capture steps. With high affinity antibody fragments, single chain variable fragments and domain antibodies, it is suited for the purification of new antibody formats that cannot be purified with protein A media.
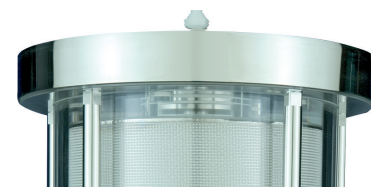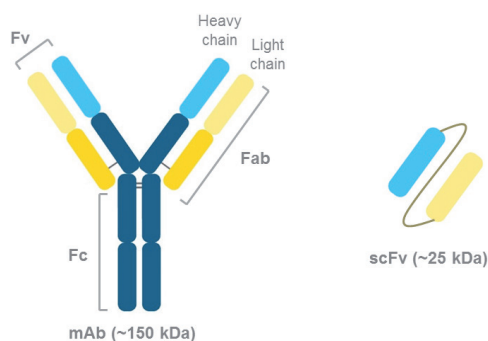
# CAPTURING OF A scFv FROM *E.COLI* USING TOYOPEARL® AF-rPROTEIN L-650F

*An application example for the general method set-up and optimization*

## scFv CAPTURING

Single chain variable fragments (scFv) are antibody derived molecules. The variable part of the light chain and the heavy chain of a mAb are connected via a peptide linker. The molecular weight of the whole construct is approximately 30 kDa. ScFvs are apt for being produced in E.coli, since they lack the glycosylation site of a full-length IgG. Other advantages over a full-length IgG include rapid target access and good tissue penetration. However, renal clearance delimits serum half-life to hours instead of days, compared to typical serum half-lives of mAbs. In some therapeutic strategies, this may be an advantage. Hence, straight-forward and efficient capturing solutions similar to Protein A for mAbs may pave the way for the future success of this class of molecules.

Fv
Heavy chain
Light chain
Fab
Fc
mAb (~150 kDa)
scFv (~25 kDa)

Herein we describe general conditions for capturing of an exemplary scFv from E.coli with TOYOPEARL AF-rProtein L-650F. Further, host cell protein (HCP) removal was optimized in a parallel chromatographic approach.

## LAB-SCALE EXPERIMENT

The κ light chain of mAbs, Fabs and scFv binds to Protein L at neutral pH and physiological conductivity. HCPs flow through the column at the applied conditions. The bound product can then be recovered at pH 2.0-3.5, depending on the particular format and molecule. As a rule of thumb, larger molecules can be eluted at comparatively higher pH. Most methods can be set-up based on these general guidelines. In detail, the herein presented experiments use 100 mM sodium phosphate buffer, pH 6.5 for column equilibration. 15 column volumes (CV) of a periplasmic scFv feedstream were loaded onto the 6.6 mm ID x 6.7 cm L column packed with TOYOPEARL AF-rProtein L-650F. The column was washed with equilibration buffer and the scFv was recovered in 100 mM glycine/HCl, pH 2.0. The method was conducted at 300 cm/h except for column loading, which was conducted at 3 min residence time.

Figure 1 shows the lab-scale capturing of scFv. 15 CV of the feedstream were loaded on the column at 3 min residence time. HCP flow through the column during loading and washing was done with 0.1 M sodium phosphate, pH 6.5. As a result the scFv eluted as a sharp peak in 100 mM glycine/HCl, pH 2.0.
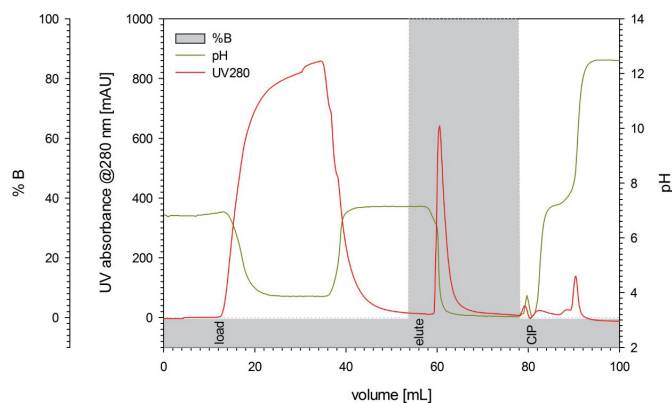
scFV LAB SCALE CAPTURING



Figure 1

THE DIFFERENT PROCESS STEPS AND CORRESPONDING
BUFFERS OF PROTEIN L CHROMATOGRAPHY

| Step | CV | Composition |
|---|---|---|
| Equilibration | 10 | 100 mM NaP, pH 6.5 |
| Load | 5 | feedstream from E.coli |
| Wash 1 | 5 | 100 mM NaP, pH 6.5 |
| Wash 2 | 5 | variable |
| Wash 3 | 5 | 100 mM NaP, pH 6.5 |
| Elute | 5 | 100 mM glycine/HCl pH 2.0 |
| CIP | 1 | 50 mM NaOH |
| Reequilibration | 10 | 100 mM NaP, pH 6.5 |

➤ Table 1

WASH SOLUTIONS APPLIED IN WASH 2, THE INTERMEDIATE POST-LOAD
WASHING STEP

| Buffer | Additive | Additive concentration |
|---|---|---|
| 100 mM NaP, pH 6.5 (reference) | - | - |
| 100 mM NaP, pH 6.5 | arginine | 1 M, 2 M |
| 100 mM NaP, pH 6.5 | guanidinium hydrochloride | 1 M, 2 M |
| 100 mM NaP, pH 6.5 | sodium chloride | 0.5 M |
| 100 mM Na acetate, pH 6.5 | sodium chloride | 0.15 M, 0.5 M |

➤ Table 2

## METHOD OPTIMIZATION

Screening of chromatographic conditions can significantly
improve performance of the corresponding process step.
In order to reduce sample consumption and to save time,
the method was down-scaled to 200 µl RoboColumn
format (P/N 0045066) and the feed loading was reduced to 5
CV. Parallel chromatography was conducted using a Tecan
Freedom Evo 150 liquid handling station equipped with a
chromatography station and a UV plate reader.

In the current study, different intermediate wash solutions
were tested with regards to their potential to improve HCP
removal compared to the use of the equilibration buffer for
post-load washing. A detailed list of the applied buffers and
solutions can be found in Table 1. Flow rates were adopted
from lab-scale chromatography. The different solutions
applied in wash 2 are listed in Table 2.

Fractions were collected in UV-readable 96 well plates and
UV absorbance was read at 280 nm. Pseudo-chromatograms
of the parallel chromatography run are shown in Figure 2.
Guanidinium hydrochloride washing and arginine washing
lead to elution of UV 280 nm absorbing material.

The different solutions applied in wash 2 are indicated in
the legend. Guanidinium hydrochloride and arginine induce
elution of UV 280 nm absorbing components during post-
load washing. The corresponding elution peaks are smaller.

PSEUDO-CHROMATOGRAMS OF TOYOPEARL AF-rProtein L-650F FOR
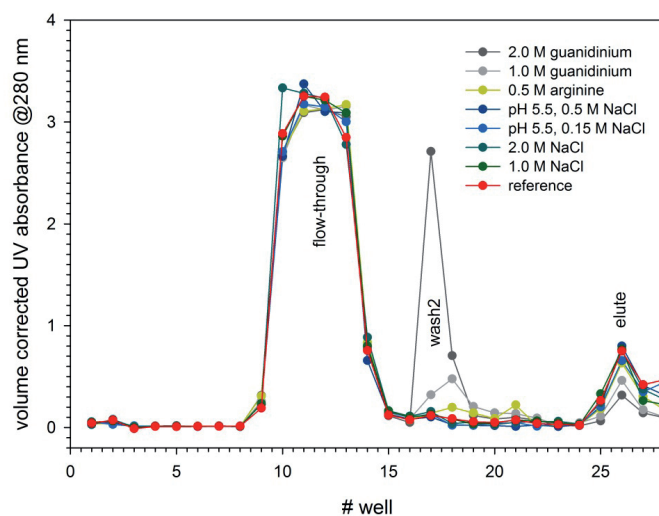CAPTURING OF SCFV FROM E.COLI



➤ Figure 2

NORMALIZED SEC CHROMATOGRAMS OF THE PARALLEL
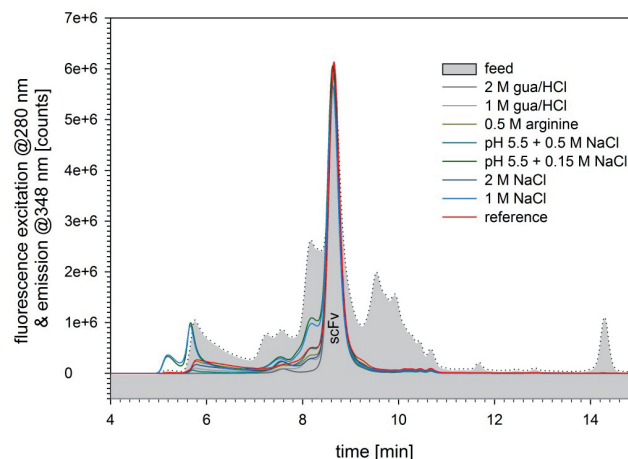CHROMATOGRAPHY ELUTION FRACTIONS AND FEEDSTREAM



➤ Figure 3

## OFF-LINE ANALYSIS

The different fractions of one process step were pooled
for off-line evaluation. The subsequent analysis included
SEC using TSKgel G2000SWxL 7.8 mm ID x 30 cm L (P/N
0008540), a Protein L leaching ELISA and an ELISA assay
for the determination of HCP from E.coli. For SEC, a 100 mM
sodium phosphate buffer, pH 6.7 was complemented with
100 mM sodium sulfate. 25 µl aliquots of the process steps
were injected and separated at 1.0 mL/min. SEC chroma-
tograms are shown in Figure 3. The scFv elutes at 8.4 min.
Guanidinium hydrochloride washings lead to highest scFv
purity.

Guanidinium hydrochloride and arginine washings
improve scFv purity. The majority of the impurities visible
in SEC elute directly upfront of the product, suggesting
these peaks may represent misfolded an aggregated scFv.
Guanidinium hydrochloride washings may lead to on-co-
lumn refolding and depletion of less specifically bound
misfolded scFv.

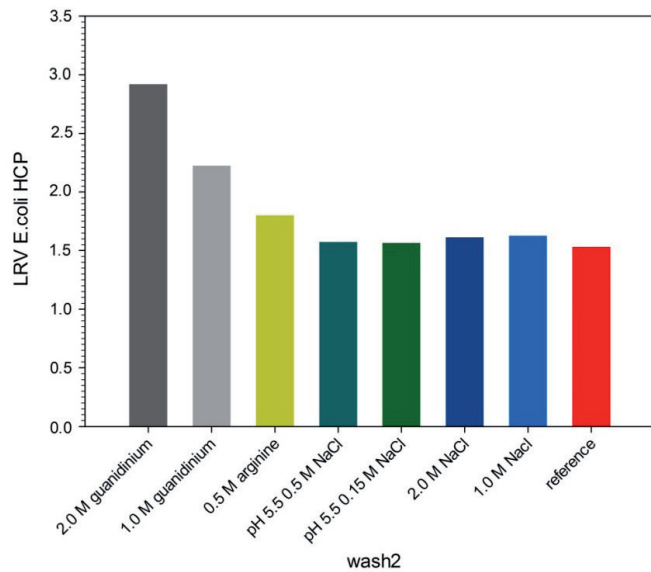LOG REDUCTION VALUES FOR HCP REMOVAL DURING
PROTEIN L CHROMATOGRAPHY



Figure 4

All elution pools contain less than 20 ng leached Protein L per mL.

CONCENTRATION OF LEACHED PROTEIN L IN THE ELUTION POOLS OF
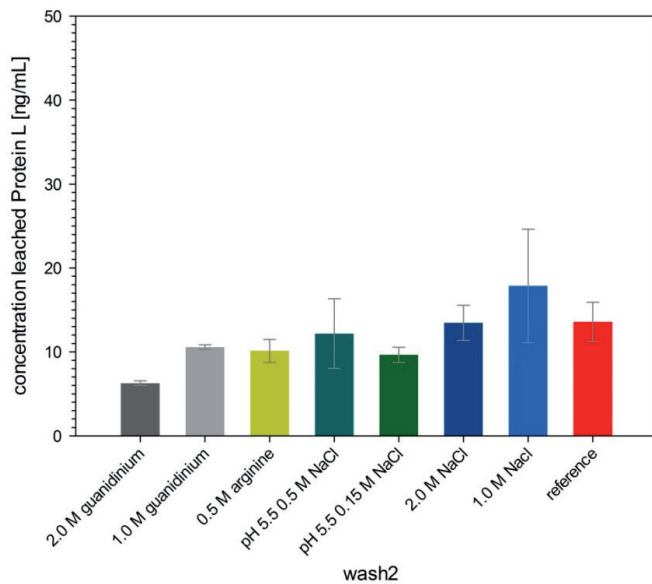THE PARALLEL CHROMATOGRAPHY EXPERIMENT



Figure 5

HCP removal is more than 10 x higher if 2 M guanidinium hydrochloride is
applied during post-load wash compared to the equilibration buffer wash,
which served as a reference in this study.

Further, the guanidinium and arginine washed elute pools
show lower HCP burden (Figure 4). Washing with 2 M
guanidinium hydrochloride increases the LRV for HCP from
1.6 to 2.9. The tested conditions did not lead to enhanced
Protein L ligand leaching, as shown in Figure 5.
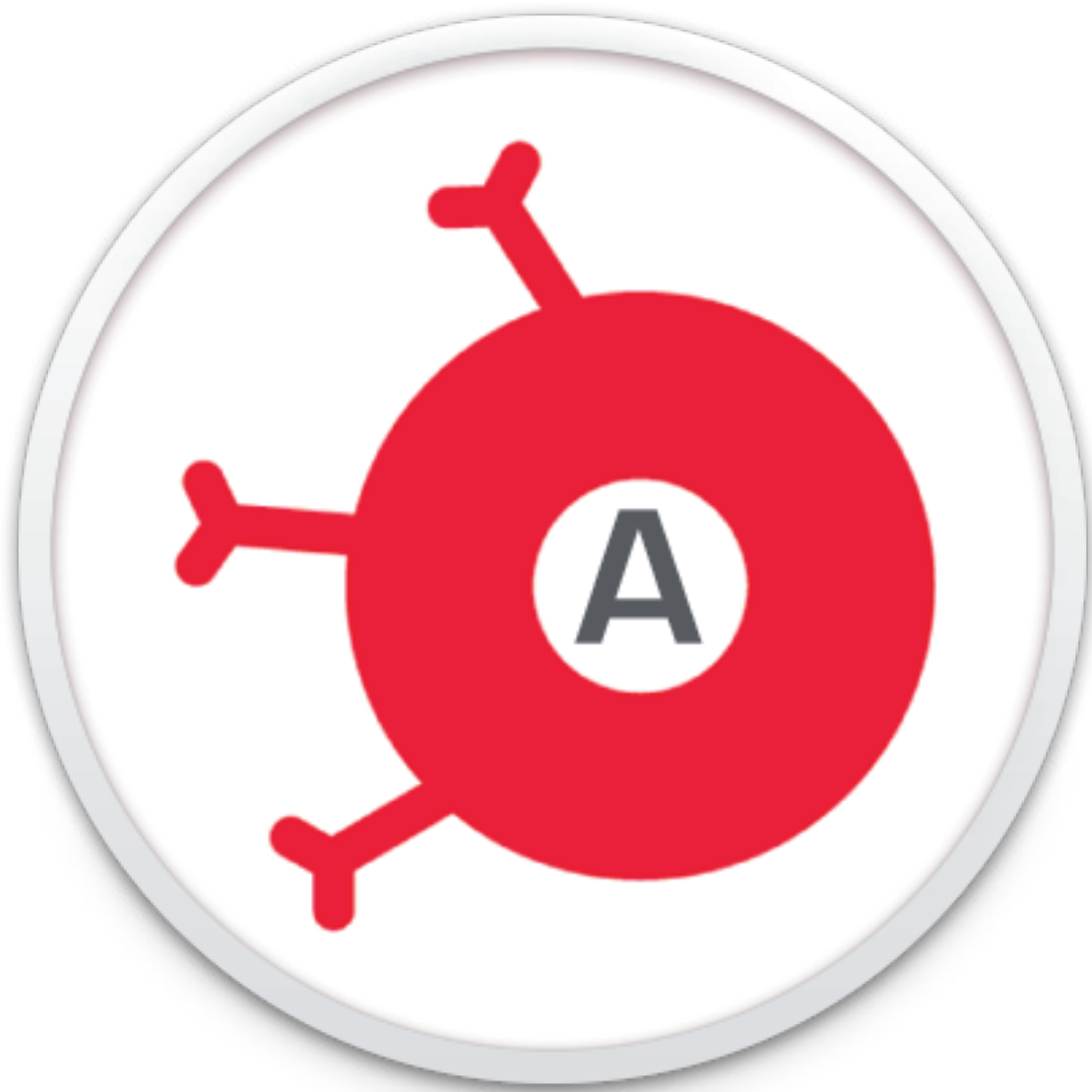
## CONCLUSION

According to the herein presented results, post-load
washing with chaotropic agents can significantly improve
HCP removal during capturing of a scFv from E.coli using
TOYOPEARL AF-rProtein L-650F. It may further support
on-column refolding of misfolded and aggregated product.

# 2. mAb
# CAPTURING

# PROTEIN A CHROMATOGRAPHY – THE PROCESS ECONOMICS DRIVER IN mAb MANUFACTURING

THE OPTIMIZATION OF THE PROTEIN A CAPTURE STEP IN DOWNSTREAM PROCESSING PLATFORMS CAN CONSIDERA-BLY IMPROVE PROCESS EFFICIENCY AND ECONOMICS OF INDUSTRIAL ANTIBODY MANUFACTURING. PARAMETERS LIKE RESIN REUSE AND ITS CAPACITY CONTRIBUTE CONSIDERABLY TO THE PRODUCTION COSTS. THE USE OF A HIGH CAPACITY PROTEIN A RESIN CAN IMPROVE THE PROCESS EFFICIENCY AND ECONOMICS. THIS PAPER PRESENTS THE KEY FEATURES OF A NEW CAUSTIC STABLE PROTEIN A RESIN PROVIDING EXTREMELY HIGH IgG BINDING CAPACITIES.

Biopharmaceuticals represent an ever growing important part of the pharmaceutical industry. The market for recombinant proteins exceeded $ 100 billion in 2011 with a contribution of 45% sales by monoclonal antibodies (mAbs) (1). The introduction of the first mAb biosimilars in Europe raised the competitive pressure in an increasingly crowded market place. The industry faces challenges, such as patent expirations accompanied by approvals of corresponding biosimilars, failures in clinical trials/rejections or the refusal of health insurers to pay for new drugs.

These challenges force the industry to minimize risk and time-to-market and to proceed more cautiously. Standardization and platform strategies in development and manufacturing, as well as process optimization become a focus for the industry. Biosimilar manufacturers need to adopt the most cost-effective manufacturing technologies including efficient and cost effective purification operations in order to realize highly competitive pricing compared to the original biologic.

## PLATFORM STRATEGY

The implementation of a platform process for mAb purification offers numerous advantages compared to a traditional "one step-at-a-time" approach. It accelerates process development while minimizing costs. Typical mAb platforms utilize a common sequence of unit operations for downstream processing, starting with Protein A affinity chromatography accompanied by ion exchange, mixed-mode, or hydrophobic interaction polishing steps.

Protein A chromatography has become a standard technique in the purification of monoclonal antibodies. Due to its selectivity, this affinity chromatography step efficiently removes host cell contaminants, while requiring comparably low method development efforts. On the other hand, Protein A affinity resins are dominating the Cost of Goods (COGs) of mAb manufacturing. Bioreactors at the 10.000 L scale operating at a titer of about 1g/L typically generate costs of $ 4-5 million (2). Therefore the Protein A capturing step is the key driver to improve process economics. Besides the capacity of the resin, life time and cycle numbers significantly contribute to the production costs in mAb manufacturing.

Today, the IgG binding capacities of most Protein A resins are in the range of 30-50 g/L, offering significant advantages for the processing of high-titer feedstreams when compared to resins with capacities of about 20 g/L. The new Protein A resin TOYOPEARL® AF-rProtein A HC-650F exhibits binding capacities of greater than 70 g/L, almost approaching level known from ion exchange chromatography. These high capacities result in even smaller in-process pools, reduced buffer consumption and potentially lower COGs.

## PROTEIN A CAPTURE STEP AND PROCESS ECONOMICS

A systematic process simulation of a standard mAb manufacturing process with 25 unit operations with SuperPro Designer® combined with design of experiments (DoE) identified a significant dependency of unit production costs on the IgG capacity of the Protein A resin (Figure 1).

The simulated downstream purification scheme comprised of Protein A, anion exchange and HIC chromatographic steps. Besides the IgG binding capacity, which had the highest impact on unit production costs, the mAb titer and the number of Protein A cycles per fermentation batch had been identified as key factors for cost optimization (3).

Figure 1 shows that the percentage share of the Protein A capturing costs in total operation costs is decreasing with rising resin capacity. This implicates that replacing a traditional Protein A resin with a dynamic binding capacity (DBC)

of 20 g/L by the new high capacity TOYOPEARL AF-rProtein A HC with a DBC of 70 g/L would reduce the relative costs of the Protein A step more than half. Similar findings were reported by Kobayashi et al. for another example comparing protein A resins with 20, 30 and 50 g/L IgG binding capacity (4).

### IgG BINDING CAPACITY AT HIGH TITER

Upstream efficiency improvements resulting in high fermentation titer have created significant DSP bottlenecks. Rising titers are a challenge for downstream processing, especially with regards to the expensive Protein A capturing step. TOYOPEARL AF-rProtein A HC-650F benefits from superior mAb capacity and beneficial mAb uptake behavior and can thereby increase capturing productivity.

Since DBC depends on flow and feed concentration, the binding capacity was tested at various residence times and various IgG and mAb titers. Figures 2 and 3 show the DBCs of TOYOPEARL AF-rProtein A HC-650F at three feed concentrations and three residence times. For both, polyclonal IgG and a monoclonal antibody, the dynamic capacities increase with rising titers.

The DBC is also a function of the residence time and is increasing with residence time, as can be expected. In contrast to other high capacity Protein A resins on the market, the binding capacity of this resin is considerably high even at a very short residence time of 1 min. This broad range of applicable flow rates enables the user to tune the capturing throughput in a way that is ideally fitting into the complete workflow. Most interesting is the observation of rising DBC with high titer. Even at short residence times the protein adsorption is high at high feedstock titer. Measured capacities of greater than 100 g/L exceed the DBCs of all other commercially available caustic stable Protein A resins.

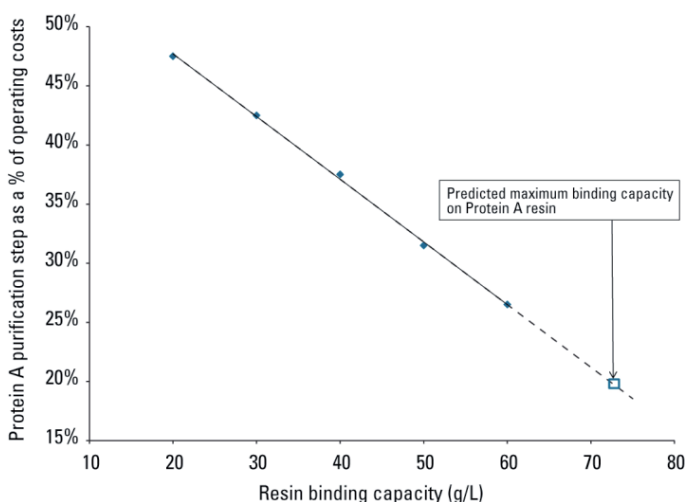EFFECT OF BINDING CAPACITY ON OPERATING COST



> Figure 1

DBC OF IgG AT DIFFERENT LOADS AND RESIDENCE TIMES



> Figure 2

Column: TOYOPEARL AF-rProtein A HC-650F  (5 mm ID × 5 cm)
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
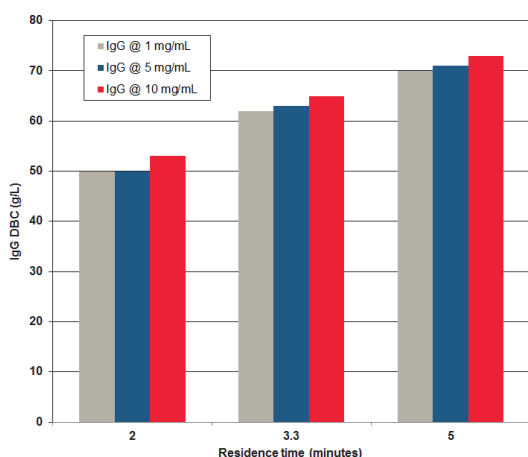
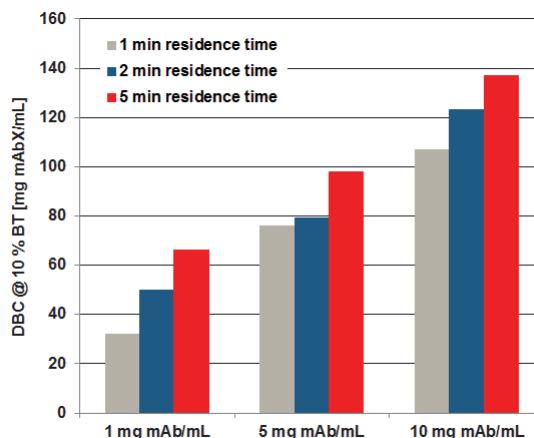DBC OF A MONOCLONAL IgG AT DIFFERENT LOADS AND RESIDENCE TIMES



> Figure 3

Column: TOYOPEARL AF-rProtein A HC-650F  (6.6 mm ID × 2 cm)
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
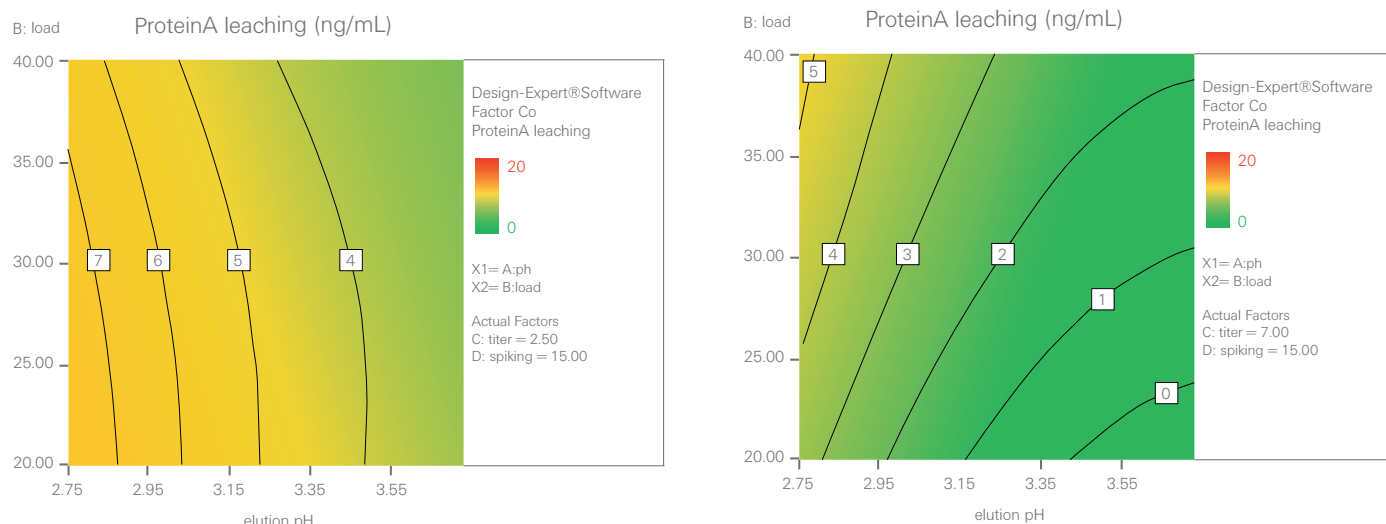
PROTEIN A LEACHING

> Figure 4

Contour plots for two different load concentrations. Protein A leaching is plotted against pH and absolute load. A: 2.5 g/L. B: 7 g/L.

