

# Hydrophobic Interaction Chromatography

## Toyopearl Resins for HIC

*650 series for most proteins*

Toyopearl Hexyl-650  
Toyopearl Butyl-650  
Toyopearl Phenyl-650  
Toyopearl Ether-650

*600 series for monoclonal antibodies*

Toyopearl Butyl-600  
Toyopearl Phenyl-600 **NEW!**  
Toyopearl PPG-600

*550 series for smaller proteins*

Toyopearl SuperButyl-550

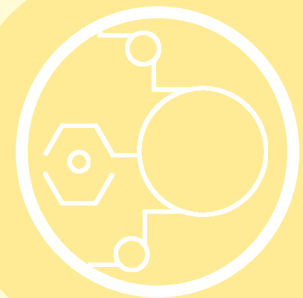
## TSK-GEL High-Performance Resins for HIC

TSKgel Phenyl-5PW  
TSKgel Ether-5PW

## ToyoScreen Process Development Columns for HIC

### Denmark

MD Scientific is authorized distributor in  
Denmark for Tosoh Bioscience  
[www.md-scientific.dk](http://www.md-scientific.dk) - [info@md-scientific.dk](mailto:info@md-scientific.dk) - Tel. 7027 8565



## Toyopearl resins for Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) is a powerful tool for the process purification of biomolecules. The technique utilizes the accessible hydrophobic regions located on protein surfaces and their interactions with a weakly hydrophobic stationary phase. HIC is an excellent complement to ion exchange and size exclusion chromatography particularly when protein isoforms exist or when feedstock impurities are of similar isoelectric point or molecular weight. The selectivity differences exploited by HIC can also be used after affinity separations in which closely related proteins with similar recognition sites are not distinguishable by the affinity ligand.

### How does HIC work?

Proteins and other molecules with hydrophobic surfaces are attracted to the hydrophobic ligands of both reversed phase (RP) and HIC resins. RP resins have higher surface coverage and/or more hydrophobic ligand compared to HIC resins. Because of this, in a RP separation the target binding readily occurs in an aqueous solution, and desorption is promoted by the addition of an increasing amount of organic solvent.

In HIC, proteins are bound to the resin by employing an aqueous high salt mobile phase. The salt conditions contribute to a lyotropic effect which allows the proteins to bind to the lower surface coverage of a hydrophobic ligand. Proteins are eluted by the simple technique of decreasing the salt concentration. Most therapeutic targets are eluted in a low salt or a no salt buffer.

During elution the energy of interaction for a HIC step is less than that of a RP step. One means of gauging the relative binding energy between the two techniques is to measure the surface tension of the two sets of binding and elution conditions. *Figure 1* provides a comparison of the surface tension generated by HIC and RP elution systems. Since HIC separates under milder eluting conditions, biological activity is typically retained.

### Five different hydrophobic surfaces and selectivities

Tosoh Bioscience offers five HIC ligands featuring different degrees of hydrophobicity and selectivity. The hydrophobicity of Toyopearl HIC resins increases through the ligand series: Ether, PPG (polypropyleneglycol), Phenyl, Butyl, and Hexyl. (*Figure 2*)

Figure 1

### The surface tension of aqueous solutions used in HIC and RPC

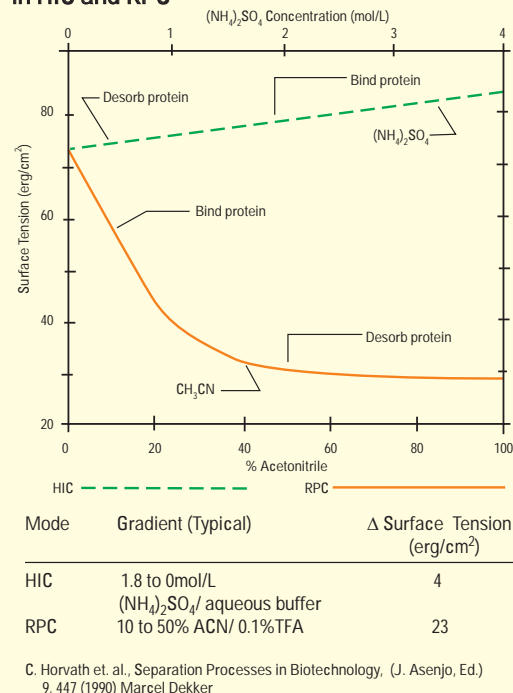
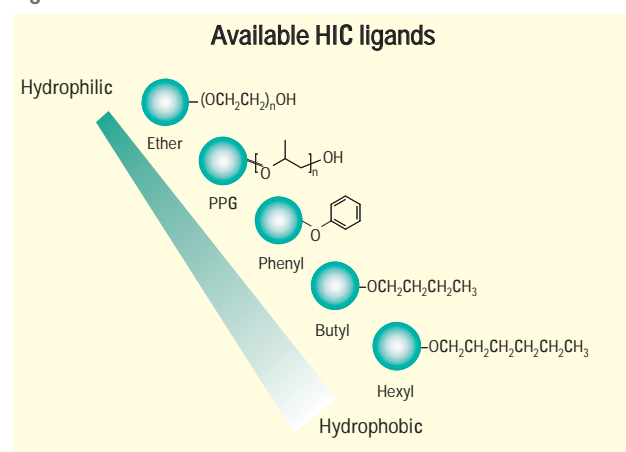