The high capacities of TOYOPEARL AF-rProtein A HC-650F at high feed concentrations enable fast and efficient capturing solutions.

mAb ELUTION AND LIGAND LEACHING AT HIGH LOAD

As high protein loads could have an impact on aggregation and ligand leaching during Protein A chromatography, the elution properties of the new resin at high protein load were analyzed. A monoclonal antibody was diluted to a final concentration of 4.75 g/L and spiked with host cell proteins. Protein A chromatography was conducted in 200 µL RoboColumns· at a RT of 2 minutes.

The total loaded mass was varied from 10 to 50 mg/mL resin and columns were washed with 20 column volumes of loading buffer prior to elution. Recovery was > 95% at pH 3.25. SEC analyses of two elution pools (10 and 50 mg/mL load) revealed similar aggregate contents (0.6%) when referring to the corresponding total protein amount. Thus, large amounts of mAb absorbed by high capacity resins do not in principle enhance the risk for mAb aggregation during elution.

The dependency between the absolute load and protein A leaching at various mAb titers was analyzed for 2.5 and 7 g/L concentrated feed streams. The contour plots (Figure 4) reveal that the absolute load has little influence on numeric Protein A leaching. Ligand leaching was even slightly lower when applying higher titers.

RESIN ROBUSTNESS AND CIP STABILITY

It goes without saying, that the repetitive use of Protein A reins in DSP of mAbs also has a considerable impact on operating costs. Therefore robust and caustic stable Protein A resins are preferable for mAb manufacturing to take advantage of repeated Cleaning-in-Place (CIP) and reuse of the resin. Figure 5 depicts the excellent caustic stability of TOYOPEARL AF-rProtein A HC against 0.2 M NaOH at 15 minute contact time per CIP cycle.

The superior caustic stability underlines the perfect ability for cleaning and reuse in capturing of mAb's. This stability exceeds the requirement for reuse, facilitates long lifetimes and features the possibility to operate highly cost-efficient. At least 300 CIP cycles without significant reduction in DBC have been approved under the conditions described in Figure 5.
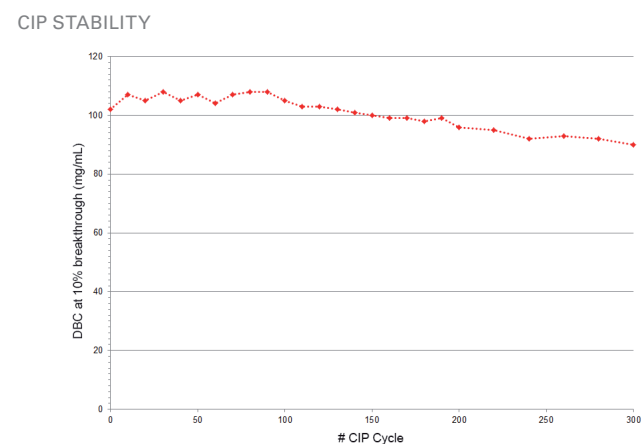
CIP STABILITY



> Figure 5

DBC at 10% breakthrough after repeated CIP cycles; 0.2 M NaOH, 15 min contact time mAb DBC at 5 g/L and 2 min. residence time.

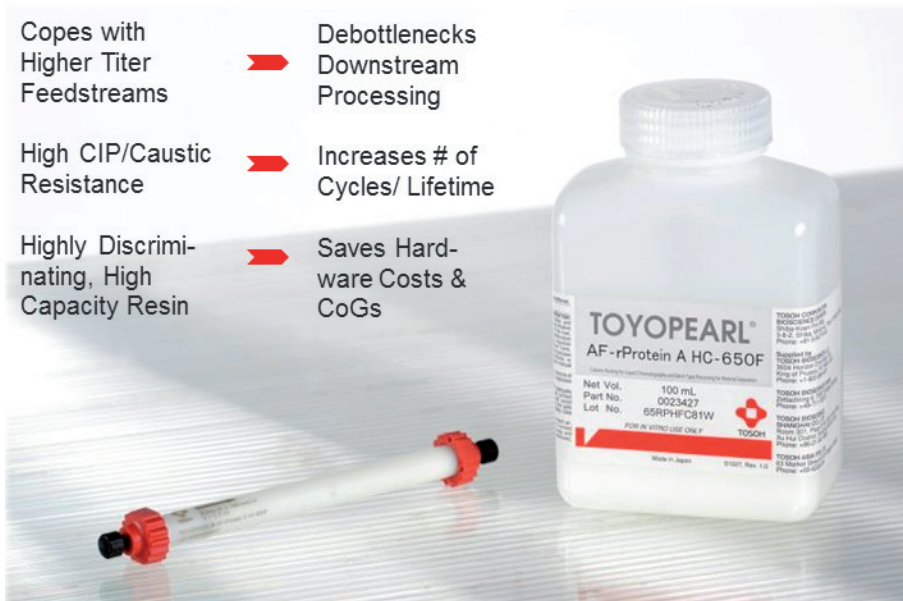## HOST CELL PROTEIN CLEARANCE AND LIGAND LEACHING

The CIP stability has also been analyzed with regard to ligand leaching and log reduction of Chinese Hamster Ovary proteins (CHOP). The results shown in Figure 6 and 7 underline the resistance of TOYOPEARL AF-rProtein A HC against 0.2 M NaOH at 15 minutes contact time. Under the described conditions more than 200 CIP cycles have been conducted and both, the ligand leaching with less than 2.5 ppm and the consistent CHOP reduction values of log > 2.5 confirm the robustness of the resin.

The main reason for the caustic stability and low ligand leaching of this resin is the multipoint attachment of an enhanced recombinant protein A ligand to the TOYOPEARL matrix. The capability of TOYOPEARL AF-rProtein A HC to keep its superior performance up to 300 CIP cycles adds to the cost reduction effect of its outstanding binding capacity and can considerably reduce operating costs of mAb manufacturing.

## CONCLUSION

The implementation of platform strategies in R&D and manufacturing of mAbs offers great opportunities to safe time, money and to improve the overall economics. Capturing with Protein A has become a standard in these platforms but traditional resins deemed to be very expensive and dominate the CoG in purification of mAbs. Binding capacities, high titer adsorption and alkaline resistance of Protein A resins have a major impact on unit production cost.

New and advanced Protein A resin technologies are addressing the current challenges like resin capacity, robustness and multiple re-use.

Copes with Higher Titer Feedstreams ➡ Debottlenecks Downstream Processing

High CIP/Caustic Resistance ➡ Increases # of Cycles/ Lifetime

Highly Discriminating, High Capacity Resin ➡ Saves Hardware Costs & CoGs

Hence, the advanced TOYOPEARL AF-rProtein HC-650F offers a multitude of benefits in development and production of antibodies.

The performance offered by TOYOPEARL AF-rProtein A HC-650F enables fast, cost effective, robust and efficient capturing solutions for R&D and manufacturing of antibodies. It is the perfect match for the Biotech Industry to improve process economics, to overcome restrictions in DSP and to face the current challenges in bioprocessing.

1. BioPlan Associates, Inc (2012); 9th Annual Report and Survey of Biopharmaceutical Manufacturing Capacity and Production
2. Uwe Gottschalk (2009); Process Scale Purification of Antibodies
3. Matthias Franzreb, Egbert Müller, Judith Vajda (2014); under publication
4. Shohei Kobayashi & Yasufumi Ueda (2013); Biopharm International

Superpro Designer is a registered trademark of Intelligen Inc.
RoboColumns are a registered trademark of Atoll GmbH
Tosoh Bioscience and TOYOPEARL are registered trademarks of Tosoh Corporation

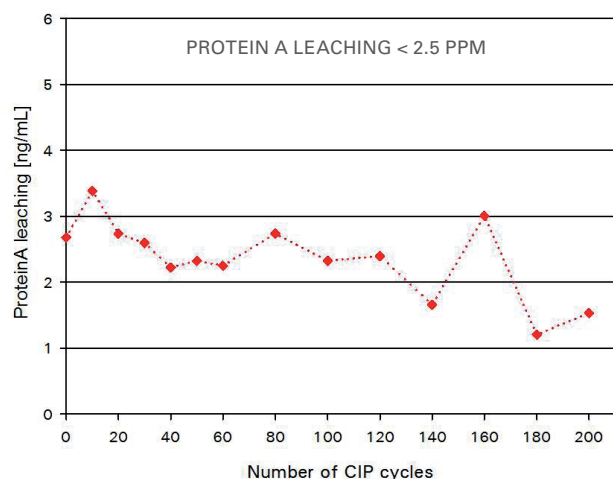PROTEIN A LEACHING AS A FUNCTION OF # OF CIP CYCLES



Figure 6
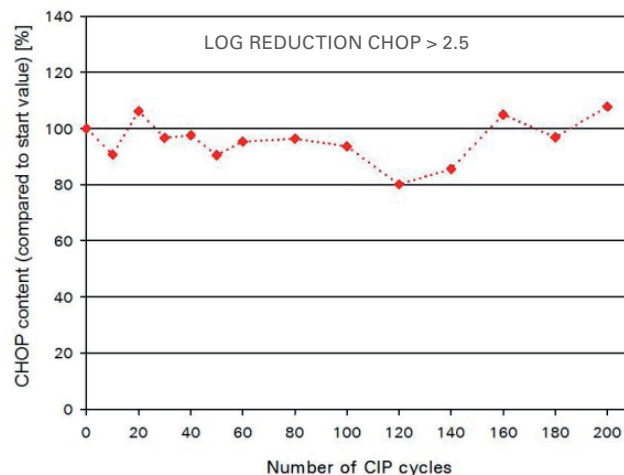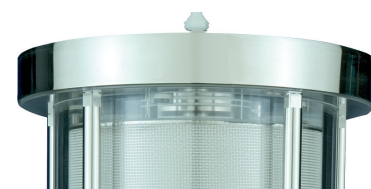
CHOP LOG REDUCTION AS A FUNCTION OF # OF CIP CYCLES



Figure 7

# A HIGH CAPACITY PROTEIN A RESIN
# DESIGNED TO REDUCE PRODUCTION COSTS

***TAKING ADVANTAGE OF HIGH CAPACITY PROTEIN A: AN ECONOMIC COMPARISON OF THE RESIN COST PER GRAM OF ANTIBODY PRODUCED***

With pressures mounting to reduce production costs at many companies, and protein A being the most expensive resin used in mAb purification, the use of a high capacity protein A resin can significantly impact the overall cost of doing business. This report details how using a high capacity protein A resin will reduce production costs, on a per-gram produced basis, for companies that implement its use in their chromatography platform.

TOYOPEARL AF-rProtein A HC-650F and two other commercially available protein A resins, one of them also a "high capacity" resin, were compared on a cost-per-gram of mAb produced basis at multiple resin prices from $7,000 to $17,000 per liter as well as three different column configurations. For in-depth comparison, the median resin price of $12,000 per liter was used as a basis to determine comparative production costs between the three resins tested.

Three configurations were examined to model what the resin costs would be in columns that were packed to have equal capacity, equal resin volume, and equal column dimensions. These three configurations were chosen to reflect the ways high capacity protein A resins could be instituted by individual companies, and also allows for a more complete look at the effects of resin capacity than just a single column configuration.
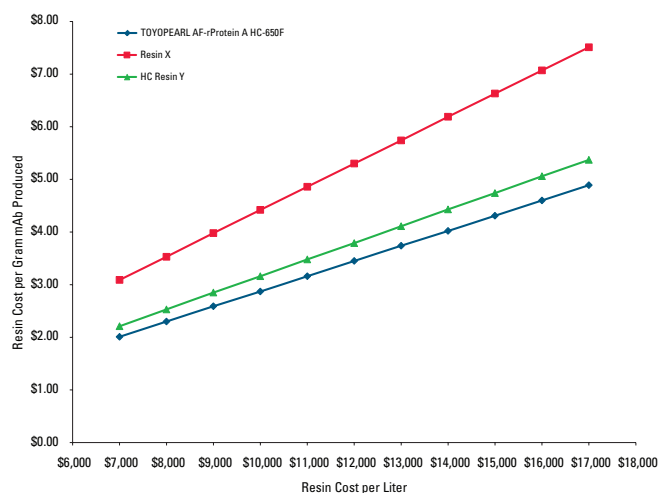
COLUMNS OF EQUAL CAPACITY



➤ Figure 1

At a column capacity of approximately 735 grams per load, the TOYOPEARL AF-rProtein A HC-650F had the lowest cost per gram of antibody purified at every similar price point.

For this evaluation, the variables examined are the resin cost per liter and the column configuration. The following values were held constant:

| | |
|---|---|
| Residence time: | 3 minutes |
| DBC: | 3 minute residence time (from product literature) |
| Column load: | 80% of stated dynamic binding capacity |
| Harvest titer: | 3 g/L |
| Harvest volume: | 2000 L |
| Column lifetime: | 100 cycles |
| Column yield: | 95% |

| Resin | Packed bed height (cm) | Column diameter (cm) | Column volume (L) | Resin compression | Resin volume to pack column | Resin cost per liter | Packed column cost | DBC at residence time (g/L) | Column capacity 80% DBC (g) | Residence time (min) |
|---|---|---|---|---|---|---|---|---|---|---|
| TOYOPEARL AF-rProtein A HC-650F | 15 | 36 | 15 | 1.25 | 20 | $12,000 | $240,000 | 60 | 732 | 3 |
| Resin X | 21 | 40 | 26 | 1.16 | 31 | $12,000 | $372,000 | 35 | 739 | 3 |
| High capacity resin Y | 18 | 36 | 18 | 1.16 | 22 | $12,000 | $264,000 | 50 | 732 | 3 |

➤ Table 1

| Resin | Flow rate (cm/hr) | Harvest titer (g/L) | Harvest volume (L) | Column cycles per harvest | Column lifetime (cycles) | Column lifetime (harvests) | Yield | Column lifetime productivity (g) | Resin cost per gram |
|---|---|---|---|---|---|---|---|---|---|
| TOYOPEARL AF-rProtein A HC-650F | 300 | 3 | 2000 | 9 | 100 | 11 | 95% | 69,587 | $3.45 |
| Resin X | 420 | 3 | 2000 | 9 | 100 | 11 | 95% | 70,160 | $5.30 |
| High capacity resin Y | 360 | 3 | 2000 | 9 | 100 | 11 | 95% | 69,587 | $3.79 |

➤ Table 2

| Resin | Resin Cost Per Gram at Median Resin Price ($12,000) | Competitor Resin Price per Liter Needed to Equal Tosoh |
|---|---|---|
| TOYOPEARL AF-rProtein A HC-650F | $3.45 | |
| Resin X | $5.30 | $7,500 |
| High capacity resin Y | $3.79 | $11,000 |

➤ Table 3

COLUMNS OF EQUAL VOLUME



➤ Figure 2

At a column capacity of appr. 732 grams per load, the TOYOPEARL AF-rProtein A HC-650F had the highest load capacity of the three resins packed at an equal column volume of 15 L.

The cost per gram of antibody purified was also lower than the other two resins at every similar price point.
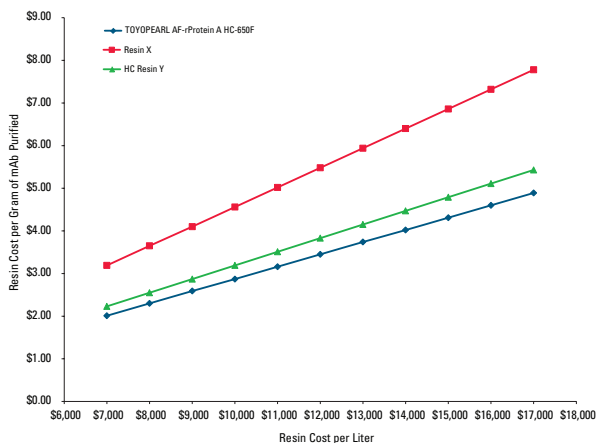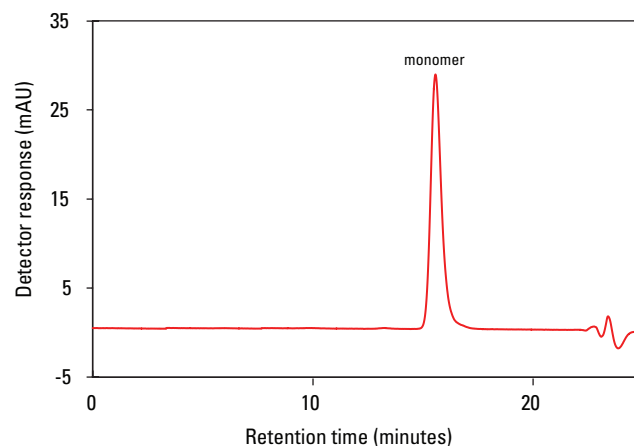
COLUMNS OF EQUAL DIMENSIONS



➤ Figure 3

At a column capacity of appr. 732 grams per load, the TOYOPEARL AF-rProtein A HC-650F had the highest load capacity of the three resins packed at equal column dimensions of 36 cm ID × 15 cm.

The cost per gram of antibody purified was also lower than the other two resins at every similar price point.

## CONCLUSIONS

As can be seen from the above comparisons, making use of a high capacity protein A resin in your purification process is an excellent way to save on production costs. At the median resin price of $12,000 per liter, the TOYOPEARL AF-rProtein A HC-650F resin would save customers almost $2.00 per gram of antibody produced over a resin with a capacity of 35 g/L and almost $0.50 per gram over a competitive high capacity resin.

With increasing pressures on producers of biopharmaceutical drugs to reduce productions costs, the use of a high capacity protein A resin is a superior way to achieve this goal without making any sacrifice to product quality or processing time.
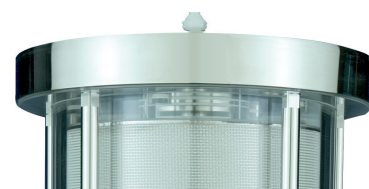
# HOW TO...IMPROVE PERFORMANCE OF THE PROTEIN A CAPTURING STEP

## DIFFERENT POST-LOAD WASH SOLUTIONS INCREASE HOST CELL PROTEIN REMOVAL OF TOYOPEARL AF-rPROTEIN A HC-650F

Protein A has become a well-established platform for capturing of monoclonal antibodies and Fc-fusion proteins. Various Protein A resins have been commercialized, one with a higher dynamic binding capacity than the other. Especially the latest generation of Protein A resins was developed to bind large quantities of mAbs and Fc fusion proteins. One drawback of these resins is that host cell protein (HCP) clearance can be - depending on the feeds-tream - comparatively lower than for standard Protein A affinity resins. When aiming for a 2-step platform process, no compromises can be made with regards to HCP removal of the capturing step. The remaining impurity burden would have to be covered by just one subsequent chromatography step. Hence, development of strategies or procedures to reduce the HCP content of the Protein A elution pool are key for the development of such purification processes. Post load washing steps during Protein A chromatography may improve HCP clearance of the capturing step. However, the employed wash buffers must not affect other product related quality criteria, such as aggregate levels and mAb activity. Besides, the impact of different wash steps on the parameters determining process economics, that is product recovery, dynamic binding capacity and resin life-time, should be evaluated.

## MATERIALS & METHODS

Different buffers, pH, and agents to reduce non-specific binding were evaluated with regards to their potential to increase HCP clearance. A parallel chromatography approach with 200 µl TOYOPEARL AF-rProtein A HC-650F RoboColumns was chosen. Columns were loaded with mAb at 3 min residence time. After laoding, columns were washed with 100 mM sodium phosphate buffer, pH 7.4. In a second wash step, columns were flushed with phosphate, citrate or acetate buffered solutions of sodium chloride, arginine or guanidinium hydrochloride. The applied pHs ranged from 5.5 to 7.4. The different wash solutions are listed in Table 1.

After washing, mAb was eluted with 100 mM acetate at pH 2.9. Columns were cleaned with 0.2 M sodium hydro-xide and re-equilibrated using 100 mM sodium phosphate buffer, pH 7.4. Except for loading, all steps were conducted at 300 cm/h. A detailed protocol can be found in Table 2.

### PROTEIN A CHROMATOGRAPHY

| Step | CV | Composition |
|------|-----|-------------|
| equil | 10 | 100 mM sodium phosphate, pH 7.4 + 150 mM sodium chloride |
| load | 26 | 2 mg/mL mAb, 1 x CHOP in equilibration buffer |
| wash 1 | 5 | 100 mM sodium phosphate, pH 7.4 + 150 mM sodium chloride |
| wash 2 | 5 | variable |
| wash 3 | 5 | 100 mM sodium phosphate, pH 7.4 + 150 mM sodium chloride |
| elute | 5 | 100 mM sodium phosphate, pH 7.4 + 150 mM sodium chloride |
| CIP | 5 | 0.2 M sodium hydroxide |
| reequil | 10 | 100 mM sodium phosphate, pH 7.4 + 150 mM sodium chloride |

### BUFFERS FOR THE PROTEIN A POST-LOAD WASH STEP

| Buffer | Additive Name | Additive Concentrations (mM) |
|--------|---------------|------------------------------|
| 100 mM sodium phosphate, pH 7.4 | sodium chloride | 150, 500, 750, 1,000, 1,500, 2,000 |
| 100 mM sodium acetate, pH 5.5 | sodium chloride | 0, 150, 250, 500 |
| 100 mM sodium citrate, pH 5.5 | n.a. | n.a. |
| 100 mM sodium phosphate, pH 7.4 | arginine | 100, 250, 500 |
| 100 mM sodium phosphate, pH 7.4 | guanidium hydrochloride | 500, 750, 1,000, 2,000 |

➤ **Table 1**

Sodium phosphate, sodium acetate and sodium citrate were substituted with different concentrations of miscellaneous additives

➤ **Table 2**

Buffers for the wash 2 step were chosen from Table 1. Buffers and solutions for the other steps are provided.
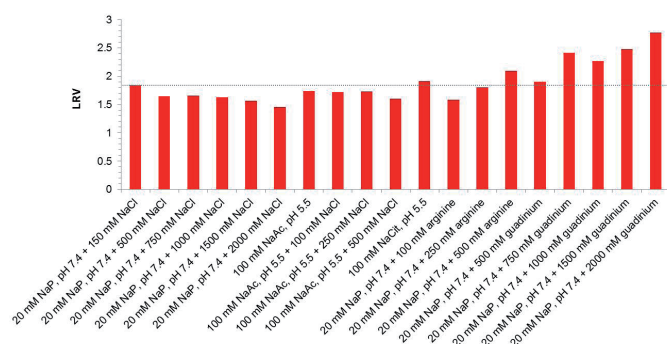
The mAb was recovered at ~10 mg/mL in total pool volumes of 1 mL ± 50 µl, which is due to drop formation at the RoboColumn outlet. Aliquots were analyzed with a CHO HCP ELISA, a protein A leaching ELISA and size exclusion chromatography. ELISAs were conducted according to the manufacturers' descriptions.

## HCP CLEARANCE

CHOP log reduction values have been calculated and are shown in Figure 1. The employed reference protocol using 20 mM sodium phosphate, pH 7.4 + 150 mM sodium chloride has a log reduction value of 1.85.

## PROTEIN A LEACHING

Protein A leaching results are shown in Figure 2. The post-load washing has no significant impact on the detected Protein A concentration. All samples have a Protein A content smaller than 1 ng/mL. Provided the mAb concentration in the elute pool is 10 mg/mL, this corresponds to 0.1 ppm of leached Protein A in the elute pools.

## AGGREGATE CONTENT

SE-UHPLC with TSKgel UP-SW3000 (4.6 mm ID x 30 cm L) was used for determination of aggregate levels. 100 mM sodium phosphate, pH 6.7 + 100 mM sodium sulfate was used as liquid phase. 5 µl of the Protein A elution pools were injected. UV absorbance was measured @280 nm. An exemplary chromatogram is shown in Figure 3. Aggregate contents of all samples are 0.85 % ± 0.05 %.

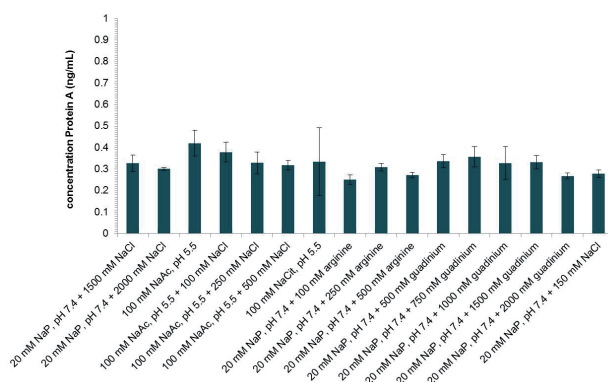CHOP LOG REDUCTION VALUES



➤ Figure 1

CHOP log reduction values of the parallel chromatography Protein A experiments using different buffers for wash 2. Comparatively higher log reduction values can be achieved using arginine and guanidinium hydrochloride for wash 2
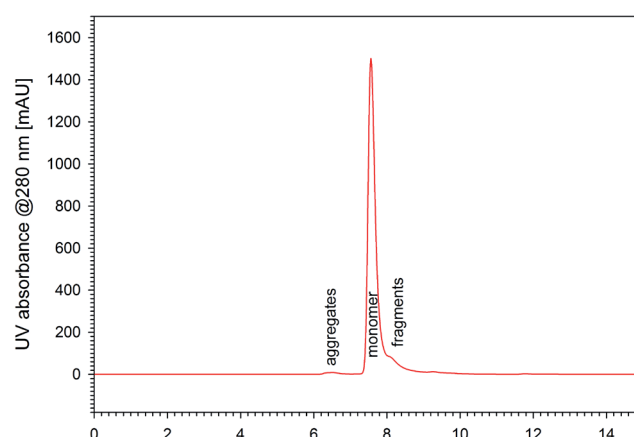
PROTEIN A CONCENTRATION IN THE ELUTE POOL



➤ Figure 2

The determined Protein A contents are well below 1 ng/mL, which corresponds to roughly 0.1 ppm.

SE-UHPLC CHROMATOGRAM OF AN EXEMPLARY PROTEIN A ELUTION POOL



➤ Figure 3

## RECOVERY

Recoveries were calculated from the UV absorbance @280 nm. A graph with the corresponding data is shown in Figure 4. Roughly, mAb concentrations of the elute pools range from 9.6 mg/mL to 11.0 mg/mL. Recovery slightly decreases in case of 100 mM sodium acetate, pH 5.5 and phosphate buffer, pH 7.4, containing high concentrations of arginine and guanidinium hydrochloride. However, recoveries exceed 90 % in all cases. Hence, these wash solutions can still be considered useful.

### RECOVERY
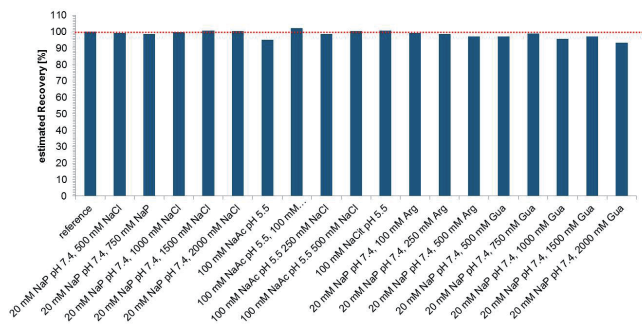


**Figure 4**

Recovery from Protein A after application of different wash buffers. Recovery is slightly affected by post-load washing with 100 mM sodium acetate, pH 5.5 and high contents of arginine or guanidinium hydrochloride. Recovery is greater than 90 % in all cases.

## CONCLUSIONS

High capacity Protein A resins are highly beneficial with regards to process economics. Potential drawbacks with regards to CHOP clearance depend on a particular feedstream and can be compensated by using post-load wash steps.

### LITERATURE:

[1] D. Ejima, R. Yumioka, K. Tsumoto, T. Arakawa, Effective elution of antibodies by arginine and arginine derivatives in affinity column chromatography., Anal. Biochem. 345 (2005) 250–7. doi:10.1016/j.ab.2005.07.004.
[2] T. Arakawa, J.S. Philo, K. Tsumoto, R. Yumioka, D. Ejima, Elution of antibodies from a Protein-A column by aqueous arginine solutions, Protein Expr. Purif. 36 (2004) 244–248. doi:10.1016/j.pep.2004.04.009.
[3] T. Arakawa, K. Tsumoto, D. Ejima, Alternative downstream processes for production of antibodies and antibody fragments, Biochim. Biophys. Acta - Proteins Proteomics. 1844 (2014) 2032–2040. doi:10.1016/j.bbapap.2014.05.005.
[4] A. Moosmann, E. Gerlach, R. Lindner, H. Böttinger, Purification of a PEGylated single chain Fv, J. Chromatogr. A. 1236 (2012) 90–96. doi:10.1016/j.chroma.2012.03.004.
[5] WO 2008031020 A2, Arginine wash in protein purification using affinity chromatography
[6] US 8263750 B2, Method for purifying a protein using protein-A affinity chromatography using an intermediate wash step
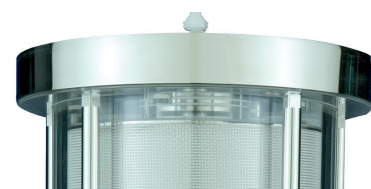
# COMPARISON OF TOYOPEARL AF-rPROTEIN A HC-650F BINDING CAPACITY AT VARIOUS BED HEIGHTS AND CONSTANT LINEAR VELOCITY

Protein A chromatography, the most widely used type of affinity chromatography, relies on the specific and reversible binding of antibodies to an immobilized ligand; in this case protein A. The protein A ligand can either bind directly to the Fc region of an antibody or to an Fc tag that has been fused to the target of interest.

Protein A chromatography is a very robust purification procedure and is used as a capture step due to its specificity. In protein A chromatography, crude feedstock is passed through a column under conditions that promote binding. After loading is complete, the column is washed under conditions that do not interrupt the specific interaction between the target and ligand, but that will disrupt any nonspecific interactions between process impurities (host cell proteins, etc.) and the stationary phase.

The bound protein is then eluted with mobile phase conditions that disrupt the target/ligand interactions. Elution of the target molecule from protein A resin is most commonly accomplished by lowering the pH of the mobile phase, creating an environment whereby the structure of the target molecule is altered in such a way as to inhibit binding. Low pH elution can have a negative effect on protein stability and it is advised that the eluted protein solution be neutralized to minimize aggregation and denaturation.

INTRODUCTION

TOYOPEARL AF-rProtein A HC-650F is a high capacity protein A resin for the purification of monoclonal antibodies (mAbs). This resin exhibits dynamic binding capacities (DBC) of 70 g/L at 5 minutes residence time. It is based on a hydroxylated methacrylic polymer particle. Table 1 lists the properties and dynamic binding capacities of this resin.

TOYOPEARL AF-rProtein A HC-650F resin remains dimensionally stable within wide extremes of pH and ionic strength. Moreover, the semi-rigid TOYOPEARL particles do not distort under flow rates that generate up to 0.3 MPa pressure. These resin properties, combined with a narrow particle size distribution, result in excellent pressure-flow characteristics for the packed TOYOPEARL bed.

Pressure is an important factor to consider throughout the separation process. Longer, thinner columns are subjected to higher pressure loads than wider columns with shorter bed heights. If it can be shown that an identical volume of

PROPERTIRES OF TOYOPEARL AF-rPROTEIN A HC-650F

| | |
|---|---|
| Particle size | 45 µm |
| Pore diameter | 100 nm |
| DBC (5 min) | 70 g/L |
| DBC (2 min) | 50 g/L |
| Caustic stability | > 200 CIP cycles (0.1 mol/L NaOH) |

➤ Table 1

resin packed in a shorter, wider column performs as well as a taller, thinner column, scientists using TOYOPEARL AF-rProtein A HC-650F can expect to see a decrease in column pressure while increasing productivity by reducing column residence time.

The data presented here demonstrates the capabilities of TOYOPEARL AF-rProtein A HC-650F to purify a human IgG1 monoclonal antibody from crude feedstock with a fixed column volume at multiple bed heights and constant linear velocity of 400 cm/hr.

EXPERIMENTAL CONDITIONS/RESULTS

Experiments were carried out on 6.6 mm ID × 19.5 cm (6.67 mL), 1.0 cm × 8.4 cm (6.60 mL), and 1.5 cm × 3.5 cm (6.20 mL) columns packed with TOYOPEARL AF-rProtein A HC-650F resin. The columns successfully passed performance tests for use in these experiments.

The columns were equilibrated with 10 mmol/L $Na_2HPO_4$, 150 mmol/L NaCl, pH 7.40, and loaded with clarified feedstock at approximately 90% of the resin DBC (63 g/L-resin)at a constant linear velocity of 400 cm/h. This linear velocity corresponds to residence times of 2.9 minutes, 1.2 minutes, and 0.5 minutes for the 19.5, 8.4, and 3.5 cm bed heights respectively.

A linear velocity of 400 cm/hr was selected for this series of experiments as it is representative of the fastest flow rates used in manufacturing scale operations. A more typical linear velocity would be in the 300 to 350 cm/hr range.

After loading, the column was washed with 5 CV of equilibration buffer to remove any unbound impurities and then eluted with 100 mmol/L citrate buffer, pH 3.0.

Post-elution, the column was washed with 3 CV of equilibration buffer and sanitized with 3 CV of 0.1 mol/L NaOH (15 minutes contact time).

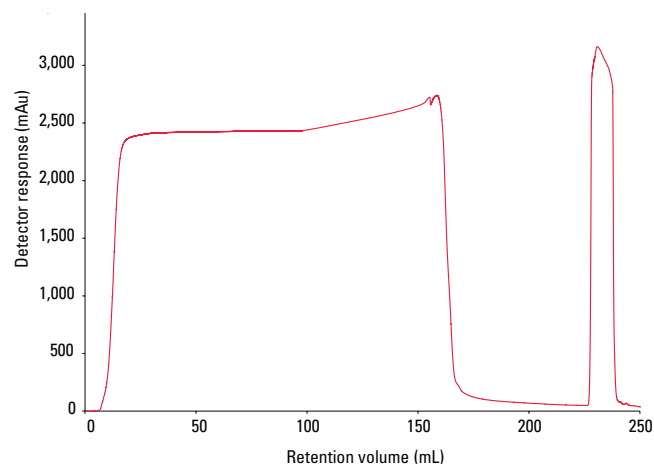Lcan be seen in Figure 1 (6.6 mm ID × 19.5 cm column size), Figure 2 (1.0 cm × 8.4 cm column size) and Figure 3 (1.5 cm × 3.5 cm column size), the elution peaks are sharply defined and exhibit minimal tailing.

Table 2 shows the capacity, yield, pressure, and purity for each of the purifications performed.

A representative HPLC analysis of the elution pool by SEC (Figure 4) indicates that column bed height has a minimal effect on the amount of aggregates present in the purified product.
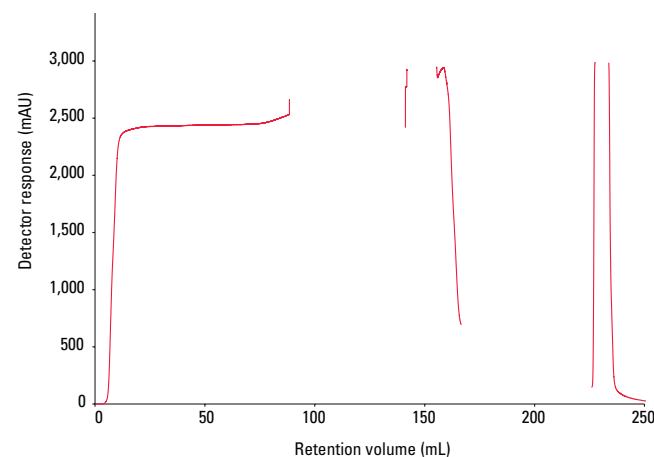
## 6.6 MM ID × 19.5 CM COLUMN



➤ **Figure 1** ⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯As

| | |
|---|---|
| Resin: | TOYOPEARL AF-rProtein A HC-650F |
| Column size: | 6.6 mm ID × 19.5 cm L (6.67 mL) |
| Mobile phase: | A: 10 mmol/L $Na_2HPO_4$, 150 mmol/L NaCl, pH 7.40 |
| | B: 100 mmol/L $Na_3C_6H_5O_7$, pH 3.0 |
| | C: 0.1 mol/L NaOH |
| Flow rate: | 400 cm/h (2.28 mL/min) |
| Residence time: | 2.9 min |
| Detection: | UV @ 280 nm |
| Sample: | crude feedstock (titer = 3.0 g/L) |
| Sample load: | 155 mL |

## 1.0 CM ID × 8.4 CM COLUMN



➤ **Figure 2** ⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯

| | |
|---|---|
| Resin: | TOYOPEARL AF-rProtein A HC-650F |
| Column size: | 1.0 cm ID × 8.4 cm L (6.60 mL) |
| Mobile phase: | A: 10 mmol/L $Na_2HPO_4$, 150 mmol/L NaCl, pH 7.40 |
| | B: 100 mmol/L $Na_3C_6H_5O_7$, pH 3.0 |
| | C: 0.1 mol/L NaOH |
| Flow rate: | 400 cm/h (5.23 mL/min) |
| Residence time: | 1.2 min |
| Detection: | UV @ 280 nm |
| Sample: | crude feedstock (titer = 3.0 g/L) |
| Sample load: | 155 mL |

## 1.5 CM ID × 3.5 CM COLUMN
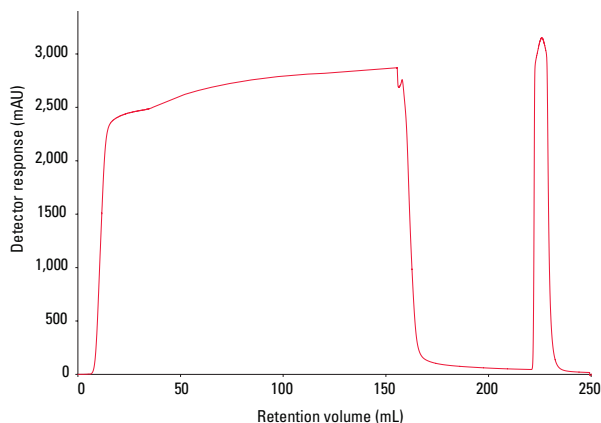


➤ **Figure 3** ⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯

| | |
|---|---|
| Resin: | TOYOPEARL AF-rProtein A HC-650F |
| Column size: | 1.0 cm ID × 8.4 cm L (6.60 mL) |
| Mobile phase: | A: 10 mmol/L $Na_2HPO_4$, 150 mmol/L NaCl, pH 7.40 |
| | B: 100 mmol/L $Na_3C_6H_5O_7$, pH 3.0 |
| | C: 0.1 mol/L NaOH |
| Flow rate: | 400 cm/h (5.23 mL/min) |
| Residence time: | 0.5 min |
| Detection: | UV @ 280 nm |
| Sample: | crude feedstock (titer = 3.0 g/L) |
| Sample load: | 155 mL |

## REPRESENTATIVE SEC HPLC ANALYSIS OF ELUTED mAb
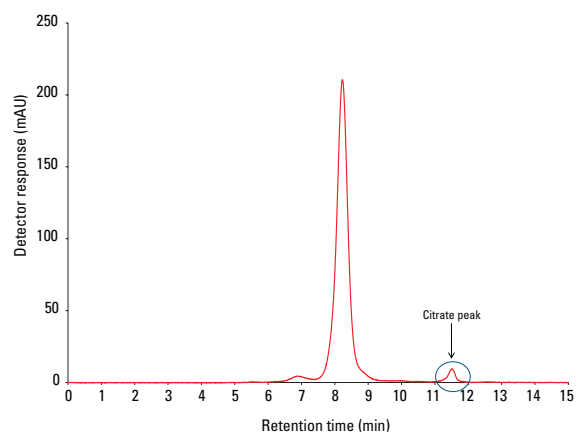


➤ **Figure 4** ⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯

| | |
|---|---|
| Column: | TSKgel G3000SW$_{XL}$, 5 µm, 7.8 mm ID x30 cm L |
| Mobile phase: | 0.1 mol/L $Na_2PO_4$, 0.1 mmol/L $Na_2SO_4$ |
| | 0.05% azide, pH 6.7 |
| Flow rate: | 1 mL/min |
| Detection: | UV @ 280 nm |
| Inj. Vol.: | 1.0 µL |
| Sample: | protein A purified mAb |

mAb PURITY AND YIELD

| Column dimension (cm ID x cm) | Residence time (min) | Sample loaded (mg) | mAb recovered (mg) | Elution volume (mL) | Yield (%) | Purity (%) | Resin capacity 400 cm/hr | Column pressure (MPa) |
|---|---|---|---|---|---|---|---|---|
| 0.66 x 19.5 | 2.9 | 464.7 | 391.6 | 37 | 84.3 | 96.2 | 58.7 g/L | 0.21 |
| 1.0 x 8.4 | 1.2 | 464.7 | 343.2 | 37 | 74.1 | 96.9 | 52.2 g/L | 0.11 |
| 1.5 x 3.5 | 0.5 | 464.7 | 224.2 | 26 | 48.2 | 94.7 | 36.2 g/L | 0.07 |

Table 2

Recovery was determined by comparing the amount of mAb present in the crude sample loaded onto the column to the amount of mAb present in the elution pool.

CONCLUSIONS

TOYOPEARL AF-rProtein A HC-650F is capable of delivering high purity monoclonal antibodies with excellent recovery at loading levels approaching the resin capacity in columns with bed heights as short as 3.5 cm with a small increase in aggregate levels at the shortest bed height.

While this study clearly shows that capacity and yield will decrease for a fixed resin volume in columns of increasing ID, the reduction in performance is relatively minor. At a bed height less than half of the 6.6 mm ID column, the difference in capacity for the 1.0 cm ID column was only 6.5 g/L and was still greater than 50 g/L overall.

Most notable was the pressure difference of 0.1 MPa (1 bar) between the 6.6 mm and 1.0 cm ID columns. These values suggest that a bed height of 20 cm is not necessary when using TOYOPEARL AF-rProtein A HC-650F in order to maximize resin performance and productivity. Columns packed to a bed height of approximately 10 cm to 15 cm will perform similarly to a 20 cm bed height column, but at reduced pressures and increased product throughput.

Further optimization of the protein A column by adjusting linear velocity and bed height may eliminate the need for chromatographers to use a 20 cm bed height for protein A process steps without any loss in column capacity.
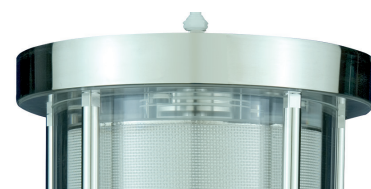
# INCREASED PRODUCTIVITY FOR DOWNSTREAM PROCESSING OF BIOTHERAPEUTICS

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.

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.

### 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 (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 (Repligen GmbH, Weingarten,

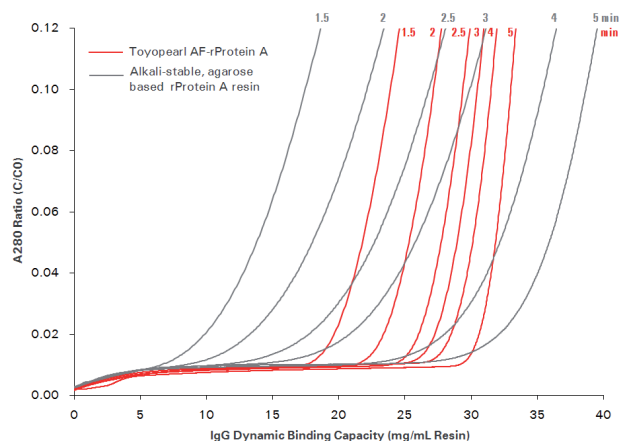### DYNAMIC BINDING CAPACITY



⮞ 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

Germany) were tested by varying the binding buffer pH, the sample load and the residence time.

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.

### 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.*

## 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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3. A-mab: A Case Study in Bioprocess Development, CMC Biotech Working Group, Version 2.1, October 2009
4. Bensch et al, Chemical Engineering & Techn 28(11): pp1,274-1,284, 2005
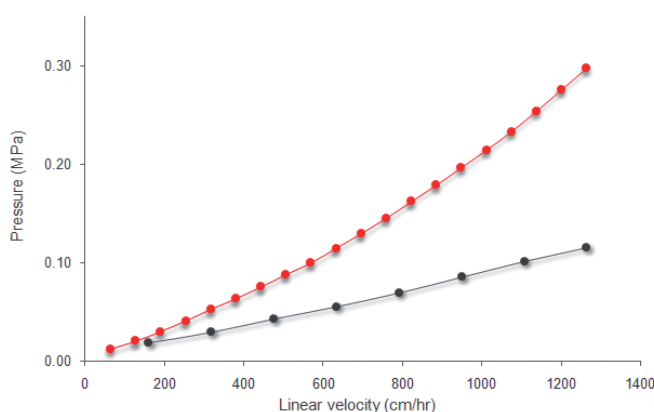
EVALUATION OF CATION EXCHANGE STEP
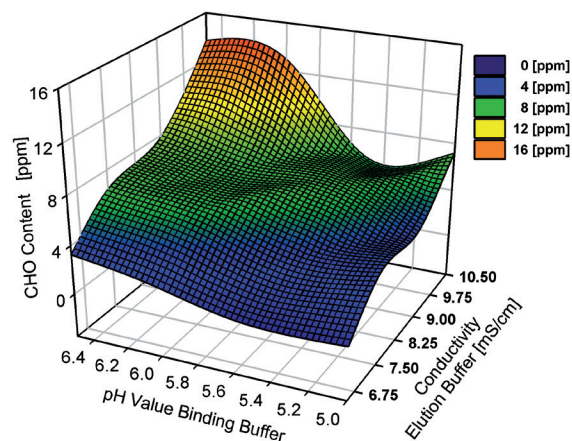


Figure 2

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.
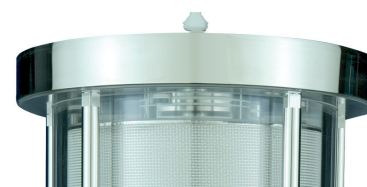
# PACKING OF TOYOPEARL AF-r PROTEIN A HC-650F IN PROCESS SCALE COLUMNS

TOYOPEARL AF-rProtein A HC is an ultra-high capacity resin for affinity purification of immunoglobulins. Particle size, porosity of the base material, as well as ligand composition and density was optimized with the aim to reach maximum binding capacity. The resulting resin offers a typical binding capacity of 70 g/L resin at 5 minutes residence time. The binding capacity is only slightly decreased when reducing the residence time/increasing linear flow.

The high binding capacity of the resin at short residence times allows using short bed heights and high flow rates. However, due to the relatively small particle size of 45 µm flow rates need to be adapted to the pressure limitations of the system/column hardware.

In order to determine the optimum conditions for packing and using this resin in process scale columns we evaluated conditions for various column dimensions and manufacturers.

## MATERIAL AND METHODS

### COLUMN HARDWARE:

A:  20 cm ID Quikscale˙ (QS 200), 10 µm mesh size, max. 0.6 MPa, MerckMillipore
B:  40 cm ID Resolute˙ 400 self-packer, 20 µm mesh size, max. 0.5 MPa, Pall Corporation
C:  CA-601S radial flow column with 6 cm bed height, 5 L bed volume, 10 µm mesh size,  Proxcys B.V.

## PACKING PROCEDURE

### A (QS 200):

Slurry concentration 40 - 50 %
Packing was performed according to the TOYOPEARL Instruction Manual:
Flow was increased stepwise, starting with 50 cm/h over 100 cm/h to 200 cm/h before lowering the piston to the top of the bed. For final bed compression the flow was increased stepwise again starting from 100 cm/h to 300 cm/h (or reaching max. 0.4 MPa) before lowering the piston 2 - 3 mm into the final bed.

### B (Resolute 400):

Slurry concentration: 30 - 40 %
Slurry transfer was performed with a diaphragm packing pump into the column at constant ~0.45 MPa pneumatic pressure. The packing speed of initially ~2,000 cm/h went down to 100 cm/h during bed formation.
Flow packing: stepwise flow increase with process pump (Δ 50 cm/h/min until max system pressure). Piston was lowered 2 mm into the bed.

### C (CA-601S):

Slurry concentration 30 - 50 %
Slurry transfer and packing with system pump into the column (Δp~0.3 MPa); packing speed ~600 cm/h.

## COLUMN TESTING

Bed stability was tested in water, 1 M NaCl and 3 M NaCl (to mimic mAb feedstock application) as a mobile phase. Packing performance was evaluated by injecting 50 mL of 5 % acetone (UV detector) or salt (conductivity detector) at 100 cm/h.

## RESULTS

Table 1 shows optimal flow rates/residence times for various bed heights in a 20 cm ID QuikScale column. Up to a bed height of 15 cm a residence time of up to 2 -3 min is feasible. For higher bed heights such as 20 cm the residence time should be increased to > 5 minutes which simultaneously allows exploiting the maximum binding capacity of the resin.

Packing qualities of 4,000 - 6,000 theoretical plates per meter for the unretained peak and symmetry values of 1.1 – 1.7 were obtained throughout all packing tests at various bed heights (~ 20 different experiments in total).

FLOW RECOMMENDATIONS FOR VARIOUS BED HEIGHTS

| Bed Height (cm) | Optimal Flow [cm/h] | Residence Time |
|---|---|---|
| 11 | < 400 (1 M NaCl) | > 2.0 min |
| 14 | < 300 (3 M NaCl) | > 2.8 min |
| 20 | < 190 (3 M NaCl) | > 6.0 min |

➤ Table 1 ............................................

PRESSURE FLOW CURVES (WATER) FOR TOYOPEARL AF-rPROTEIN A HC-650F IN A QS 200 COLUMN AT VARIOUS BED HEIGHTS
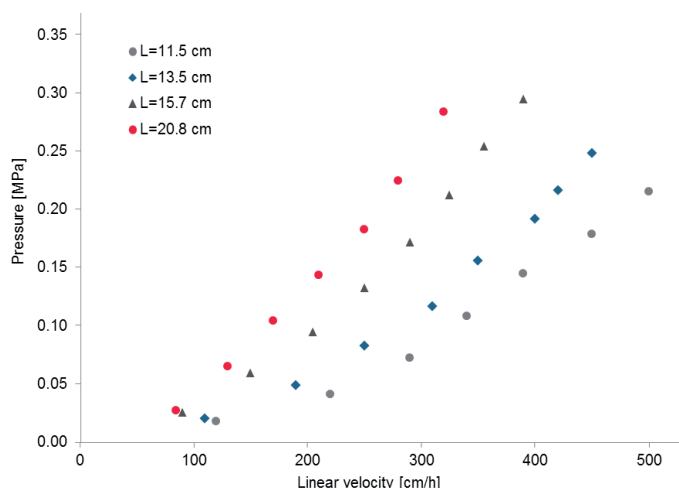


➤ Figure 1

Figure 1 shows the pressure flow curves in water as mobile phase for various bed heights for the 200 mm ID QuickScale column.

Figure 2 shows a comparison of the pressure flow curve for water at 14 cm bed height and 3 M NaCl, which was used to mimic the viscosity of a mAb feedstock.

COMPARISON OF PRESSURE FLOW CURVES (WATER VS. 3 N NACI) FOR TOYOPEARL AF-rPROTEIN A HC-650F AT 14 cm BED HEIGHT IN QS 200
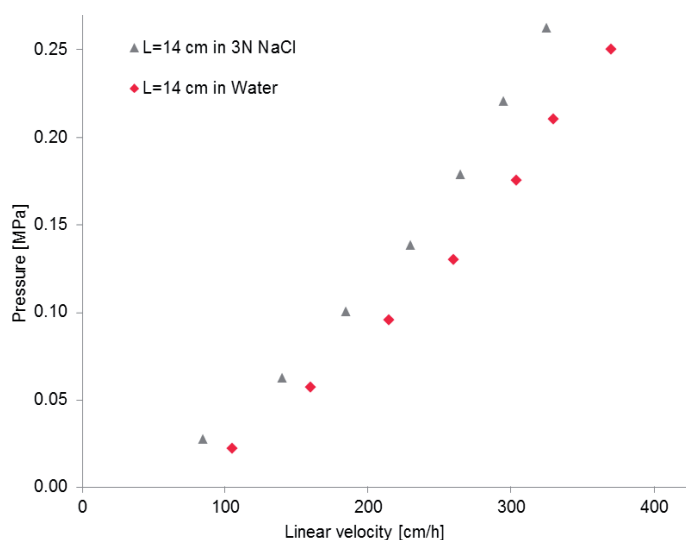


➤ Figure 2

Packing experiments with a 40 cm ID Resolute columns show that at a bed height of about 15 cm is ideal to run at a residence time of 3 min minimum (with linear flow rates between 200 and 250 cm/h). Figure 3 shows pressure flow curves in water as mobile phase for various bed heights for the 40 cm ID Resolute column.

PRESSURE FLOW CURVES (WATER) FOR TOYOPEARL AF-rPROTEIN A HC-650F IN A RESOLUTE 400 COLUMN AT VARIOUS BED HEIGHTS
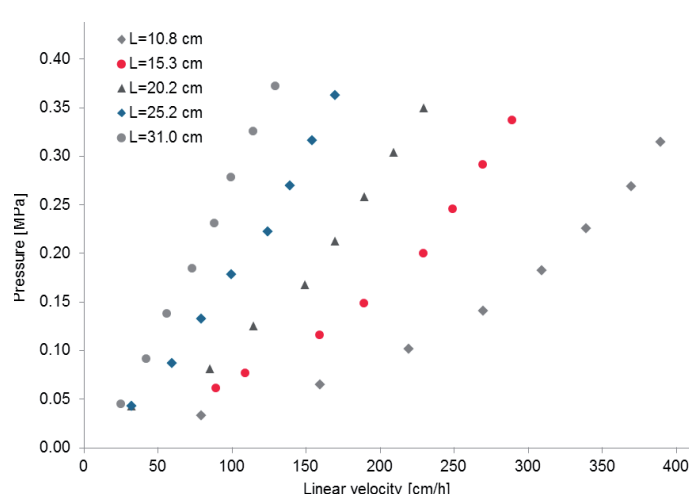


➤ Figure 3

In the CA-601S radial flow column from Proxcys TOYOPEARL AF-rProtein A-650F HC compresses ~22 % in water. A 28 % excess of the resin is recommendable to fill the column. The tests performed in water yielded reproducible plate numbers of 3,500 N/m and symmetry values of 1.1. Pressure at 1.3 minutes residence time was 0.12 MPa. Packing in 1 N NaCl yielded 4.,500 N/m and Af=1.2, when 2 N NaCl was injected. The packed bed remained consistent at 1.5 minutes residence time in 1N NaCl at 0.12 MPa.

This demonstrates that this column type is suitable for capturing processes of mAb solutions at high flow rates/short residence times.

CONCLUSION

TOYOPEARL AF-rProtein A HC-650F can be used in a variety of process column types. Its small particle diameter of 45 µm contributes to the high binding capacities of up to 100 g/L for IgG. At the same time it requires balancing flow rate and bed height to obtain best results. To fully exploit the fast adsorption kinetics of TOYOPEARL AF-rProtein A HC-650F it is favorable to use short bed heights with fast flow rates/short residence times. This is easily applicable in radial flow columns.

Other than the column types, dimensions and system setups described here, might require the adaption of the proposed conditions to the respective pressure specifications. Ask our experienced Technical Support Team to support your individual column packing approach.

To screen and optimize method parameters the resin is also available in prepacked ToyoScreen°, RoboColumn° and 5 mL MiniChrom format. Larger prepacked columns with TOYOPEARL AF-rProtein A HC-650M can be supplied in cooperation with Repligen GmbH, Weingarten.

# 3. SALT-TOLERANT
# ION EXCHANGE INTERMEDIATE PURIFICATION
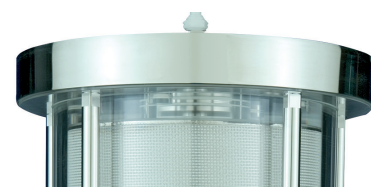
# INCREASE MONOCLONAL ANTIBODY PURITY WITH TOYOPEARL SULFATE-650F RESIN FOR CAPTURE AND REMOVAL OF mAb AGGREGATES

## INTRODUCTION

Ion exchange chromatography is often used as an intermediate purification step in monoclonal antibody (mAb) purification for the removal of protein aggregates, host cell proteins (HCP), and leached protein A ligand. Industry trends are focusing on the development of continuous downstream processing. Typically scientists in biopharmaceutical settings use cation exchange (CEX) and anion exchange (AEX) chromatography steps in series to further polish a purified mAb after the protein A capturing step. In this study, we focus on the development of a post protein A CEX step for the removal of aggregates, host cell proteins and leached protein A to improve the purity of the mAb eluate in a single polishing step.

A strong cation exchange resin, TOYOPEARL Sulfate-650F, is used in this study. It is a novel resin with the following benefits: strong capture of mAb aggregates, high salt-tolerance, wide working pH range, and high dynamic binding capacity. The protocol used with this resin has a minimal pH adjustment of the purified mAb eluate after the protein A step.

## MATERIALS AND METHODS

TOYOPEARL Sulfate-650F resin, 45 µm, 100 nm and TOYOPEARL AF-rProtein A HC-650F, 45 µm, 100 nm resins were used in this study. The resins were packed into Omnifit® Benchmark columns, (25 mm ID × 5 cm or 6.6 mm ID × 10 cm). A TSKgel G3000SWxL SEC column was used for analyzing collected $IgG_1$ fractions

### Purify $IgG_1$ using TOYOPEARL AF-rProtein A HC-650F

As shown in the steps below, CHO clarified cell supernatant (CCS) containing $IgG_1$ was passed through a TOYOPEARL AF-rProtein A HC-650F column (25 mm ID x 15 cm) to purify the $IgG_1$. The elution peak was collected starting and ending at 100 mAU.

The $IgG_1$ eluate from the TOYOPEARL AF-rProtein A HC-650F column was adjusted to pH 5.0 with 1 mol/L Tris base and quantified by UV absorbance at 280 nm.

| | |
|---|---|
| 1 | Equilibrate (6 CV, 225 cm/h): 20 mmol/L Tris-acetate, 150 mmol/L NaCl, pH 7.4 |
| 2 | Load (48 mg/mL-resin, 225 cm/h): TBL-mAb-01 CCS |
| 3 | Wash (10 CV, 225 cm/h): 20 mmol/L Tris-acetate, 150 mmol/L NaCl, pH 7.4 |
| 4 | Elute (5 CV, 225 cm/h): 50 mmol/L acetic acid, pH 3.0 |
| 5 | Sanitize (4 CV, 225 cm/h): 0.1 mol/L NaOH |
| 6 | Regenerate (4 CV, 225 cm/h): 20 mmol/L Tris-acetate, 150 mmol/L NaCl, pH 7.4 |

### Static Binding Capacity Screening

To obtain static binding capacity (SBC) for the mAb on TOYOPEARL Sulfate-650F, the collected purified $IgG_1$ eluate was dialyzed and adjusted to various pH and sodium chloride conditions and a final total $IgG_1$ concentration of 10 mg/mL. The $IgG_1$ was bound to TOYOPEARL Sulfate-650F resin in Resin Seeker plates (20 µL/well) as described below. Adjusted protein A $IgG_1$ eluate was bound to the resin by batch adsorption for 1 hour at ambient temperature. Following incubation, resin was removed from each well by vacuum filtration, and 75 µL samples of each well were read for UV absorbance to determine concentration of unbound protein. Static binding capacity was determined. SBC data was analyzed with SAS JMP 12 software.

| | |
|---|---|
| 1 | Equilibrate (3 ×200 µL/well): 50 mmol/L Tris-acetate*, various NaCl conc. and pH |
| 2 | Bind (200 µL/well, 1 h, RT) : TBL-mAb-01, 10 g/L, various NaCl conc. and pH |

*Note: for pH 4.0 – 5.6, 50 mmol/L acetic acid was titrated with Tris base. For pH 7.2 – 8.4, 50 mmol/L Tris base was titrated with acetic acid.

### Dynamic Binding Capacity Optimization

Dynamic binding capacity (DBC) at 10% breakthrough was determined for TOYOPEARL Sulfate-650F. Dialyzed protein A eluate was adjusted to various pH and sodium chloride concentrations and a final $IgG_1$ concentration of 5 mg/mL. DBC determination was done as shown below. Chromatograph was primed with protein solution to determine UV absorbance (280 nm) at 100% breakthrough.

Protein was bound to column until UV absorbance at 10% breakthrough was reached, and DBC was determined based on the volume of protein solution loaded. DBC data was analyzed with SAS JMP 12 statistical software.

| 1 | Equilibrate (10 CV, 180 cm/h): 50 mmol/L acetate-Tris, various NaCl conc. and pH |
|---|---|
| 2 | Load (45 cm/h): TBL-mAb-01, 5 g/L to ca. 136 mAU |
| 3 | Wash (5 CV, 45 cm/h): equilibration buffer |
| 4 | Elute (5 CV, 45 cm/h): equilibration buffer + 1 mol/L NaCl |
| 5 | Sanitize (5 CV, 60 cm/h): 0.5 mol/L NaOH |
| 6 | Regenerate (5 CV, 60 cm/h): water |

**Elution Optimization**

Collected $IgG_1$ eluate was adjusted to pH 5.2 with 1 mol/L acetic acid and/or 1 mol/L Tris base and 12.1 mS/cm conductivity with 4 mol/L NaCl and/or water. Sample was loaded onto a 6.6 mm ID × 3.0 cm column of TOYOPEARL Sulfate-650F as shown below. Conductivity at elution peak was determined.

| 1 | Equilibrate (10 CV, 180 cm/h): 50 mmol/L acetate-Tris, 100 mmol/L NaCl, pH 5.2 |
|---|---|
| 2 | Load (45 cm/h): TBL-mAb-01, ca. 10 g/L |
| 3 | Wash (5 CV, 45 cm/h): equilibration buffer |
| 4 | Elute (20 CV, 45 cm/h): 50 mmol/L acetate-Tris, 100 – 500 mmol/L NaCl, pH 5.2 |
| 5 | Sanitize (5 CV, 60 cm/h): 0.5 mol/L NaOH |
| 7 | Regenerate: (5 CV, 60 cm/h): water |

Separation was repeated with a 98 mg/mL-resin load, and step gradient elution at 260, 290, or 320 mmol/L NaCl for 10 CV. Throughout step gradient elution, 1-CV fractions were collected and analyzed for protein concentration, aggregate content (SEC chromatography with TSKgel G3000SW$_{XL}$), CHO-HCP (ELISA), and protein A content (ELISA).

| 1 | Equilibrate (10 CV, 180 cm/hr): 50 mmol/L acetate-Tris, 100 mmol/L NaCl, pH 5.2 |
|---|---|
| 2 | Load (45 cm/h): TBL-mAb-01, ca. 10 g/L |
| 3 | Wash (5 CV, 45 cm/h): equilibration buffer |
| 4 | Elute (10 CV, 45 cm/h): equilibration buffer + 260 – 320 mmol/L NaCl |
| 5 | Strip (5 CV, 45 cm/h): 50 mmol/L acetate-Tris, 1.0 mol/L NaCl, pH 5.2 |
| 6 | Sanitize (5 CV, 60 cm/h): 0.5 mol/L NaOH |
| 7 | Regenerate: water (5 CV, 60 cm/h) |

RESULTS AND DISCUSSIONS

**Purification of $IgG_1$ with TOYOPEARL AF-rProtein A HC**

The crude sample containing $IgG_1$ was passed through the protein A column and fractions of $IgG_1$ were collected for further work. Fig. 1 demonstrates that the $IgG_1$ was purified by protein A chromatography. The eluate peak was collected and further analyzed by size exclusion chromatography using a TSKgel G3000SW$_{XL}$ SEC column for monomer and aggregate yield, host cell protein (HCP) content and protein A ligand leaching (see Table 1).

PURIFICATION OF $IgG_1$ FROM CHO SUPERNATANT
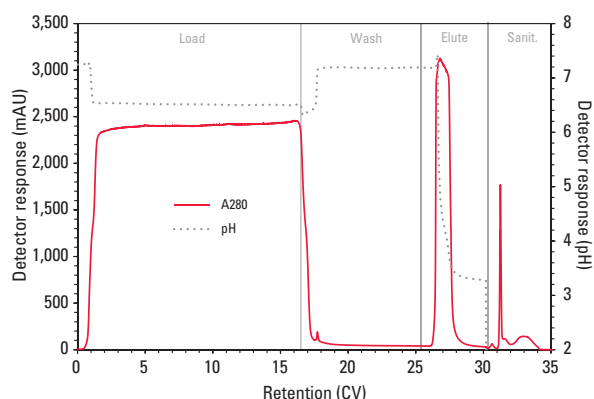


Figure 1

Resin:         TOYOPEARL AF-rProtein A HC-650F
Column:        25 mm ID × 15 cm  (74 mL)
Mobile phase:  A: 20 mmol/L Tris-acetate, 150 mmol/L NaCl, pH 7.4
               B: 50 mmol/L acetic acid
               C: 0.1 mol/L NaOH
Flow rate:     225 cm/h (4 min residence time)
Detection:     UV @ 280 nm (mAU), pH
Temperature:   ambient
Injection vol.: 1200 mL (48 mg/mL-resin load ratio)
Sample:        TBL-mAb-01 CSS @ 2.95 g/L

ANALYSIS DATA FOR THE COLLECTED $IgG_1$ ELUATE PEAK

| Protein A Eluate Analysis | |
|---|---|
| Yield (total IgG) | 99% |
| Aggregate | 4.4% (0.5% HMW, 3.9% dimer) |
| HCP | 1260 ppm |
| Protein A | 1.2 ppm |

Table 1

**Static binding capacity (SBC) screening for TOYOPEARL Sulfate-650F**

To optimize the binding capacity of TOYOPEARL Sulfate-650F resin, the SBC screening was carried out to find out the maximum amount of protein bound to a chromatography medium at given solvent and protein concentration conditions. Fig. 2 shows a binding map that was created for TOYOPEARL Sulfate-650F resin at pH values from 4.0 – 5.6 or 7.2 – 8.4, and 0 – 500 mmol/L NaCl. Maximum protein binding was noted between pH 4.8 and 5.6, from ca. 100 mmol/L – 200 mmol/L NaCl. At lower pH values, NaCl is necessary for protein binding. At higher pH values, significant binding is noted with little to no NaCl.

**Dynamic Binding Capacity (DBC) Optimization**

DBC was optimized by DoE with a three-level, full-factorial method at pH 4.8 – 5.6 and 100 – 200 mmol/L NaCl (results as shown in Figure 3 with data points consolidated in Table 2). A maximum DBC of >120 mg/mL-resin was noted between pH 4.8, 150 mmol/L NaCl, and pH 5.2, 100 mmol/L NaCl. Conditions of pH 5.2, 12.1 mS/cm were used for the elution optimization experiments for maximum binding.

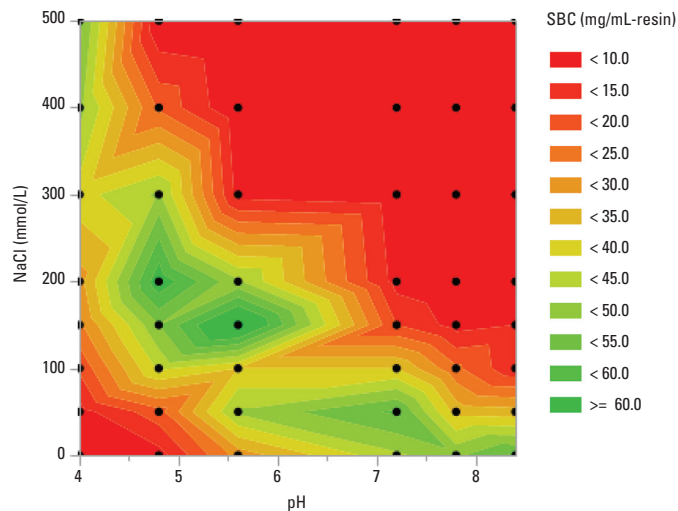SBC SCREENING FOR TOYOPEARL SULFATE-650F USING THE COLLECTED IgG₁ ELUATE



➥ Figure 2

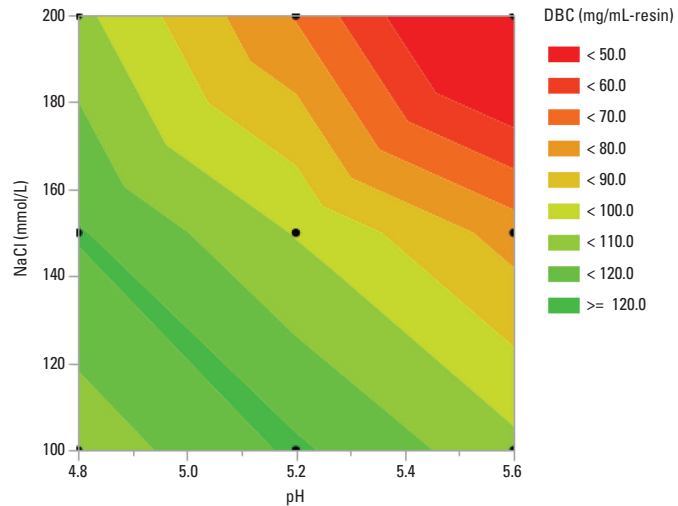DYNAMIC BINDING CAPACITY OPTIMIZATION FOR TOYOPEARL SULFATE-650F USING THE COLLECTED IgG₁



➥ Figure 3

DYNAMIC BINDING CAPACITY DATA AT VARIOUS CONDITIONS (RUN ORDER OF CONDITIONS RANDOMIZED)

| Exp. | Run | Load pH | Load NaCl (mmol/L) | DBC (mg/mL) |
|------|-----|---------|--------------------|-------------|
| 1 | 5 | 4.8 | 100 | 104 |
| 2 | 1 | 5.6 | 100 | 103 |
| 3 | 2 | 4.8 | 200 | 103 |
| 4 | 4 | 5.6 | 200 | 23 |
| 5 | 8 | 4.8 | 150 | 121 |
| 6 | 9 | 5.6 | 150 | 76 |
| 7 | 7 | 5.2 | 100 | 122 |
| 8 | 6 | 5.2 | 200 | 69 |
| 9 | 3 | 5.2 | 150 | 99 |
| 10 | 10 | 5.2 | 150 | 102 |

➥ Table 2

## Optimize conditions for separation and elution of monomer and high molecular weight peaks

To optimize elution conditions, a gradient elution was performed at pH 5.2. A peak conductivity of 30.1 mS/cm was noted (ca. 288 mmol/L NaCl). Experiment was repeated as a step gradient at 260, 290, or 320 mmol/L NaCl (see Fig 4). Due to peak tailing during elution, 1-CV fractions were collected throughout elution. Fractions were analyzed for IgG₁ concentration, aggregate, HCP and protein A, and results were analyzed to determine optimum NaCl concentration and peak volume.

Peaks were analyzed for recovery, aggregates, HCP and protein A content (Figure 5). Data analysis suggests the optimum aggregate and HCP removal are obtained at 260 mmol/L NaCl in elution buffer and maximum (9 CV) elution volume. Protein A ligand content at these conditions (40 ppb) is significantly lower than that found in the load material (1200 ppb). Data is consolidated in Table 3.

PROFILING O THE COLLECTED IgG₁ ELUATE PEAK SEPARATED BY TOYOPEARL SULFATE-650F AT VARIOUS CONDITIONS
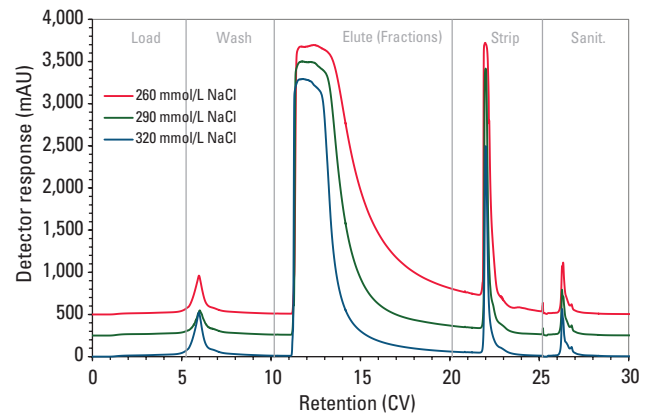


➥ Figure 4

Resin:   TOYOPEARL Sulfate-650F Column;  6.6 mm ID × 3.0 cm (1.0 mL)
Mobile phase: A: 50 mmol/L acetate-Tris, 100 mmol/L NaCl, pH 5.2
        B: 50 mmol/L acetate-Tris, pH 5.2, NaCl as indicated
        C: 50 mmol/L acetate-Tris, 1.0 mol/L NaCl, pH 5.2
        D: 0.5 mol/L NaOH
Flow rate:   45 cm/h (4 min residence time)
Detection:   UV @ 280 nm (mAU)
Temperature: ambient
Injection vol.: 5.3 mL (97 mg/mL-resin load ratio)
Sample:   TBL-mAb-01, 19.1 mg/mL

| Exp. | Run | Elution NaCl (mmol/L) | Pool volume (CV) | Recovery (% monomer) | Aggregate (% dimer/ HMW) | HCP (ppm) | Protein A (ppm) |
|------|-----|------------------------|-------------------|-----------------------|---------------------------|-----------|------------------|
| | | Load | | | 3.9/0.54 | 1260 | 1.2 |
| | | 260 | 3 | 63.1 | 2.6/0.09 | 141 | 0.009 |
| 1 | 2 | 260 | 6 | 77.5 | 2.4/0.07 | 133 | 0.033 |
| | | 260 | 9 | 82.6 | 2.4/0.07 | 134 | 0.040 |
| | | 290 | 3 | 78.3 | 3.0/0.13 | 161 | 0.007 |
| 2 | 1 | 290 | 6 | 86.3 | 3.0/0.12 | 165 | 0.042 |
| | | 290 | 9 | 88.7 | 3.1/0.12 | 170 | 0.060 |
| | | 320 | 3 | 83.4 | 4.3/0.20 | 171 | 0.003 |
| 3 | 3 | 320 | 5 | 87.7 | 4.4/0.19 | 181 | 0.039 |
| | | 320 | 7 | 90.2 | 4.5/0.19 | 185 | 0.067 |

➥ Table 3

## ANALYSIS DATA OF THE COLLECTED ELUATE PEAKS FROM TOYOPEARL SULFATE-650F
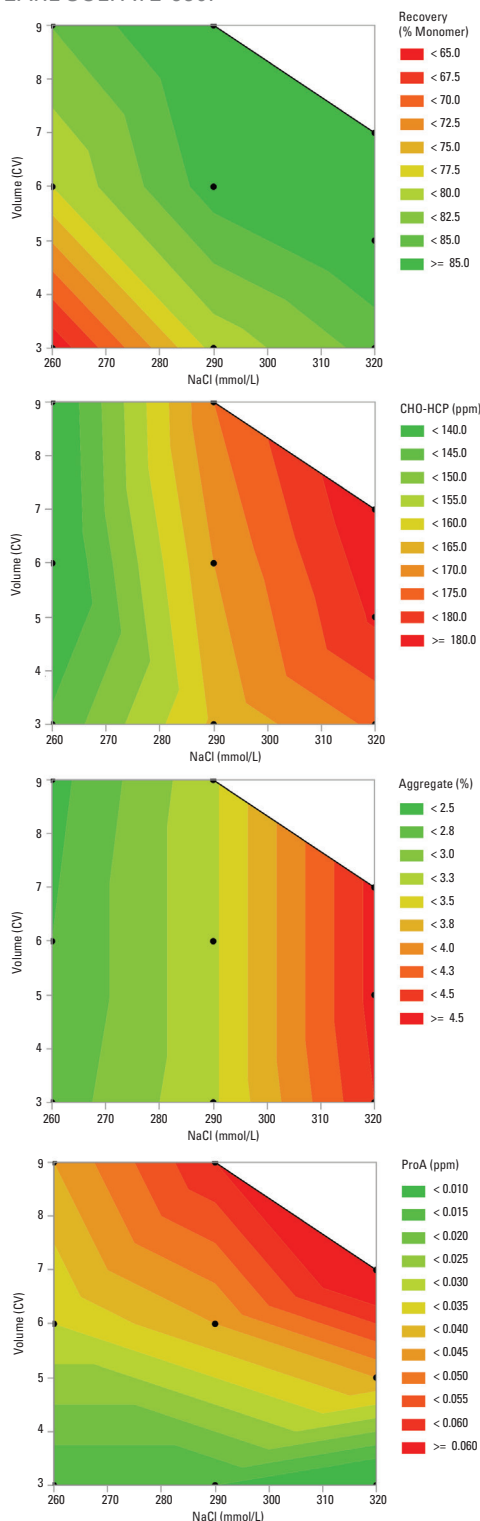


➤ Figure 5

## INTEGRATED PEAK DATA FROM SEC COLUMN

| Impurity | ProA eluate | Sulfate eluate |
|---|---|---|
| Dimer % | 3.9 | 2.4 |
| HMW % | 0.54 | 0.07 |
| HCP (ppm) | 1260 | 134 |
| ProA (ppm) | 1.2 | 0.6040 |

➤ Table 4

## COLLECTED MONOMER PEAKS ANALYZED BY SEC COLUMN
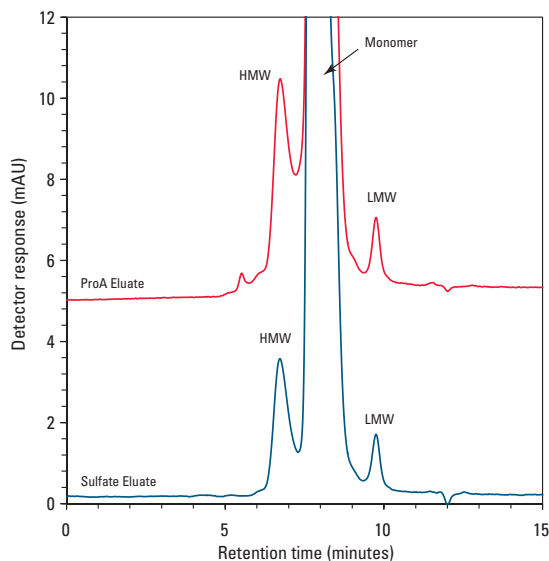


| Column: | TSKgel G3000SW$_{XL}$ (7.8 mm ID x 30 cm) |
|---|---|
| Mobile phase: | 0.1 mol/L phosphate, 0.1 mol/L sodium sulfate, 0.1% sodium azide, pH 6.7 |
| Gradient: | isocratic |
| Flow rate: | 1.00 mL/min |
| Detection: | UV @ 280 nm (mAU) |
| Temperature: | 25° C |
| Injection vol.: | 10 µL (100 µg total IgG) |
| Sample: | ProA or Sulfate eluate as indicated |

➤ Figure 6

Figure 6 shows data from the SEC analysis of the eluate pool at 260 mmol/L NaCl, 9 CV volume. Data shows there is a reduction in aggregate content (in particular HMW impurities), relative to the collected IgG$_1$ eluate peak material from the protein A resin eluate peak.

The peaks from the SEC column were analyzed for high molecular weight, HCP and protein A ligand content. Table 4 shows that after passing through the TOYOPEARL Sulfate-650F resin, the collected IgG$_1$ peak has significantly reduced amounts of HMW, HCP and protein A ligand. This suggests that TOYOPEARL Sulfate-650F resin can effectively remove and reduce impurities of IgG$_1$.

### CONCLUSIONS

The TOYOPEARL Sulfate-650F resin offers a high dynamic binding capacity (>120 mg/mL-resin) with DBC maxima at pH 4.8, 150 mmol/L NaCl and pH 5.2, 100 mmol/L NaCl. With elution at pH 5.2, recovery and impurity removal (aggregate, HCP, leached protein A) is optimal. In fact, analyzed data of the collected IgG$_1$ monomer peak from the TOYOPEARL Sulfate-650F resin column showed that its purity was significantly improved with an acceptable amount of HMW proteins and HCP while nearly no protein A ligand was detected in the collected IgG$_1$ peak. By selecting this strong cation exchange resin as a step after mAb post-protein A purification, only a minimal adjustment to pH or salt concentration to the sample is needed.
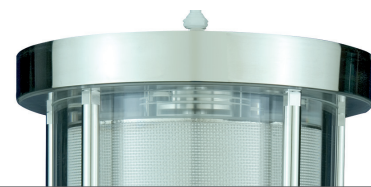
# TOYOPEARL NH2-750F AGGREGATE REMOVAL FROM MONOCLONAL ANTIBODIES

Given the current drive to reduce the cost of manufacturing for biological therapeutics, it is incumbent upon chromatographers and process engineers to streamline the manufacturing process wherever possible. As a result of this need, the demands on downstream unit operations have increased to the point where a single processing step is expected to accomplish a multitude of purification objectives.

Anion exchange chromatography is a widely used technique for protein capture or impurity removal. Typically, anion exchange resins with quaternary amine or DEAE ligands are used. However, these more conventional resins have the disadvantage of reduced capacity for proteins in relatively high salt concentrations associated with post-protein A purification monoclonal antibodies (mAbs) or undiluted biological feedstock. In order to use a DEAE or quaternary amine resin at these process stages, the column load material must be diluted to adjust its conductivity to approximately 5 mS/cm. TOYOPEARL NH2-750F is a new salt tolerant anion exchange resin for process scale applications. This resin is based on the TOYOPEARL HW-75F size exclusion resin functionalized with primary amine groups. This allows the TOYOPEARL NH2-750F resin to maintain its capacity at conductivities up to 15 mS/cm. Table 1 lists the properties of the TOYOPEARL NH2-750F resin.

This new resin uses the same polymethacrylate backbone as all other TOYOPEARL chromatography resins. Thus, it has similar pressure-flow and chemical stability characteristics. TOYOPEARL NH2-750F resin is ideal for the intermediate purification of mAbs where aggregates and other impurities are removed from the target of interest without having to dilute or buffer exchange the product prior to loading.

The data presented here demonstrate the capabilities of TOYOPEARL NH2-750F to remove dimer and higher order aggregates from the monomer of a protein A purified IgG1 monoclonal antibody.

SEC ANALYSIS OF mAb PRIOR TO AGGREGATE REMOVAL BY TOYOPEARL NH2-750F
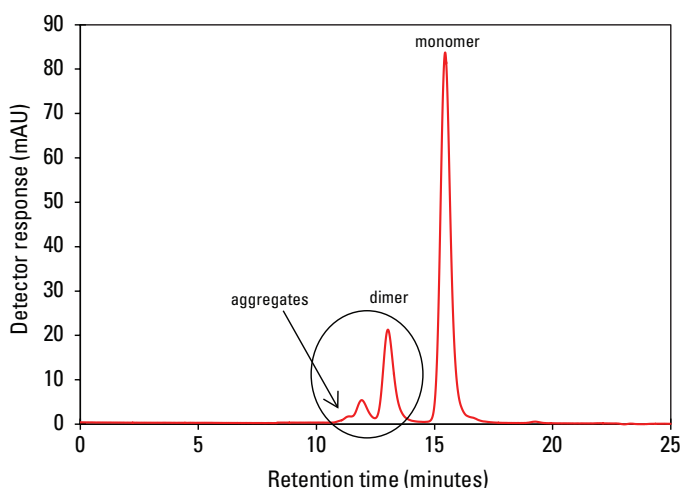


➤ Figure 1

Column: TSKgel G3000SW$_{XL}$, 7.8 mm ID × 30 cm L; Mobile phase: A. 0.1 mol/L sodium phosphate, 0.2 mol/L arginine, pH 6.8; Flow rate: 0.5 mL/min; Detection: UV @ 280 nm; Sample: mAb (IgG$_1$), post protein A

## EXPERIMENTAL CONDITIONS/RESULTS

Figure 1 is a size exclusion chromatography analysis of an aliquot of the protein A purified IgG$_1$ mAb on a TSKgel G3000SW$_{XL}$ column. This sample is representative of the material that will be loaded onto the TOYOPEARL NH2-750F column. In this Figure, the presence of monomer, dimer, and higher order aggregates can be seen.

For the dimer/aggregate removal experiment, a 5 mm ID × 5 cm column was packed with TOYOPEARL NH2-750F resin. The column was equilibrated with 20 mmol/L Tris-HCl, pH 8.0 (mobile phase A) for 5 CV.  A 0.5 g/L sample of protein A purified mAb (Ig$_1$) was then loaded onto the column, and the  column was washed with mobile phase A.

PROPERTIES OF TOYOPEARL NH2-750F

| | |
|---|---|
| Particle size | 30-60 µm |
| Pore size | > 100 nm |
| Ion Exchange Capacity | 0.07 – 0.13 eq/L resin |
| Shipping buffer | 20% ethanol |
| SBC | ≥ 70 g/L resin |

➤ Table 1

A 60 minute linear gradient from 0-100% mobile phase B (mobile phase A + 1.0 mol/L NaCl) was used to separate the mAb monomer (peak 1) from mAb dimer and aggregates (peak 2). The individual peaks were collected as fractions 1 and 2 (Figure 2). After elution, the column was sanitized using 0.5 mol/L NaOH.

The purified mAb monomer (fraction 1) and the mAb aggregate (fraction 2) peaks were analyzed by size exclusion chromatography using a TSKgel G3000SW$_{XL}$ column to verify the separation of dimer and aggregates from the mAb monomer.

There was no dimer or higher order aggregate present in the mAb monomer peak, as can be seen in Figure 3. Analysis of the aggregate peak in Figure 4 does indicate that a small amount of mAb monomer was co-eluted with the dimer and aggregates in this experiment.
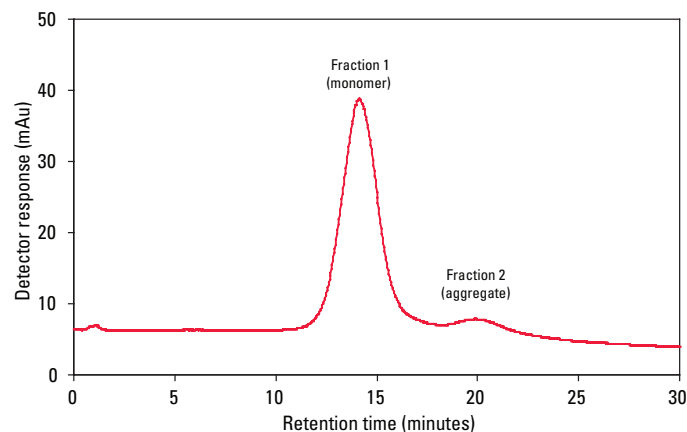
PURIFICATION OF mAb ON TOYOPEARL NH$_2$-750F COLUMN



➤ Figure 2 ························································

Resin: TOYOPEARL NH$_2$-750F; Column: 5 mm ID × 5 cm L (1 mL); Mobile phase: A. 20 mmol/L Tris-HCl, pH 8.0, B. mobile phase A + 1 mol/L NaCl; Gradient: 0 – 100% B (60 minutes); Flow rate: 300 cm/h (1 mL/min); Detection: UV @ 280 nm; Sample: mAb (IgG$_1$), 0.5 g/L

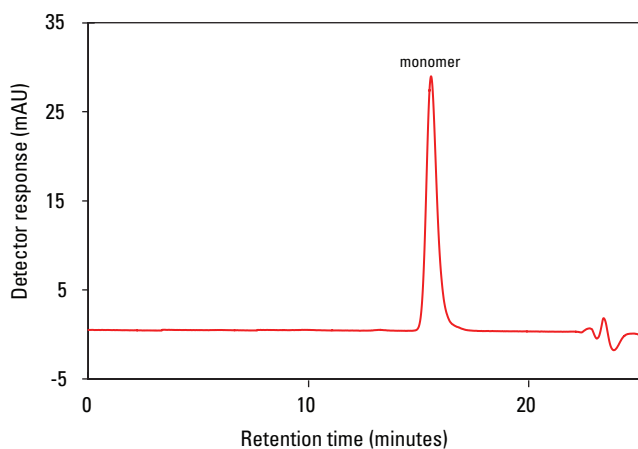SEC ANAYLSIS OF PURIFIED mAb MONOMER FROM TOYOPEARL NH$_2$-750F (FRACTION 1)



➤ Figure 3 ························································

Column: TSKgel G3000SW$_{XL}$, 7.8 mm ID × 30 cm L; Mobile phase: A. 0.1 mol/L sodium phosphate, 0.2 mol/L arginine, pH 6.8; Flow rate: 0.5 mL/min; Detection: UV @ 280 nm; Sample: fraction 1, mAb monomer peak

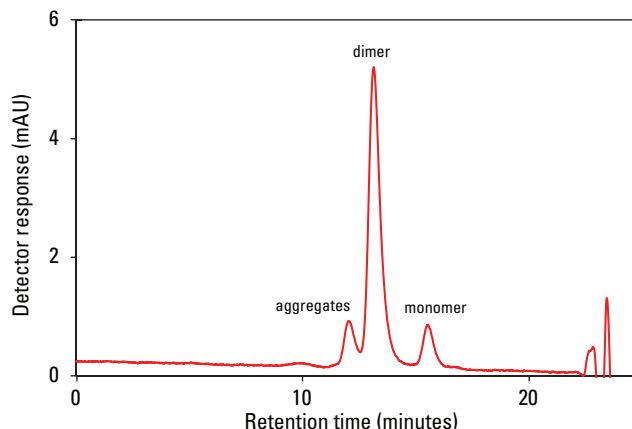SEC ANAYLSIS OF mAb AGGREGATE PEAK FROM TOYOPEARL NH$_2$-750F (FRACTION 2)



➤ Figure 4 ························································

Column: TSKgel G3000SW$_{XL}$ 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

CONCLUSIONS

TOYOPEARL NH$_2$-750F is an effective anion exchange resin for the removal of dimer and higher order aggregates from mAb monomer in a protein A purified antibody without the need for sample dilution or buffer exchange. Though some monomer was co-eluted with the dimer and aggregates in this experiment, further method development may reduce the amount of carryover and increase product yield in the monomer peak.
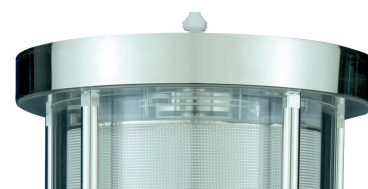
# MAb AGGREGATE REMOVAL THROUGH IEC IN BIND/ELUTE AND FLOW-THROUGH MODE

Purification schemes for monoclonal antibodies typically consist of three chromatographic steps accompanied by filtration steps. The common Protein A capturing is typically followed by ion exchange (IEC), hydrophobic interaction (HIC) or mixed-mode polishing steps. Residual DNA, viruses, and host cell proteins are usually removed by flow-through anion exchange chromatography while aggregates can be reduced through a cation exchange, mixed-mode, or HIC step.

The salt tolerant anion exchange resin TOYOPEARL NH2-750F provides a unique selectivity compared to other anion exchange resins and was found to be suited for aggregate removal, too. Herein we describe the development of an anion exchange polishing step for the purification of a monoclonal antibody by using TOYOPEARL NH2-750F. In general, anion exchange resins can be used in bind and elute (B/E) mode as well as in flow-through (FT) mode. Both options were evaluated. To increase the amount of aggregates of the test sample, a monoclonal antibody was aggregated by acidic incubation and subsequently diluted to 1 g/L in loading buffer.

## EXPERIMENTAL

**Ion Exchange Bind/Elute Mode:**

| | |
|---|---|
| Column: | TOYOPEARL NH2-750F (P/N 0023438), 2.0 mL Omnifit column (6.6 mm ID) |
| Mobile Phase: | A: 10 mM Tris/HCl pH 8.0 B: 0.35 M NaCl in A |
| Gradient: | 60 column volumes (CV) to 100%B |
| Linear Flow: | 300 cm/h |
| Detection: | UV @ 280 |
| Load: | 5 mg aggregated mAb |

**Ion Exchange Flow-Through Mode:**

| | |
|---|---|
| Column: | TOYOPEARL NH2-750F, 2.0 mL Omnifit column (6.6 mm ID) |
| Loading Buffer: | 10 mM Tris/HCl pH 7.0, 250 mM NaCL |
| Load: | 100 mg aggregated mAb |

**SEC Analysis of Collected Fractions:**

| | |
|---|---|
| Column: | TSKgel G3000SWXL, 7.8 mm ID x 30 cm L |
| Eluent: | 100 mM sodium phosphate pH 6.7, 100 mM sodium sulfate, 0.05 % sodium azide |
| Volumetric flow: | 1 ml/min |
| Detection: | UV @ 280 nm |

ELUTION PROFILE OF AGGREGATED ANTIBODY ON TOYOPEARL NH2-750F IN BIND AND ELUTE MODE
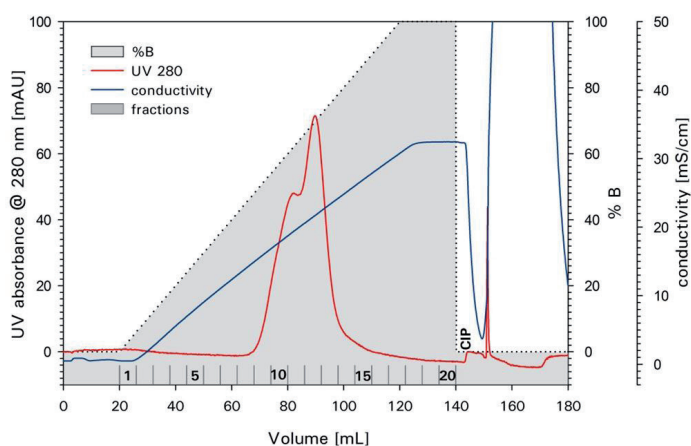


➤ Figure 1

SEC ANALYSIS OF THE BIND/ELUTE OF FRACTIONS OF THE AGGREGATED mAb SAMPLE
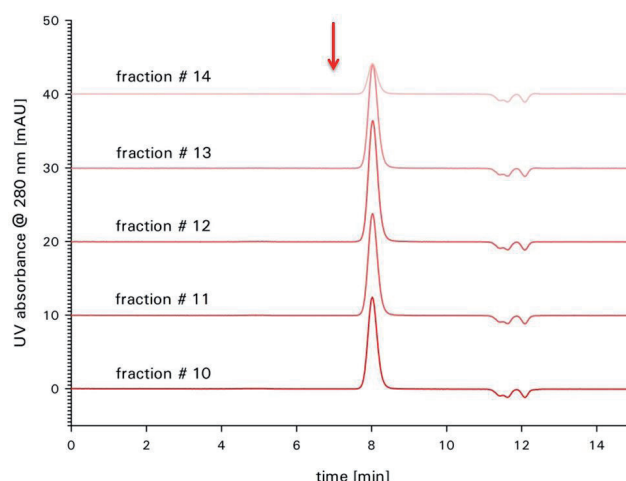


➤ Figure 2

ELUTION PROFILE OF AGGREGATED ANTIBODY ON TOYOPEARL NH2-750F IN FT MODE
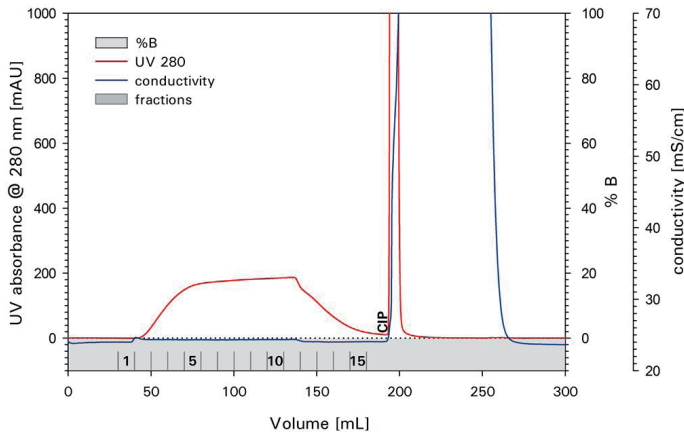


➤ Figure 3

SEC ANALYSIS OF THE AGGREGATED mAb SAMPLE (RED LINE AT 0 MAU) AND FLOW-THROUGH FRACTIONS IN INCREASING FRACTION ORDER FROM BOTTOM TO TOP
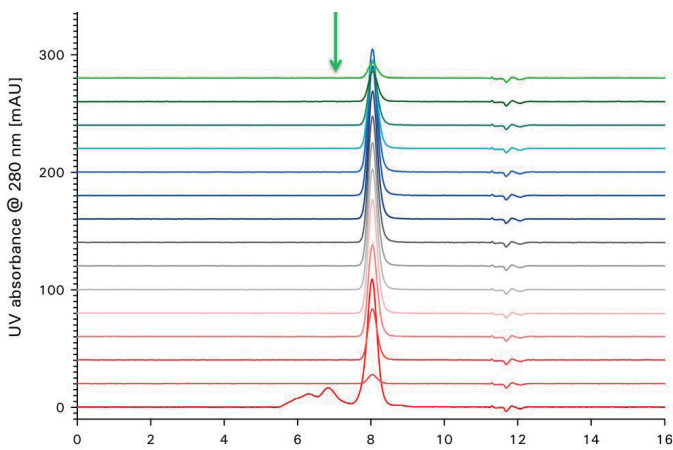


➤ Figure 4

## RESULTS

The dynamic binding capacity of TOYOPEARL NH2-750F for the mAb used in this study was evaluated and a value of 95 mg/ml could be reached with 10 mM Tris/HCl, pH 8.0. Figure 1 shows the elution profile of the aggregated anti-body on TOYOPEARL NH2-650F in B/E mode. Fractions were collected and analyzed by SEC (Figure 2). The results prove that fractions 10 to 14 have an aggregate content below the limit of detection of SEC. The aggregates did not elute in the salt gradient and remained bound until the column was CIPed with sodium hydroxide.

In order to establish a FT polishing step, buffer conditions were evaluated to optimize non-binding conditions for the monomer by varying pH (pH 7 to 8) and salt content (250 -500 mM NaCl). Best results were obtained with 10 mM Tris/HCl, pH 7.0 at a sodium chloride concentration of 250 mM (Figure 3). To analyze the aggregate removal, 100 mg aggregated antibody were loaded on a 2 mL column and fractions of the flow through were analyzed by SEC.  All FT fractions are essentially aggregate free (Figure 4).

## CONCLUSION

TOYOPEARL NH2-750F is a salt tolerant anion exchang resin for downstream processing with high binding capacity for immunoglobulin. The resin is ideally suited to develop a polishing step for monoclonal antibodies by either using the resin in BE mode or in FT mode. For both modes ideal conditions for aggregate removal could be established. An additional benefit when using this resin in FT mode is the delivered excellent viral clearance. Typical virus log reduction exceeds five for enveloped and non-enveloped DNA and RNA viruses.
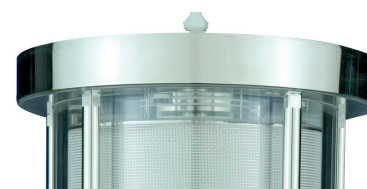
# EFFECTIVE REMOVAL OF ENDOTOXINS WITH AEX RESIN TOYOPEARL NH2-750F

Endotoxins are remnants of bacterial cell walls that may contaminate drug products and cause an immunogenic response. They are often referred to as "pyrogens" due to their fever-inducing effects. Endotoxins may be found in drug products either due to contamination from host cells used to produce a drug product in a bacterial expression system or due to adventitious bacterial contamination in non-microbial products. Thus, endotoxin clearance is a requirement of downstream processing of biologics, especially those derived from microbial expression systems that contain endogenous host cell endotoxin. In this study, we evaluate the ability of TOYOPEARL NH2-750F for the removal of endotoxins by anion exchange chromatography.

## INTRODUCTION

TOYOPEARL NH2-750F, a salt-tolerant anion exchange resin for process scale applications, is based on the TOYOPEARL HW-75F size exclusion resin functionalized with primary amine groups. Table I lists the properties of the TOYOPEARL NH2-750F resin.

|  | TOYOPEARL NH2-750F |
| --- | --- |
| Particle size (µm) | 30-60 |
| Pore size (nm) | > 100 |
| Ion Exchange capacity (eq/L resin) | 0.07 – 0.13 |
| SBC (g/L resin) | ≥ 70 |

➤ Table I ........................................................

TOYOPEARL NH2-750F resin is ideal for the intermediate purification of mAbs and other proteins where aggregates and other negatively charged impurities, such as DNA and endotoxins, are removed from the target of interest without having to dilute or buffer exchange the product prior to loading.

The data presented here demonstrate the capabilities of TOYOPEARL NH2-750F to remove endotoxin in a flow-through chromatography process.

## EXPERIMENTAL CONDITIONS

An ÄKTA® system was flushed with 75 mL of 0.1 mol/L phosphoric acid, followed by 75 mL of E-pure water. The system was then flushed with 0.5 mol/L sodium hydroxide and incubated for a minimum of 1 hour at ambient temperature. Following the incubation, the system was flushed with 75 mL of E-pure water, and then with 20 mmol/L Tris base, pH 7.4, until a stable conductivity and pH baseline was noted. Adequate system cleaning was verified by Limulus amebocyte lysate (LAL) assay.

A TOYOPEARL NH2-750F MiniChrom column (8 mm ID × 10 cm) was cleaned-in-place with 5 CV of 0.5 mol/L sodium hydroxide with a 15 minute contact time. The column was regenerated with 2 CV of 20 mmol/L Tris base, 1 mol/L NaCl, pH 7.4, and then equilibrated with 20 mmol/L Tris base, pH 7.4, until a stable conductivity and pH baseline was obtained (ca. 3 CV).

All solutions used in this experiment were tested for background endotoxin levels by LAL assay and were found to be endotoxin free, though the strip buffer did show some trace amounts of endotoxin with the LAL assay. Therefore the strip sample was diluted with endotoxin-free water prior to testing.

A 5 mg aliquot of lipopolysaccharide (LPS) was suspended in 1.0 mL endotoxin-free water with vigorous mixing for 5 minutes. Dilutions of the LPS solution were tested for endotoxin activity by LAL assay. The endotoxin stock solution was protected from light and refrigerated until use.

The column was equilibrated with 20 mmol/L Tris base, pH 7.4, until a stable conductivity and pH baseline was obtained. A 50 mL sample of equilibration buffer (20 mmol/L Tris base, pH 7.4) was spiked with standardized LPS solution to a final concentration of approximately 100,000 EU/mL. The column was then loaded with spiked equilibration buffer and 2 CV (10 mL) flow-through fractions were collected. Following loading, the column was washed with 3 CV of equilibration buffer and stripped with 3 CV of a high-salt buffer (20 mmol/L Tris base, 1 mol/L NaCl, pH 7.4). Fractions were collected for both wash and strip steps. The column was cleaned-in-place, as described previously, prior to subsequent runs or storage in 20% ethanol.

The load material, flow-through, wash, and strip fractions were all tested for endotoxin by LAL assay.

## RESULTS

For the endotoxin clearance, a solution of *E. coli* lipopolysaccharide was prepared in water. A 5 mg/mL LPS solution gave an endotoxin activity of $5.6 \times 10^7$ EU/mL when tested at a 1:1 x $10^8$ dilution. The column loading material was prepared to have an endotoxin content of approximately 100,000 EU/mL in column equilibration buffer. A direct assay of this solution gave a starting concentration of 89,000 EU/mL with a total load of 4,450,000 EU (89,000 EU/ mL × 50 mL).

To calculate the endotoxin clearance, the following method was used:

Sample endotoxin concentration ($C_i$) was determined to be 89,000 EU/mL as stated above. The log endotoxin content ($L_i$) for the load sample was determined from the sample endotoxin concentration and the load volume ($V_i$):

$$L_i = log_{10} \, C_i + log_{10} \, V_i$$

For the load sample:

$$L_{Load} = log_{10} \, C_{Load} + log_{10} \, V_{Load} = log10 \, 89{,}000 \, EU/mL + log_{10} \, 50.0 \, mL = 6.65$$

Endotoxin clearance for a given fraction ($A_i$) was determined by subtraction.

$$A_i = L_{Load} - L_i$$

For the wash sample:

$$A_{Wash} = L_{Load} - L_{Wash} = 6.65 - 0.83 = 5.82$$

As can be seen in Figure 1, there was some minor breakthrough of endotoxin during the wash phase, and the log reduction value for this fraction was 5.82. Please note that this represents a breakthrough of less than 0.0002% of endotoxin from the original load material.

The endotoxin concentration of the flow-through fractions was less than the limit of detection for the assay (0.1 EU/mL); therefore, the minimum log reduction value for each flow-through fraction was 6.7. Figure 1 shows a graphical representation of the log endotoxin clearance for each step in the process.

## CONCLUSIONS

TOYOPEARL NH2-750F is a very effective anion exchange resin for the removal of endotoxin in a flow-through chromatography mode. This experiment shows that TOYOPEARL NH2-750F is capable of reducing endotoxin in a sample to levels below the limit of detection for an LAL assay, in this instance greater than 6.7 logs.

ENDOTOXIN CLEARANCE SUMMARY
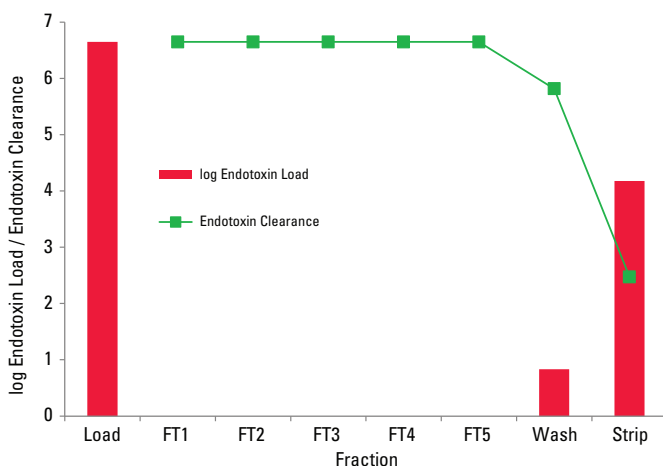


⮂ Figure 1

Resin: TOYOPEARL NH₂-750F
Column: MiniChrom, 8.0 mm ID × 10.0 cm (5.0 mL)
Mobile phase: A: 20 mmol/L Tris base, pH 7.4
B: 20 mmol/L Tris base, 1 mol/L sodium chloride, pH 7.4
Flow rate: 150 cm/hr (1.25 mL/min, 4 min residence time)
Detection: LAL endotoxin assay
Temperature: ambient
Injection vol.: 5 × 2 CV (10 mL) injections, 10 CV (50 mL) total
Sample: 89,000 EU/mL LPS in equilibration buffer
Instrument: ÄKTA Explorer 100

# 4. HYDROXYAPATITE FOR AGGREGATE REMOVAL
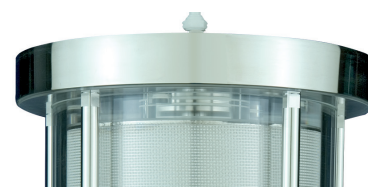
# EFFECTIVE REMOVAL OF mAb AGGREGATE USING Ca⁺⁺Pure-HA® MEDIA WITH POTASSIUM SALTS

## INTRODUCTION

Downstream process chromatography scientists are constantly seeking for better and more selective ways to remove aggregates and other process related impurities from a monoclonal antibody (mAb) monomer. Making use of chromatography resins with better selectivity, resolution and capacity is one approach to solving the problem of aggregate removal in monoclonal antibody production.

Ca⁺⁺Pure-HA (hydroxyapatite: $Ca(PO_4)(OH)$) is a form of calcium phosphate used in the chromatographic separation of biomolecules. Unlike other resins available from Tosoh Bioscience, Ca⁺⁺Pure-HA is both the ligand and the base bead. Hydroxyapatite has unique separation properties for biomolecules and Ca⁺⁺Pure-HA offers unparalleled selectivity and resolution for process scale operations. Its highly selective nature often separates proteins otherwise shown to be homogeneous by electrophoresis and other chromatographic techniques.

Ca⁺⁺Pure-HA is a spherical, macroporous form of the hexagonal crystalline structure of hydroxyapatite. It has been sintered at high temperatures for increased mechanical and chemical stability, allowing it to withstand the rigors of industrial-scale applications. Table 1 lists the properties of Ca⁺⁺Pure-HA.

The data presented here demonstrates the capabilities of Ca⁺⁺Pure-HA media operated with potassium salts such as potassium phosphate and potassium chloride, to remove dimer and higher order aggregates from the monomer of a protein A purified IgG₁ monoclonal antibody.

PROPERTIES OF Ca++Pure-HA

|  | Ca⁺⁺Pure-HA |
| --- | --- |
| Particle size (mean): | 39 µm |
| Shipped as: | dry powder |
| pH stability: | 6.5 – 14 |
| Shelf life (estimated): | 10 years |

➤ Table 1

## EXPERIMENTAL CONDITIONS/RESULTS

**Purification of IgG₁ using TOYOPEARL AF-rProtein A HC-650F Resin**

A crude sample containing IgG₁ was passed through a protein A column and fractions of IgG₁ were collected for further work (Figure 1). The eluate peak was collected and further analyzed by size exclusion chromatography using a TSKgel G3000SWₓL SEC column for monomer and aggregate yield, host cell protein (HCP) content and protein A ligand leaching (see Table 2).

PURIFICATION OF IgG₁ FROM CHO SUPERNATANT CRUDE SAMPLE USING TOYOPEARL AF-RPROTEIN A HC-650F RESIN
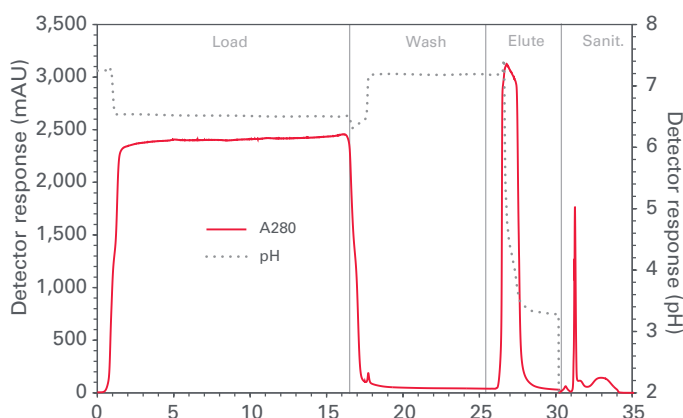


➤ Figure 1

| Resin: | TOYOPEARL AF-rProtein A HC-650F |
| --- | --- |
| Column: | 25 mm ID × 15 cm (74 mL) |
| Mobile phase: | A: 20 mmol/L Tris-acetate, 150 mmol/L NaCl, pH 7.4 |
|  | B: 50 mmol/L acetic acid |
|  | C: 0.1 mol/L NaOH |
| Flow rate: | 225 cm/hr (4 min residence time) |
| Detection: | UV @ 280 nm (mAU), pH |
| Temperature: | ambient |
| Injection vol.: | 1200 mL (48 mg/mL-resin load ratio) |
| Sample: | TBL-mAb-01 CSS @ 2.95 g/L |
| Instrument: | ÄKTA® avant 25 |

ANALYSIS DATA FOR THE COLLECTED IgG₁ ELUATE PEAK

|  | Protein A Eluate Analysis |
| --- | --- |
| Yield (total IgG) | 99% |
| Aggregate | 4.4% (0.5% HMW, 3.9% dimer) |
| HCP | 1260 ppm |
| Protein A | 1.2 ppm |

➤ Table 2

**Removal of mAb aggregates using Ca⁺⁺Pure-HA media**

To remove mAb aggregates from a post-protein A purified sample, Ca⁺⁺Pure-HA media was used in a polishing chromatography step. The below protocol was used.

| | |
|---|---|
| Column: | 5 mm ID × 5 cm (1.0 mL) |
| Mobile phase: | A: 50 mmol/L HEPES, 5 mmol/L $KPO_4$, pH as indicated |
| | B: mobile phase A + 2.0 mol/L potassium chloride, pH as indicated |
| | C: 500 mmol/L KPO4, pH as indicated |
| | D: 1.0 mol/L KOH |
| Gradient: | 69.4% B (chloride), 10 CV |
| | gradient delay, 5 CV |
| Flow rate: | 300 cm/hr (1 min residence time) |
| Detection: | UV @ 280 nm (mAU), |
| | Conductivity (mS/cm), pH |
| Temperature: | ambient |
| Injection vol.: | 5 µL |
| Sample: | 2.0 mg/mL-media partially-purified mAb-01 (0.2 mL injection) |
| Instrument: | ÄKTA avant 25 |
| Method: | Pre-equilibrate, mobile phase C, 3 CV |
| | Equilibrate, mobile phase A, 10 CV |
| | Load |
| | Wash, mobile phase A, 5 CV |
| | Elution, gradient as indicated, 25 CV |
| | Strip, mobile phase C, 5 CV |
| | Sanitize, mobile phase D, 5 CV |

Data from Figure 2 shows a high resolution separation between the monomer peak and the aggregate peak across three different pH conditions. The elution of the monomer peak at pH 6.5 was delayed and broader.

REMOVAL OF mAb AGGREGATES FROM THE POST-PROTEIN A PURIFICATION SAMPLE USING CA⁺⁺PURE-HA MEDIA
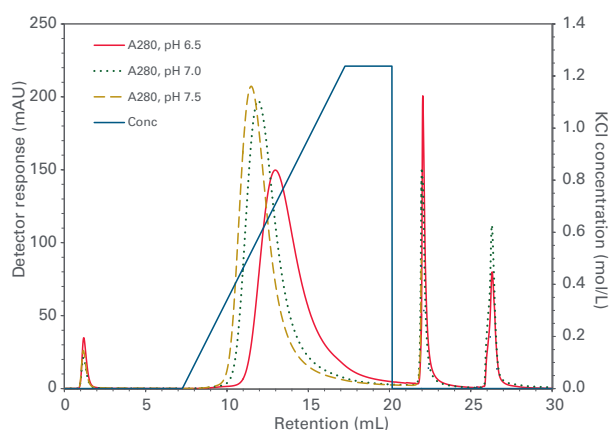


➤ Figure 2

Figure 3 shows a size exclusion chromatography analysis of pooled monomer samples from Figure 2 for the analysis of aggregate content. These samples were injected onto a TSKgel G3000SWxL column. Data analysis show that after the sample passed through Ca⁺⁺Pure HA media under potassium phosphate buffer and potassium chloride operating conditions, mAb aggregates were reduced significantly. In fact, at pH 6.5 operating conditions, the aggregate amount

AGGREGATE ANALYSIS OF POOLED MAB MONOMER PEAKS ELUTED FROM DIFFERENT PH BUFFERS USING SIZE EXCLUSION CHROMATO-GRAPHY
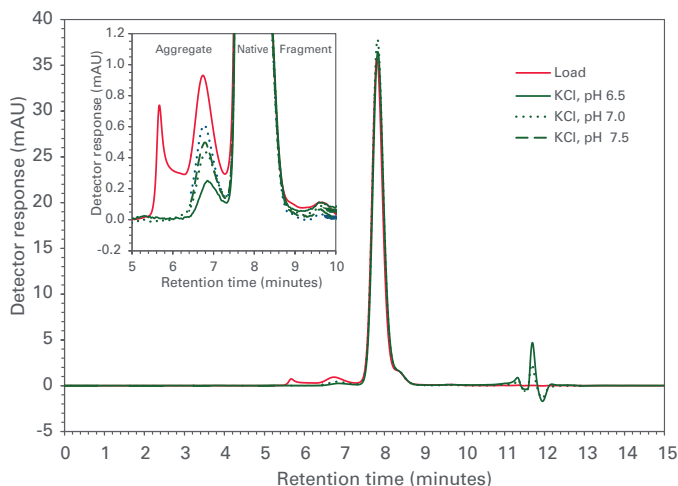


➤ Figure 3

| | |
|---|---|
| Column: | TSKgel G3000SWxL, 7.8 mm ID × 30 cm |
| Mobile phase: | 0.1 mol/L phosphate, 0.1 mol/L $Na_2SO_4$, 0.3% sodium azide, pH 6.7 |
| Flow rate: | 1.0 mL/min |
| Gradient: | isocratic |
| Detection: | UV @ 280 nm |
| Temperature: | 25 °C |
| Injection: | 10 µg native |
| Instrument : | HPLC (400 bar pressure) |

was reduced from 6.6% to as low as 1.3% (Table 3)

| Salt | pH | Peak molarity (mmol/L) | Recovery (% native) | Aggregate (%) | Fragment (%) |
|---|---|---|---|---|---|
| | | Load | | 6.6 | 0.6 |
| KCl | 6.5 | 814 | 72.9 | 1.3 | 0.5 |
| | 7.0 | 615 | 80.0 | 1.8 | 0.3 |
| | 7.5 | 509 | 81.0 | 2.2 | 0.3 |

➤ Table 3

CONCLUSIONS

Ca⁺⁺Pure-HA media is effective for the removal of dimer and higher order aggregates from a purified mAb sample, post-protein A purification step. In fact, when Ca++Pure-HA media was operated using potassium phosphate buffer as a loading buffer and potassium chloride as an elution buffer, the aggregate content was reduced from 6.6% to as low as 1.3%.

# 5. MIXED-MODE CHROMATOGRAPHY

# PROCESS ANALYTICS AND INTERMEDIATE PURIFICATION OF BISPECIFIC ANTIBODIES WITH A NON-AFFINITY PLATFORM

## INTRODUCTION

The therapeutic benefit of monoclonal antibodies (mAb) has been demonstrated in the past decades with uncontestable success as treatments for human disease. Despite mAbs' key features such as specificity, selectivity, and safety, the format has limitations [1][2]. Bispecific antibodies may overcome these difficulties [3].

Multiple formats of bispecific antibodies have been developed, although only the κλ body is fully human and devoid of linkers or mutations; it does not require any genetic modifications of heavy and light chains and results in bispecific antibodies with natural sequences [4]. Different affinity chromatography steps have been developed for the purification of bispecific mAbs.

However, development of a non-affinity-based platform leads to more cost-effective production processes. The advent of hydrophobic cation exchange resins, often referred to as "mixed mode", provides opportunities towards reducing the number of affinity steps in a platform process. Establishing protocols for such chromatography media in the past required extensive screenings. However, the advent of automated parallel chromatography, application of design of experiments, and use of pre-packed columns, accelerate this process.

## MATERIAL AND METHODS

### Bispecific antibodies
A κλ body was produced from recombinant Chinese Hamster Ovary (CHO) cells in a fed-batch fermentation process. The clarified fermenter content, containing the three species κκ monospecific mAb, λλ monospecific mAb and κλ body with a theoretical distribution of 25%, 25%, and 50%, respectively, was partially purified by Protein A chromatography. The pH of the Protein A eluate was readjusted with 2.0 M Tris to pH 6.5 and the material was 0.22 μm filtered.

### Analytical chromatography
Analytical SEC was conducted using a TSKgel G3000SWxl, 7.8 mm ID x 30 cm length column. 100 mM sodium phosphate buffer, pH 6.7, containing 100 mM sodium sulfate was used as liquid phase. The injected volume was 20 μl, and samples were injected without further sample preparation. The UV signal was traced at 280 nm.

nalytical CEX was conducted using a TSKgel SP-STAT, 4.6 mm ID x 10 cm length column. The injected volume was 20 μl, and samples were injected without further sample preparation. The different monospecific and bispecific species were separated in a linear gradient from 5 mM sodium phosphate, pH 6.0, to 100 mM sodium phosphate + 500 mM sodium chloride, pH 6.0.

All columns were connected to analytical HPLC instruments. The applied flow rate was 1 mL/min. UV absorbance at 280 nm was recorded.

### Preparative chromatography
TOYOPEARL Butyl-600M and TOYOPEARL Phenyl-600M were used for preparative scale HIC. 1 M ammonium sulfate in 100 mM sodium phosphate; pH 7.0 was used for column loading. The Protein A elute pool was diluted 1:1 (v/v) with a 2x concentrated stock solution of the loading buffer. 10 mM sodium phosphate at pH 7.0 was used for elution.

TOYOPEARL MX-Trp-650M (TOSOH Bioscience) is a hydrophobic cation exchanger, herein simply referred to as mixed mode resin. 100 mM sodium acetate or sodium phosphate were chosen according to the applied pH. Product elution in linear gradient experiments was accomplished by addition of 500 mM sodium chloride to the binding buffer at constant pH. Buffers for step elution experiments were substituted with sodium chloride to reach the required conductivity.

Dynamic binding capacities were determined with 6.6 mm ID x 2 cm L Omnifit columns (Diba Industries, Cambridge, UK), which were packed with the different resins. The feed stream was diluted to 1 g/L protein and loaded at 150 cm/h.
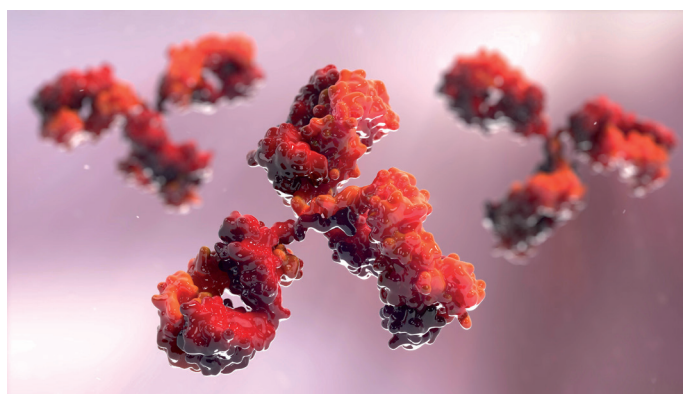
UV absorbance was monitored at 280 nm. The resulting breakthrough curves were used to calculate the dynamic binding capacity at 10 % breakthrough.

Subsequent scouting experiments were conducted with 1 cm ID x 7.5 cm L Omnifit columns. Alternatively, 8 mm IDx 10 cm L MiniChrom pre-packed columns were used. A 50-column volume linear gradient was applied with all resins and different conditions. Buffers for preparative HIC and mixed mode were adapted from analytical chromatography. Collected fractions were analyzed with analytical CEX. Subsequent step elution gradients were developed according to the scouting results. All resins were flushed with 200 mM sodium hydroxide after every cycle, followed by re-equilibration.

RESULTS AND DISCUSSION

**Process analytical chromatography**
Monospecific mAbs in the Protein A elute pool eluted in a uniform peak in SEC. The retention of this peak corresponded to a molecular weight of 150 kDa. The presence of κ- or λ-light chains do not alter the average molecular weight of a mAb to an extent visible in SEC. The relative aggregate content of the Protein A elute pool was below the target of 1 %. Thus, optimization for further aggregate removal was not pursued.

Baseline separation of the three mAb variants was achieved with CEX chromatography using TSKgel SP-STAT (Figure 1). A comparison with the reference ratios indicated that the λλ-monospecific mAb eluted first, followed by the κλ body and the κκ-monospecific mAb. Analytical CEX was used as the method of choice for process analysis during the scouting and evaluation of the preparative chromatography.

**Scouting of intermediate downstream processing**
Because a one-step purification of bispecific mAbs using HIC has previously been described [5], HIC was the first chromatographic mode to be evaluated for the separation of the κλ body from the monospecific mAbs after an initial Protein A capture step. Linear gradient scouting experiments were performed on TOYOPEARL Phenyl-600M and TOYOPEARL Butyl-600M for the separation of κκ-monospecific mAb, λλ-monospecific mAb, and the κλ body present in the Protein A eluate pool. Peak elution from Phenyl-600M and Butyl-600M started when approximately 50 % of the gradient was reached. Resolution using Butyl-600M was greater than Phenyl-600M, with the κκ-monospecific mAb and the κλ body being resolved to baseline. Thus, Butyl-600M was selected for subsequent optimization experiments.

By contrtast with analytical CEX chromatography experiments, baseline resolution could not be achieved at process scale using CEX chromatography resins. A combination of both HIC and IEC interactions may provide sufficient selectivity to accomplish separation of both monospecific mAbs and the κλ body. Hydrophobic CEX (mixed mode) resin was therefore evaluated.

Chromatographic scouting runs of the separation of the κκ-monospecific mAb, the λλ-monospecific mAb, and the κλ body on the mixed mode resin TOYOPEARL MX-Trp-650M have been performed in a linear sodium chloride gradient at pH 5.0, pH 5.5 and pH 6.0. At pH 5.0 and pH 5.5 all components of the Protein A elute pool adsorbed to the resin and protein eluted in the linear sodium chloride gradient in three separate peaks. At pH 6.0, the flow-through fraction contained λλ-monospecific mAb, while the κλ body and the κκ-monospecific mAb were adsorbed to the resin (Figure 2).

ANALYTICAL SEPARATIONS OF THE PROTEIN A ELUTE POOL CONTAINING THE κλ-MONOSPECIFIC mAb, THE κλ-BODY, AND THE λλ-MONOSPECIFIC mAb BY CEX CHROMATOGRAPHY MODE
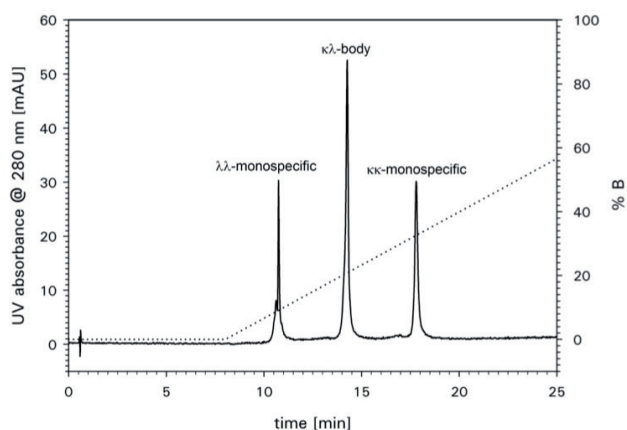


➤ Figure 1

MIXED-MODE SCOUTING RUN ON TOYOPEARL MX-Trp-650M WITH LOADING AT PH 6.0
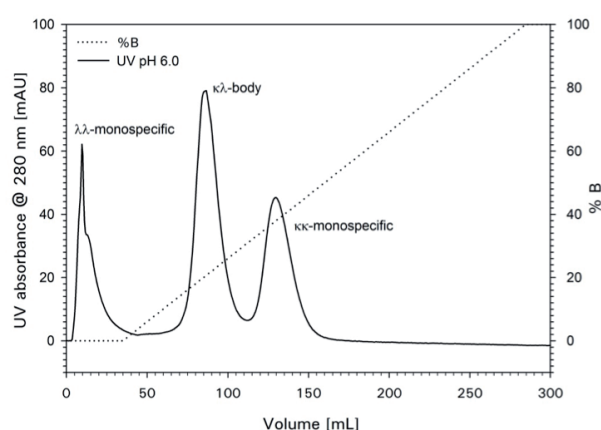


➤ Figure 2

Dynamic binding capacities of TOYOPEARL Butyl-600M and the mixed mode resin TOYOPEARL MX-Trp-650M are presented in Table 1.

**Step-gradient elution Mixed-Mode and Hydrophobic interaction chromatography**

Mixed-mode chromatography and HIC discriminated the κλ body and the monospecific mAbs with orthogonal retention criteria. The selective flow-through of the λλ-monospecific mAb observed at pH 6.0 in the scouting experiments with TOYOPEARL MX-Trp-650M provided an opportunity to develop an efficient step elution protocol for the purification of the κλ body. Figure 3 shows a chromatogram of a step elution separation at pH 6.0. 50-mg protein/mL of the mixed mode resin were loaded. Loaded amounts of bispecific and monospecific mAbs were well above load amounts reported for a strong CEX resin [6]. The λλ-monospecific mAb flowed through the column without adsorbing to the mixed mode resin. The κλ body was recovered in the first sodium chloride step elution. The κκ-monospecific mAb was washed off during cleaning-in-place. The chromatography was performed at a linear flow rate of 300 cm/h, which allowed for fast processing. κλ body purity was approximately 65 %. The majority of the remaining contamination was κκ-monospecific mAb.

Hence, a subsequent purification step was required. Selectivity of TOYOPEARL Butyl-600M is less susceptible to variations in conductivity and pH. Besides, the use of HIC adds another orthogonal separation criterion to the process. This is advantageous with regards to other process-related impurities, such as viruses and DNA. The TOYOPEARL MX-Trp-650M step 1 eluate pool containing κλ body was loaded onto a TOYOPEARL Butyl-600M column. The chromatogram of a step gradient using 1 M ammonium sulfate is shown in Figure 4. The κλ body was recovered at a purity of 99.5 %. Although the Protein A elute pool did not contain significant aggregate levels, even after a low pH hold for virus inactivation, both TOYOPEARL MX-Trp-650M and TOYOPEARL Butyl-600M can be used for aggregate removal at conditions similar to the operating conditions applied here [7]. Hence, it can be expected that the applied conditions would provide aggregate removal, in case a particular κλ body candidate would contain a higher level of aggregates. This is especially important with regards to platform applicability.

DYNAMIC BINDING CAPACITIES OF TOYOPEARL Butyl-650M AND MX-Trp-650M

| Resin | Adsorption buffer | Dynamic binding capacity at 10 % breakthrough |
|---|---|---|
| TOYOPEARL Butyl-600M | 1 M ammonium sulfate + 100 mM sodium phosphate, pH 7.0 | 20 mg/mL |
| | 100 mM sodium acetate, pH 5.0 | 76 mg/mL |
| TOYOPEARL MX-Trp-650M | 100 mM sodium acetate, pH 5.5 | 87 mg/mL |
| | 100 mM sodium phosphate, pH 6.0 | n/a |

Table 1

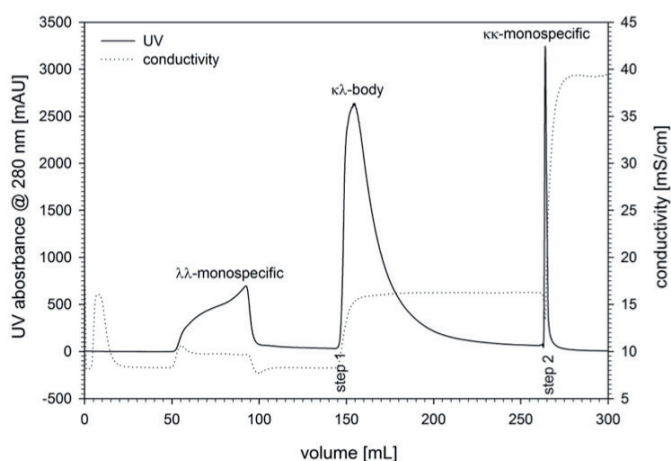SEPARATION OF THE PROTEIN A ELUATE POOL ON THE MIXED MODE RESIN TOYOPEARL MX-TRP-650M IN A STEP-GRADIENT ELUTIO



Figure 3

PURIFICATION OF THE κλ-BODY FROM THE MIXED MODE STEP 1 ELUATE POOL WITH TOYOPEARL BUTYL-600M IN A STEP-GRADIENT APPROACH; 99.5 % PURE κλ-BODY IS RECOVERED DURING STEP 2
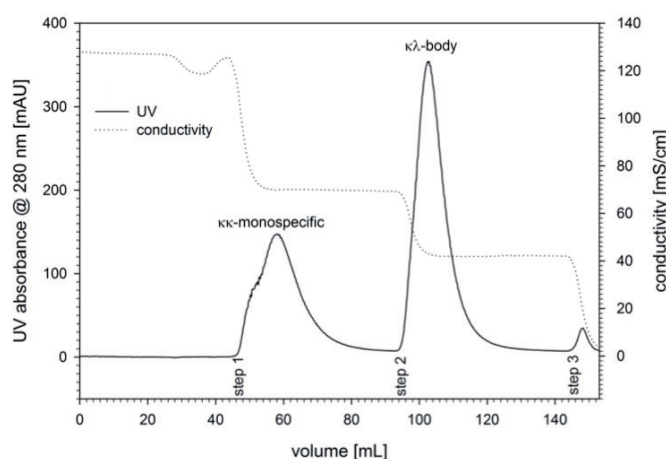


Figure 4

## COMPARABLE PURITY, LOWER COSTS

Modern chromatography resins were evaluated for the purification of a κλ body. Hydrophobic CEX and HIC can replace two subsequent affinity chromatography steps for the purification of a κλ body from the monospecific mAb by-products (Figure 5). The three-step process using mixed mode and HIC chromatography showed comparable yields to a 3-step affinity platform process currently used to purify a κλ body. The excellent selectivity of TOYOPEARL MX-Trp-650M and TOYOPEARL Butyl-600M paves the way for future implementation at research, clinical and commercial manufacturing scales. This approach combining reduced cost of goods and higher binding capacities offers an attractive new version of the purification process for the future manufacture of κλ-bodies.

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[7]      J. Vajda, E. Mueller, E. Bahret, Biotechnol. J. 9 (2014) 555–565.

STATE-OF-THE-ART DOWNSTREAM PROCESSING WORKFLOW FOR THE PURIFICATION OF mAbs (A) AND κλ BODIES (B), COMPARED WITH THE NEW PURIFICATION PROCESS FOR κλ BODIES (C)
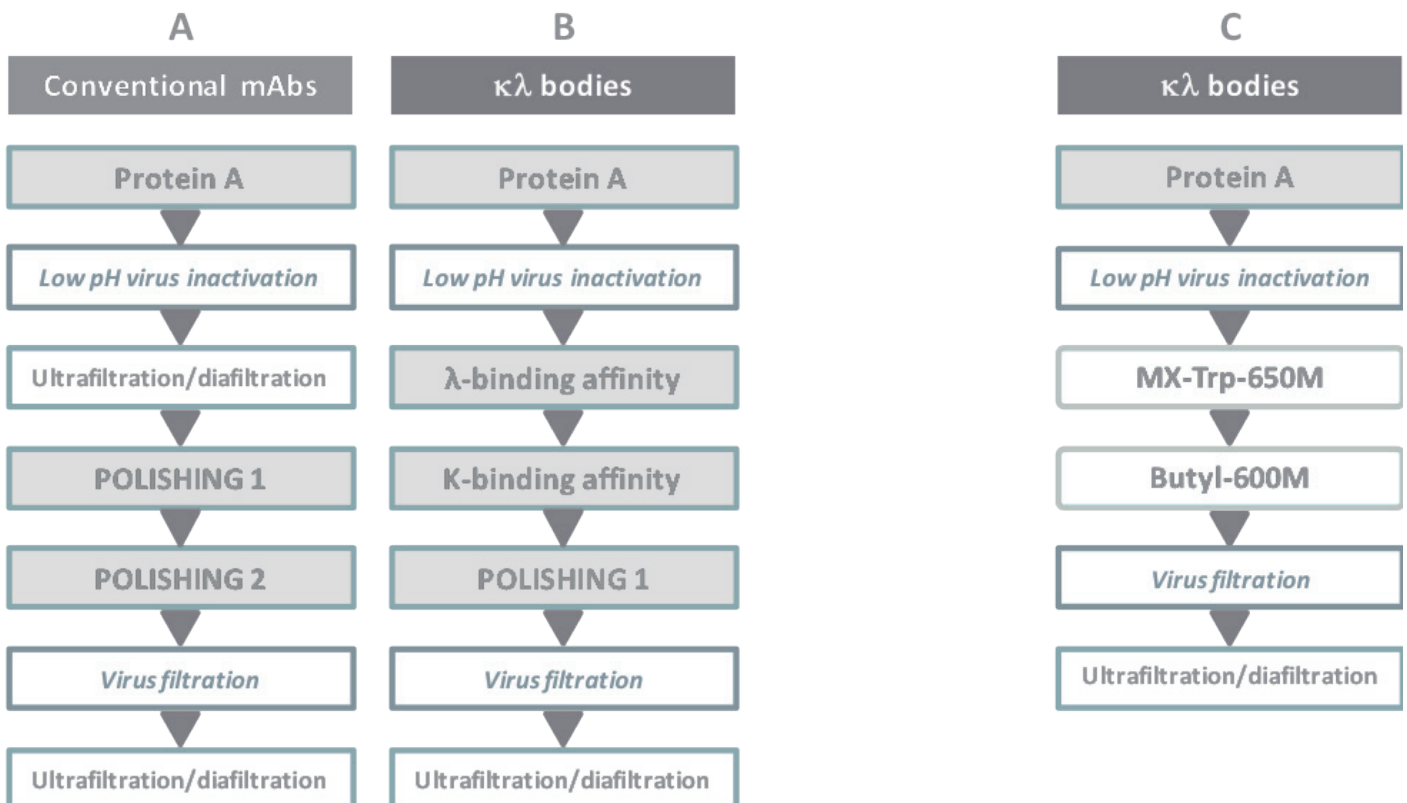


➤ Figure 5

# 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.
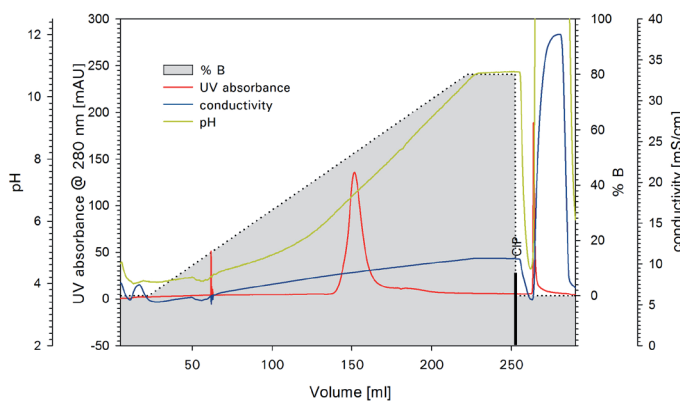
ELUTION BY pH GRADIENT

➤ 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)

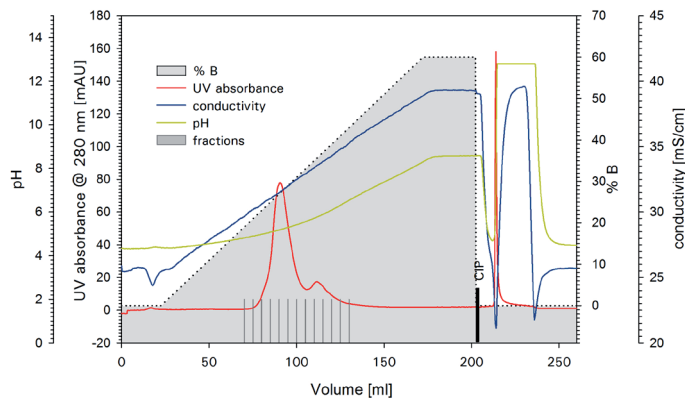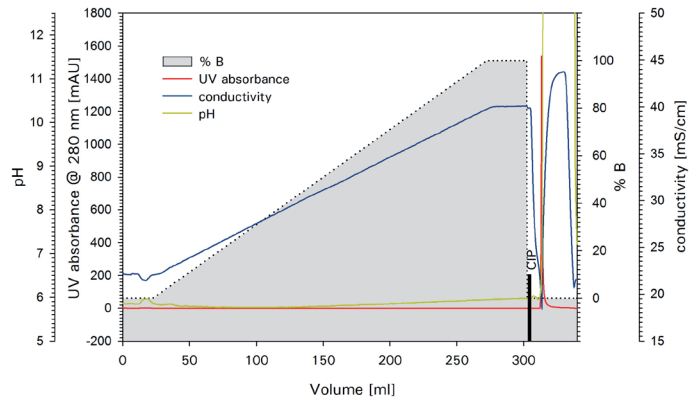ELUTION BY COMBINED pH AND SALT GRADIENT

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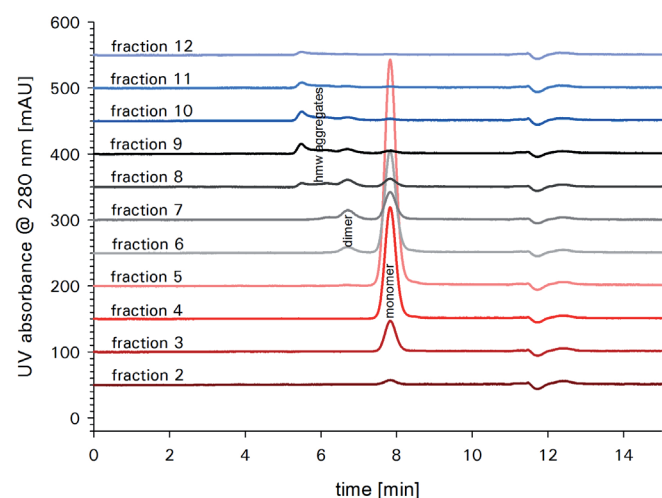
While the salt gradient does not allow protein recovery, the pH gradient leads to the elution of one protein peak. In contrast, the combined pH and salt gradient recovers the protein in two peaks, a monomer peak in the front, followed by the aggregates. Quantitative and qualitative analysis of the collected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

mAb AGGREGATE REMOVAL APPLYING AN UP-SCALA-BLE 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.

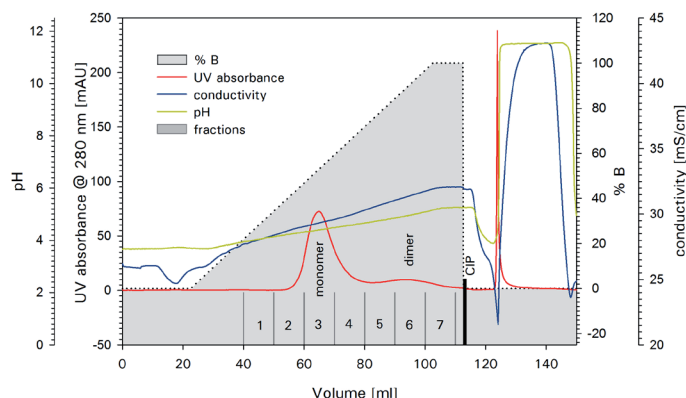From these results, we conclude that TOYOPEARL MX-Trp-650M can be utilized as a highly efficient tool for aggregate

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) |

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).
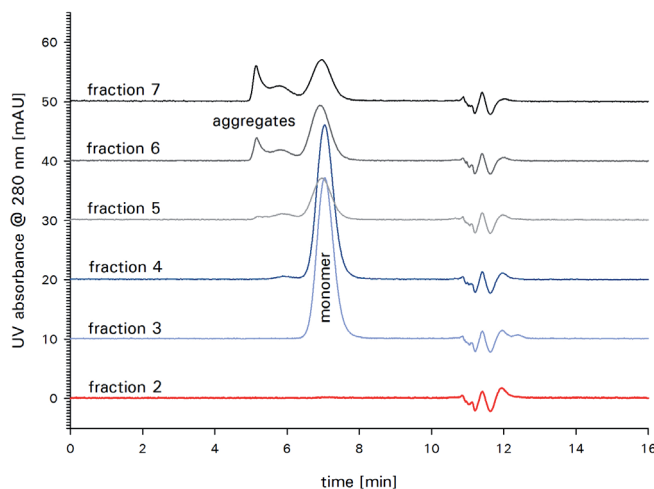


➤ Figure 4

| 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 |
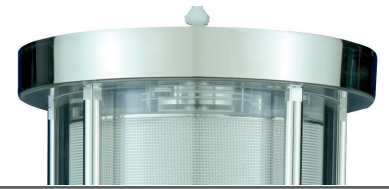
# DEVELOPMENT OF A HIGH CAPACITY MIXED-MODE RESIN FOR HIGH CONDUCTIVITY mAb FEEDSTOCKS

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

TOYOPEARL MX-Trp-650M STRUCTURE
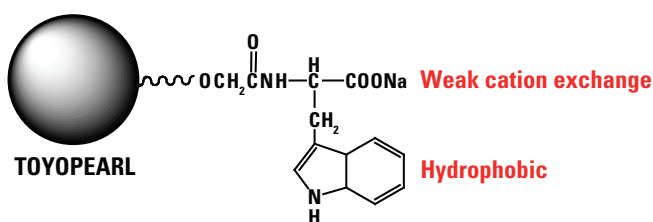


Product name: TOYOPEARL MX-Trp-650M
Particle size: 50-100 µm

➤ Figure 1 ...................................................

## BC 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.

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).

DYNAMIC BINDING CAPACITIES AT HIGH CONDUCTIVITIES

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

➤ Table 1 ===============================================

Resins: TOYOPEARL MX-Trp-650M, Brand M;
Column size: 6 mm ID × 4 cm;
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 @ 280 nm;
Sample: human polyclonal IgG (1 mg/mL);
DBC calculated from 10% height of breakthrough curve.

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.
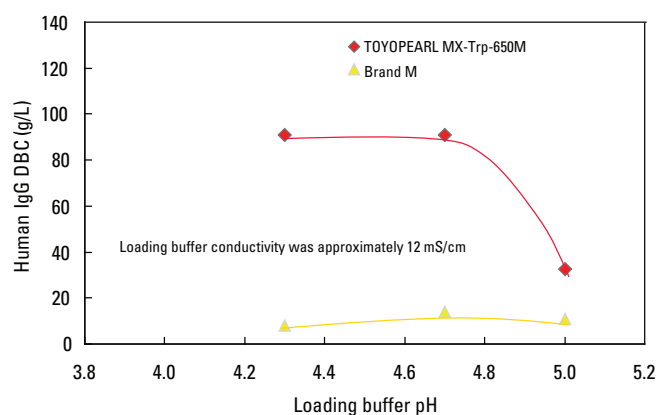
DBC AT VARYING pH



**Figure 2**

Resins: TOYOPEARL MX-Trp-650M, Brand M;
Column size: 6 mm ID × 4 cm;
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 @ 280 nm;
Sample: human polyclonal IgG (1 mg/mL)
DBC calculated from 10% height of breakthrough curve.
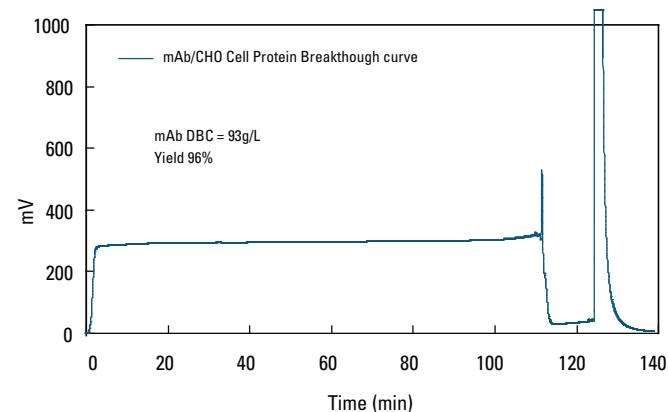
NARROW ELUTION PEAK WIDTHS



**Figure 3**

Resin: TOYOPEARL MX-Trp-650M; Column size: 6 mm ID × 4 cm;
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 @ 280 nm;
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; 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
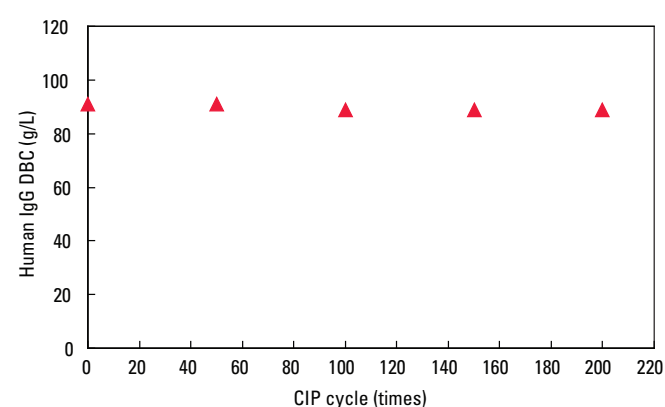


**Figure 5**

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; 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

# 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

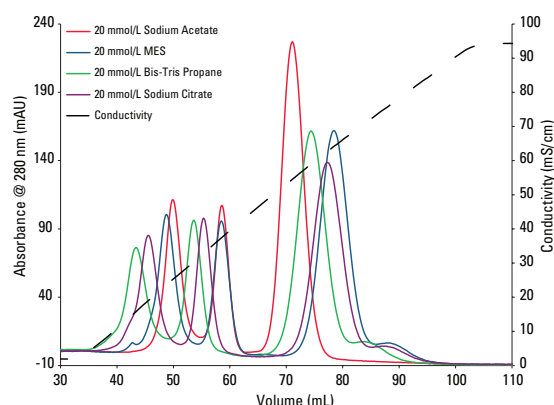TOYOPEARL MX-Trp-650M MULTIPLE BUFFERING SALTS AT PH 6.0



➤ 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)

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 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

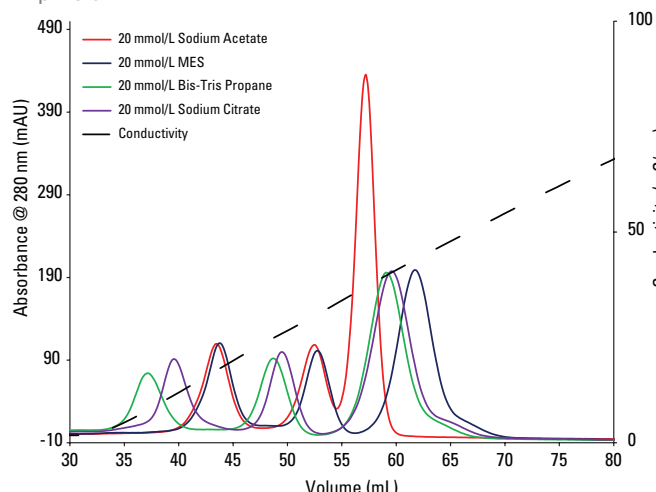TOYOPEARL GigaCap S-650M MULTIPLE BUFFERING SALTS AT pH 6.0



➤ 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 respect 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).

# 6. ADCS PURIFICATION
# AND SALT INFLUENCE ON HIC

# ANTIBODY-DRUG CONJUGATE MIMIC PURIFICATION WITH TOYOPEARL PPG-600M HIC RESIN FOR DAR-SEPARATION

## INTRODUCTION

Antibody-drug conjugates (ADCs) are promising anti-cancer biopharmaceuticals with one of the highest annual growth rates. Four ADCs received market approval to this point. They combine the high selectivity and affinity of an antibody to cancer cells with the toxicity of chemotherapeutics in one molecule.

ADCs consist of a monoclonal antibody, covalently bound via a linker to a highly potent cytotoxic drug. Due to the highly toxic payload, very high safety standards should be implemented during method and process development. ADC-mimic contain a non-toxic payload with similar structure and physicochemical properties as the toxic payload of an ADC. Therefore, they can be used as a model to develop a suitable purification process or analytical method. The ADC-mimic in this work consists of Adalimumab bound to Fluorescein 5-isocyanate (FITC).

The purification process of ADCs is complex due to the heterogeneity of the conjugates. The main challenges are in the isolation of the unconjugated antibody and free drug, and the separation in different Drug-Antibody-Ratio (DAR), which correlates with the potency of the ADC. High DARs are associated with high cytotoxic levels and can cause aggregation, affecting the stability of the ADC. On the other hand, low DARs affect the efficacy of the therapeutics.

The mass spectrometric evaluation shows, that the ADC-mimic developed at Tosoh Bioscience displays a similar drug-to-antibody ratio as real ADCs. Thus, this ADC-mimic is a useful tool for analytical and preparative method development.

Due to the very hydrophobic payload, ADCs are more hydrophobic than normal monoclonal antibodies. An increasing DAR results in an increase in the hydrophobicity of the ADC, which can be used for the separation of different DAR.

A relatively hydrophilic HIC resin, TOYOPEARL PPG-600M, is used in this study. This resin has the benefits of a high recovery due to the relatively hydrophilic ligand together with a high binding capacity, and a wide working pH range.

**TOYOPEARL PPG-600M: the HIC resin for the purification of ADCs with specific DARs**

## MATERIALS AND METHODS

The antibody used in this study is Adalimumab, a biosimilar of Humira®. The ADC-mimic consists of a heterogenic, randomized coupling of fluorescein-5-isothiocyanate through the lysine group to the antibody.

TOYOPEARL PPG-600M, 65 µm, 50 nm hydrophobic interaction resin was used in this study. The resin was packed into an Omnifit® Benchmark column (6.6 mm ID x 10 cm).

A TSKgel Butyl-NPR analytical HIC column (4.6 mm ID x 3.5 cm, 2.5 µm) was used for analyzing collected ADC-mimic fractions.

## PURIFYING ADC-MIMIC USING TOYOPEARL PPG-600M

The ADC-mimic was loaded to a TOYOPEARL PPG-600M column to separate the ADC-mimic in fractions of low, medium and high DAR (Figure 1).

CHROMATOGRAM OF THE ADC-MIMIC BY A THREE-STEP GRADIENT FROM 70-80-100 % B IN TOYOPEARL PPG-600M (6.6 MM ID X 10 CM)
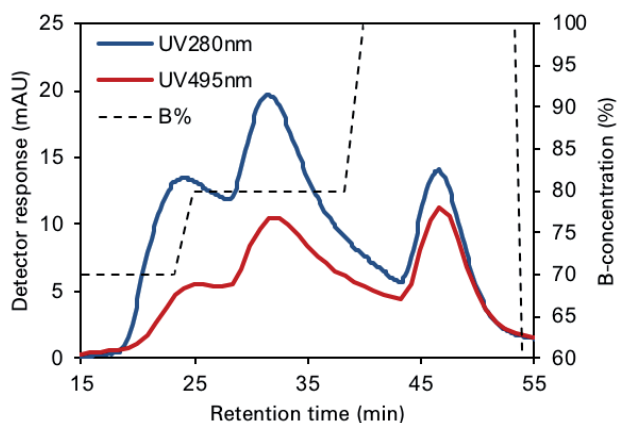
Figure 1

The concentration of low salt buffer in each step can be adjusted to modify the separation:

1. Equilibrate (5 CV, 250 cm/h): 100 mmol/L sodium-phosphate, 1.5 mol/L ammonium sulfate, pH 6.5
2. Load (5 mg/mL-resin, 150 cm/h): FITC-Adalimumab-Miimic
3. Wash (5 CV, 250 cm/h): 100 mmol/L sodium-phosphate, 1.5 mol/L ammonium sulfate, pH 6.5
4. Elute 1 (5 CV, 175 cm/h): 30% 100 mmol/L sodium-phosphate, 1.5 mol/L ammonium sulfate, pH 6.5 + 70 % 100 mmol/L sodium-phosphate pH 6.5
5. Elute 2 (5 CV, 175 cm/h): 20% 100 mmol/L sodium-phosphate, 1.5 mol/L ammonium sulfate, pH 6.5 + 80 % 100 mmol/L sodium-phosphate pH 6.5
6. Elute 3 (5 CV, 175 cm/h): 100 mmol/L sodium-phosphate pH 6.5
7. Sanitize (5 CV, 250 cm/h): 500 mmol/L sodium hydroxide
8. Equilibrate (5 CV, 250 cm/h): 100 mmol/L sodium-phosphate, 1.5 mol/L ammonium sulfate, pH 6.5

## DAR-ANALYSIS USING TSKgel BUTYL-NPR

The eluate for each elution step was fractionated and analyzed on TSKgel Butyl-NPR. Due to the different absorption maxima between FITC (495 nm) and antibody (280 nm), it is possible to calculate an UV-estimated DAR according to the following equation (Figure 2):

$$DAR_{estimated} = \frac{2.77 * A_{495}}{A_{280} - (0,35 * A_{495})}$$

ABSORBANCE DIFFERENCE OF THE ADC-MIMIC AND ESTIMATED DAR FOR EACH ELUTION STEP
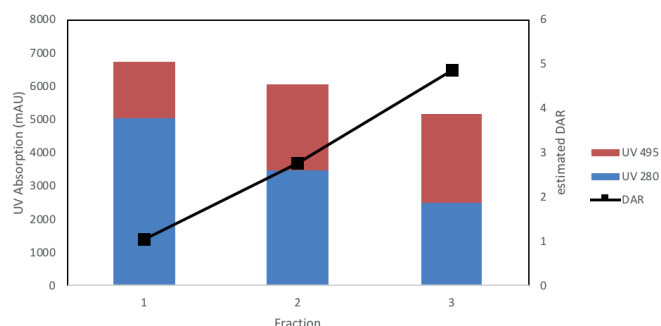
Figure 2

Absorbance difference of the ADC-mimics and estimated DAR for each elution step on PPG-600M. Each bar represents the absorbance of the ADC-mimics in blue for the antibody and red for FITC. The fractions from the step gradient were analyzed on TSKgel Butyl NPR (4.6 mm ID x 3.5 cm)

## CONCLUSIONS

Due to the randomized and heterogenic coupling, the purification process is more complex than the separation of site-directed ADC, since we see a variation on DAR from 0 to 6.

Nevertheless, the TOYOPEARL PPG-600M resin offers sufficient selectivity in step gradient to separate the ADC-Conjugate into groups with low, medium and high DARs. The low DAR fraction has an average DAR of 1, the medium DAR fraction of 3 and the high DAR fraction a medium DAR of 5.

All approved ADCs exhibit DARs between 2 and 4. By slight adjustments of the concentration during the step gradient, the process can be adjusted to isolate ADCs within target DAR ranges.

# 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, Phenyl-600M and 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/mL resin 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 behaviour is observed for TOYOPEARL Phenyl-600M (Figure 2) and Butyl-600M (Figure 3).
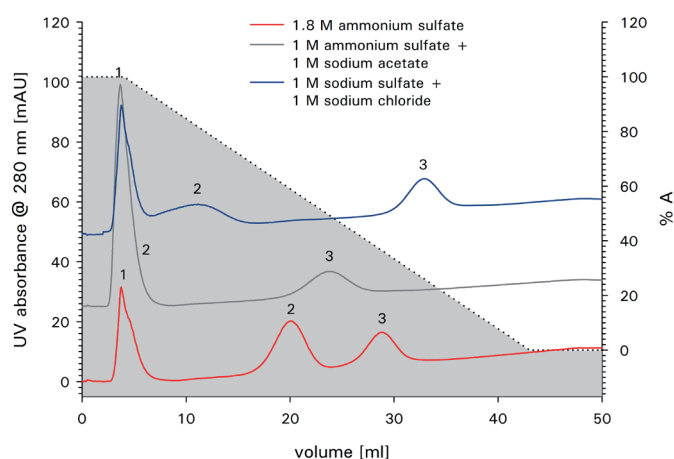


➤ Figure 1

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.
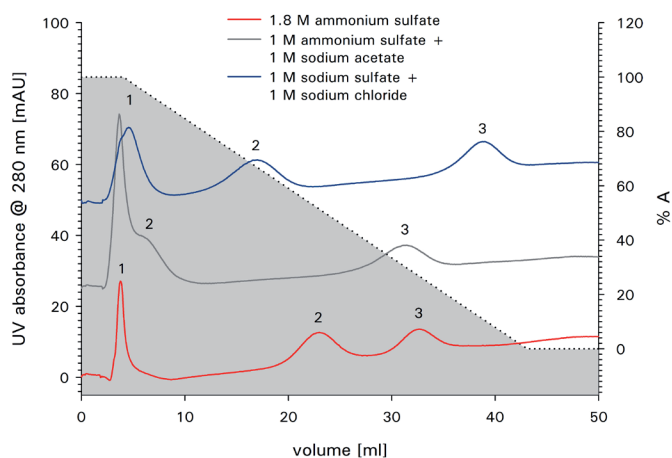


➤ Figure 2

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.
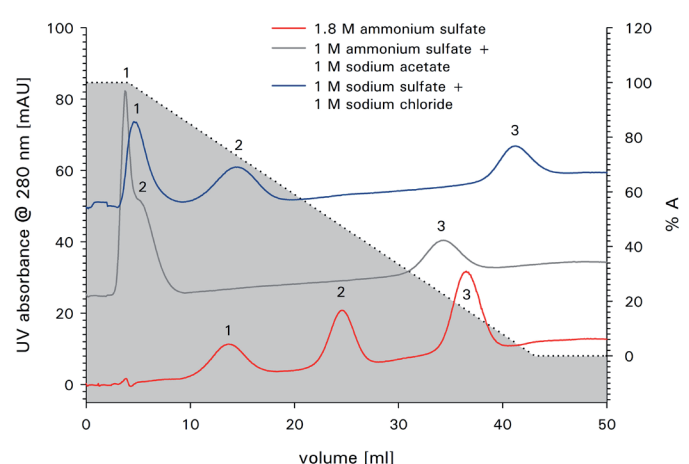


➤ Figure 3

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.

# 7. OLIGONUCLEOTIDE PURIFICATION
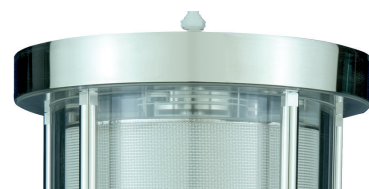
# PURIFICATION OF DNA-BASED OLIGONUCLEOTIDE AT 60°C ON TSKgel SuperQ-5PW (20) RESIN

## INTRODUCTION

TSKgel SuperQ-5PW (20) resin is a 20 µm particle size, strong anion exchange chromatographic resin used for large and small biomolecules. In downstream processing it can be used for intermediate purification and polishing steps. When used for oligonucleotides it does an excellent job of separating the oligonucleotide away from the "n-1" and "n+1" impurities.

The use of higher temperatures in a chromatographic separation can improve the resolution of the target molecule from closely eluting and similar chemistry impurities. In this report we compare data for an oligonucleotide separation at both ambient and 60 °C temperatures.

## METHODS AND RESULTS

An unpurified, lyophilized, 20-mer oligonucleotide of the following sequence: 5' - GAA TTC ATC GGT TCAS GAG AC - 3' was purchased from Trilink Biotechnology, San Diego, CA. Two equivalent lots of crude oligonucleotide were used, one lot estimated at 64.9% purity by HPLC, and the second lot estimated at 61.6% purity by HPLC.

A 6.6 mm ID x 15 cm column was packed (as described in *"Packing and Use Guide, TOYOPEARL and TSKgel-5PW Instruction Manual"* available from Tosoh Bioscience)

The sample for injection was prepared by diluting the crude oligonucleotide into the column equilibration buffer (Buffer A) before loading onto the column. For a 1 mg load, 38 mL of crude oligonucleotide was diluted to 10 mL with Buffer A and loaded into the sample loop.

Two sets of gradient conditions (shown in Figure 1) were investigated for optimum target resolution using the following buffers:

- ➤ Buffer A: 20 mmol/LTris, 1 mmol/L EDTA pH 9.0
- ➤ Buffer B: 20 mmol/LTris, 1 mmol/LEDTA, 1 mol/L NaCl pH 9.0

The gradient conditions selected for subsequent pH screening were:

- ➤
- ➤ 40% B (5 CV)
- ➤ 40%-65% B (15 CV)
  100% B (5 CV)

TSKgel SuperQ-5PW (20) RESIN USING DIFFERENT GRADIENTS AT 60 °C



40%B (5CV)
40%-65%B (12CV)
100%B (5CV)



40%B (5CV)
40%-65%B (15CV)
100%B (5CV)
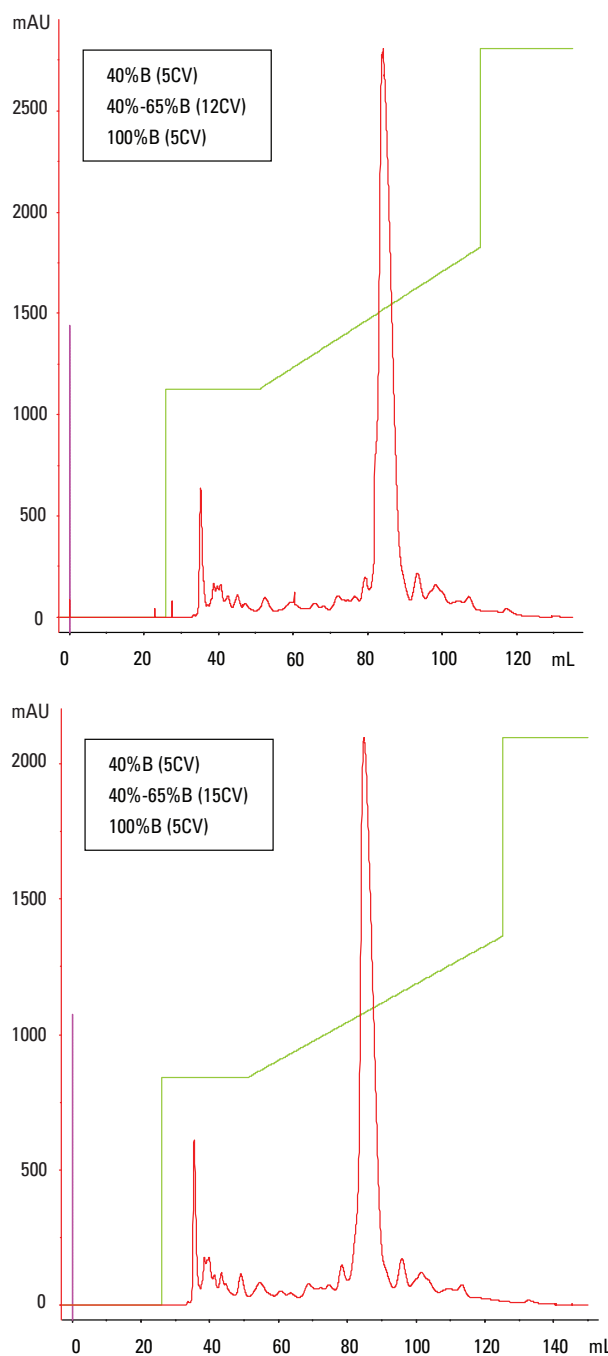
➤ Figure 1

TSKgel SuperQ-5PW (20) visually resolved N-1 peak and N+1 peak from the main oligonucleotide peak at 60 °C. The gradient that best resolved these peaks was: Step to 40% B (5 CV), Gradient 40%-65% B (15 CV), Step to 100% B (5 CV)

PURIFICATION OF OLIGONUCLEOTIDE AT VARIOUS pH ON TSKgel SuperQ-5PW (20) RESIN AT 60 °C
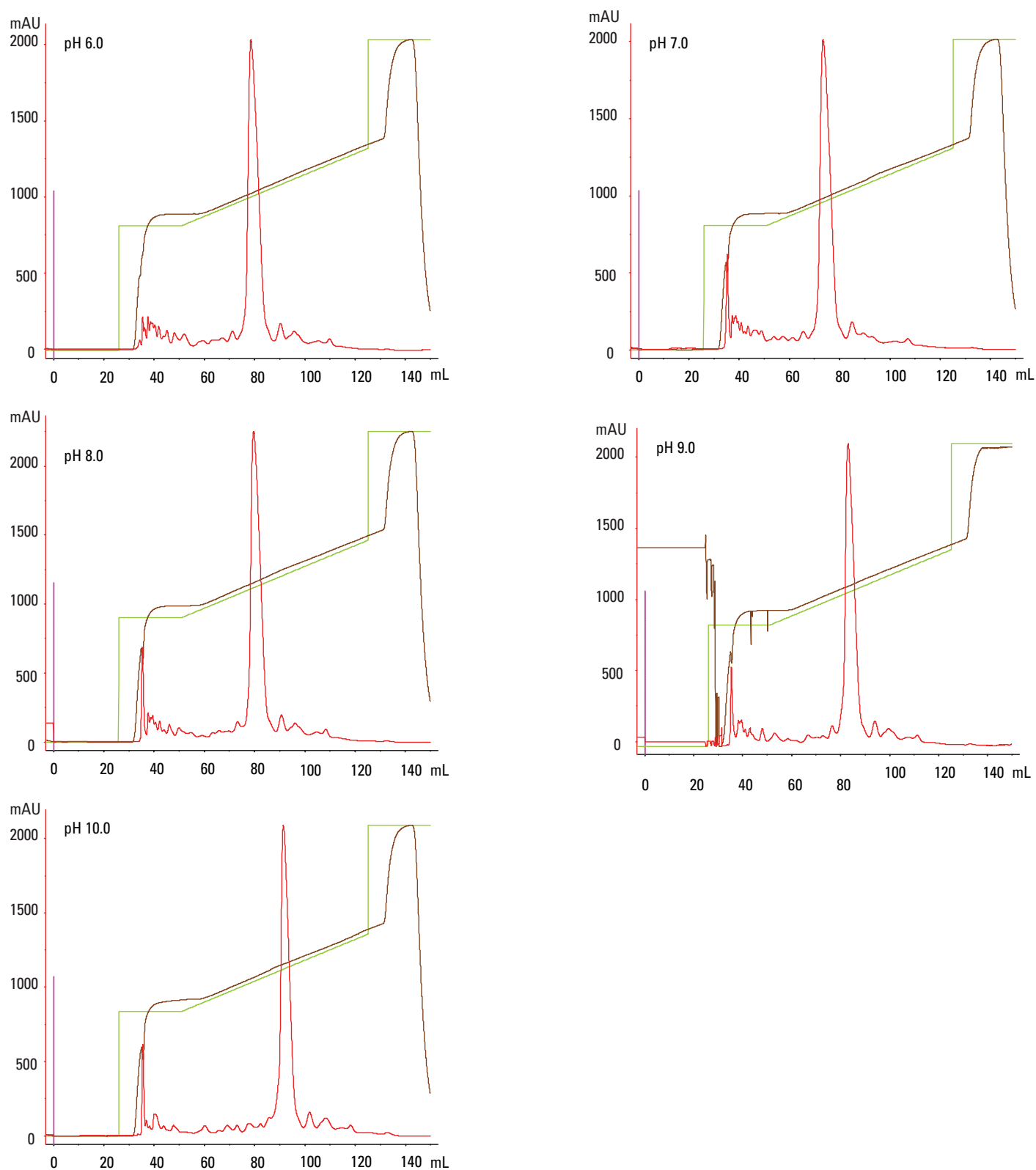


➤ Figure 2 ................................................................................................................

Column size: 6.6 mm ID x 15 cm; Flow rate: 250 cm/hr; Detection: Ab @ 254 nm; Buffer A: 20 mmol/L Tris + 1 mmol/L EDTA, various pH; Buffer B: Buffer A + 1.0 mol/L NaCl; Sample loaded: 1 mg/column; Separation conditions: Column is washed with 5 CV 100% Buffer A followed by 11 mL injection. Column is then washed with 3 CV 100% Buffer A followed by a step gradient to 40% Buffer B for 5 CV. This is followed by a linear gradient to 65% Buffer B over 15 CV. Finally, column is washed with 5 CV 100% Buffer B.

For the series of pH experiments, chromatographic runs were performed at pH values of 6.0, 7.0, 8.0, 9.0, 10.0. The operational conditions for each pH are as described in Figure 2. The peak purities and recoveries at the noted pH conditions are reported in Table 1.

CONCLUSION

The data shows that TSKgel SuperQ-5PW (20) resin can be used at 60 °C with varying pH conditions to successfully purify oligonucleotides. For this study pH 9.0 was ideal.

MAIN OLIGONUCLEOTIDE PEAK PURITY AND RECOVERY FOR TSKgel SuperQ-5PW (20) RESIN FOR VARIOUS pH VALUES AT 60 °C

| pH VALUE | MAIN PEAK PURITY | RECOVERY |
|---|---|---|
| pH 6.0 | 92.7% | 68.8% |
| pH 7.0 | 91.8% | 65.2% |
| pH 9.0 | 96.1% | 62.0% |
| pH 10.0 | 95.9% | 50.7% |

➤ Table 1

All pH levels except pH 8.0 showed an ability to purify oligonucleotides adequately. The purification performed at pH 9.0 showed the best purity and recovery of those values that were evaluated on the HPLC. The purification performed at pH 8.0 was not included due to poor separation between the main oligonucleotide peak and the N-1 peak seen by HPLC analysis.

A15P27A
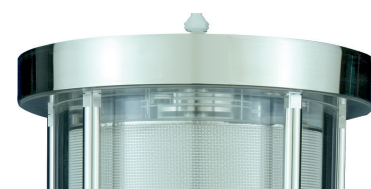
# PURIFICATION OF OLIGONUCLEOTIDES ON TOYOPEARL GigaCap Q-650S

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.

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 consideration 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).

PROPERTIES OF TSKgel SuperQ-5PW AND TOYOPEARL GigaCap Q-650S

| | TSKgel Super Q-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

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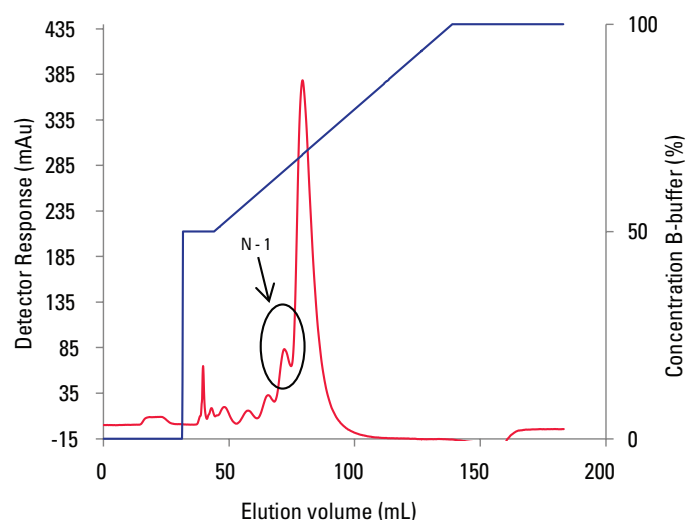


➤ Figure 1

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

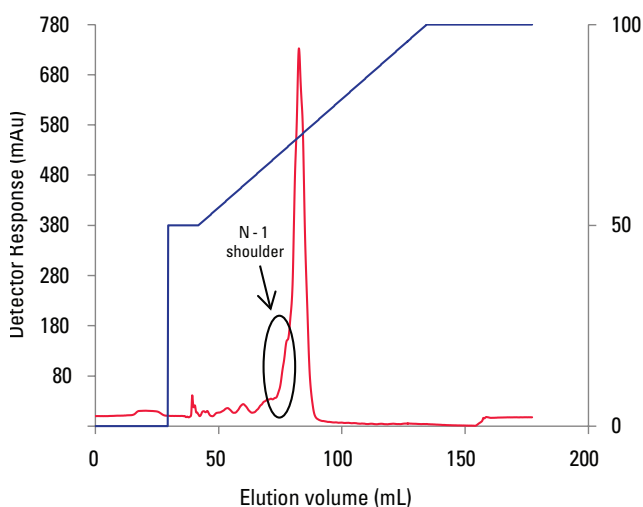TOYOPEARL GigaCap-Q-650S, 1.0 mg LOAD



**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

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.

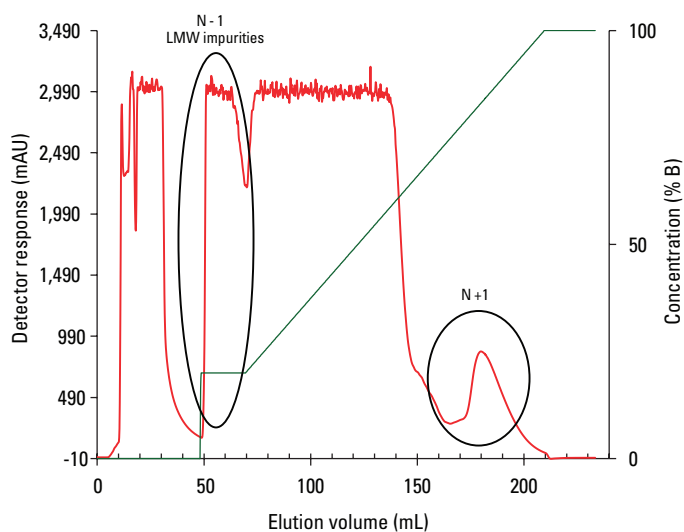PURIFICATION OF OLIGONUCLEOTIDE AT 80% DBC ON TSKgel SuperQ-5PW (20) RESIN



**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

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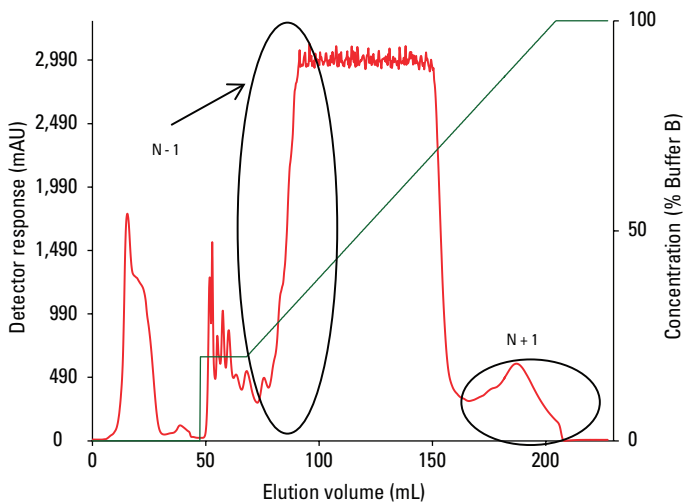


**Figure 4**

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
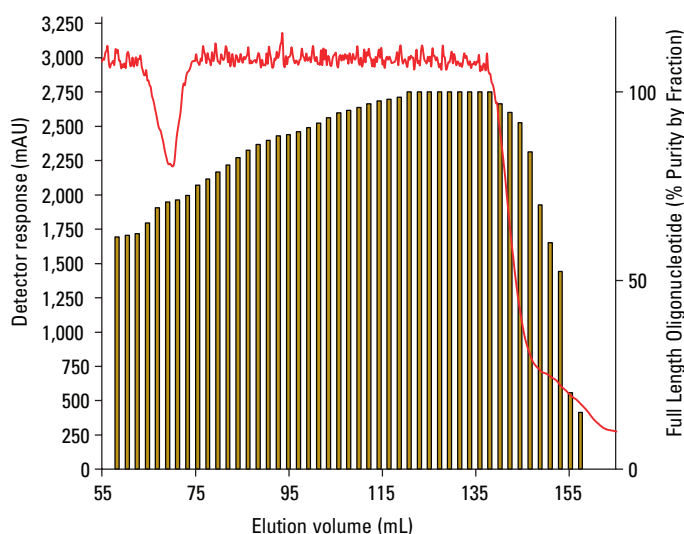WITH FRACTION PURITY HISTOGRAM



➤ Figure 5 ······································

Resin: 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

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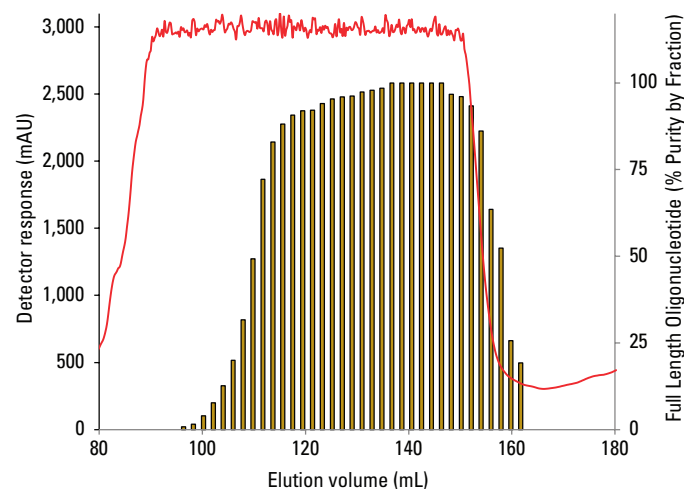FRACTION PURITY HISTOGRAM



➤ Figure 6 ······································
An enlarged image of the main oligonucleotide peak, over-

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

laid 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 reso-
lution at 80% DBC loading conditions.

OLIGONUCLEOTIDE PURITY AND YIELD FROM 80% DBC
PURIFICATIONS

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

➤ Table 1 ······································

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 oligonucleo-
tide 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.

# LOOKING FOR MORE?

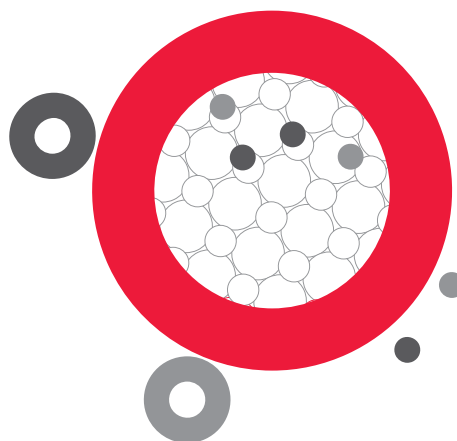## YOU HAVE PLENTY OF OPTIONS TO GET SUPPORT AND INSIGHTS FOR YOUR CHROMATOGRAPHY PROJECTS!
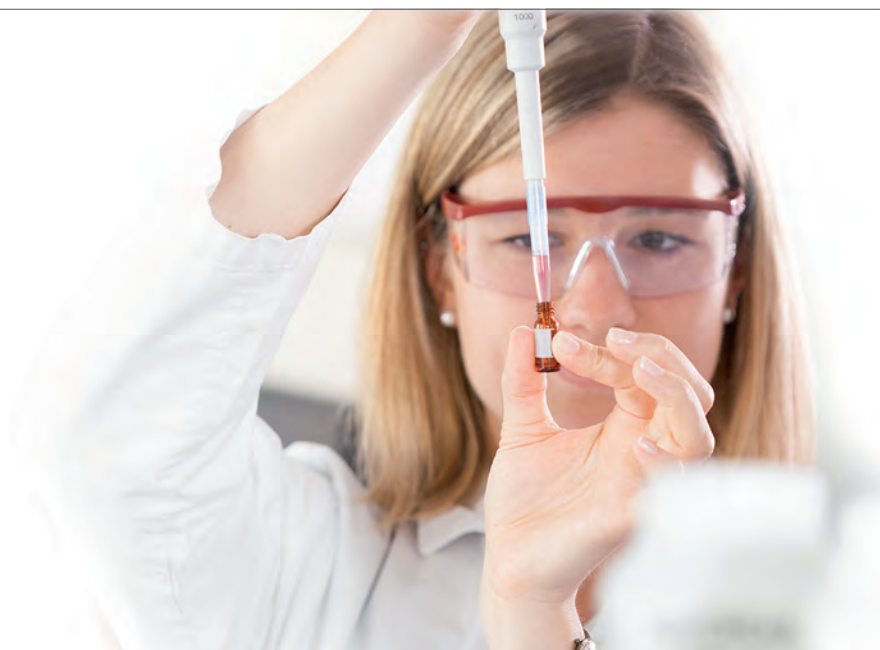
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