Figure 2



### Features

hydrophilic polymer resin matrix



good mechanical stability



### Benefits

- robust chemical stability between pH 1-13
- temperature range 4° -60 °C autoclavable at 121 °C
- compatible with organic solvents
- constant bed volume over a wide range of salt concentrations
- low non specific protein binding
- superior protein recovery

- excellent flow characteristics in large industrial size columns
- direct scale-up from TSK-GEL HIC HPLC columns

Coordinating the hydrophobicity of the therapeutic target to the resin hydrophobicity is critical for the best overall purification performance. Too hydrophobic a resin for a given protein can result in its irreversible binding to the resin or a loss of enzymatic activity. *Table I and II* show typical mass recovery and biological activity recovery data for Toyopearl HIC resins.

An optimum HIC process step will balance high dynamic binding capacity, adequate selectivity, good mass recovery and retention of biological activity.

The wide range of Toyopearl selectivities enables a developer to optimize protein separations at the extremes of the hydrophobic spectrum. Highly retentive Toyopearl Hexyl-type and Toyopearl Butyl-type resins are used to separate hydrophilic proteins. These two resins should also be considered for separations requiring a low salt environment.

Toyopearl Ether-type resin is used for the purification of very hydrophobic targets such as certain monoclonal antibodies and membrane proteins. These proteins may bind irreversibly to other more hydrophobic resins.

Toyopearl PPG-type and Toyopearl Phenyl-type phases complement the other HIC ligands available in the Toyopearl series and offer alternatives for mid-range hydrophobic Toyopearl proteins.

Table I

High mass recovery (%) of proteins	Toyopearl HIC resin		
	Ether -650M	Phenyl -650M	Butyl -650M
Bovine serum albumin	84	62	76*
$\alpha$ -Chymotrypsinogen	96	88*	90
Cytochrome C	—	81*	87*
IgG	91	—	—
$\alpha$ -Lactalbumin	90	—	—
Lysozyme	94	92	85
Ovalbumin	83	88	73
Ribonuclease A	—	72*	82*

*Procedure: A 200mL sample containing 200mg of protein was loaded onto a 7.5mm ID x 7.5cm column and eluted with a 60 minute gradient of 1.8mol/L (\*1.5 mol/L) to 0.0mol/L ammonium sulfate in 0.1mol/L sodium phosphate (pH 7.0). The mass recovery was determined spectrophotometrically at UV 280nm and 25°C.*

Table II

Recovery of enzymatic activity of proteins		
Toyopearl HIC resin	Protein	% Activity recovery
Phenyl-650	Phytochrome	79
Butyl-650	Halophilic protease	85
Butyl-650	Poly (3-hydroxybutyrate) depolymerase	88
Butyl-650	Aculeacin-A acylase	82
Butyl-650	Opine dehydrogenase	81

### Toyoscreen prepacked columns for process development

Toyoscreen columns packed with the full range of our Toyopearl HIC products are available in 1mL and 5mL resin volumes. They provide a convenient way to screen different resins for both target retention and recovery. Multiple columns can be connected in series for additional separation. Please see the ordering information at the end of this section or contact us for more information on these products.

The retention and selectivity of protein standards on Toyopearl HIC resins using the Toyoscreen process development columns are shown in *Figure 3*.

### Influence of salt type

In addition to the hydrophobicity of the ligand, the selectivity in HIC is influenced by the eluent salt type. *Figure 4* demonstrates the effect of salt type on the resolution factor of different protein pairs.

Figure 3

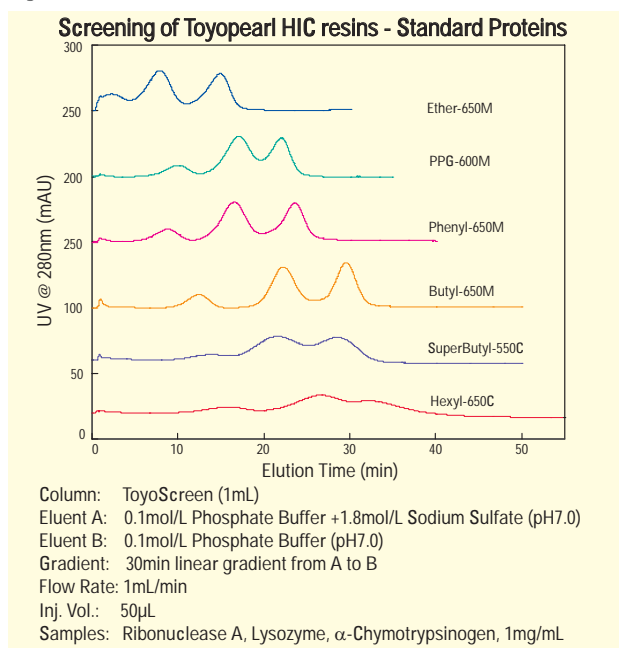
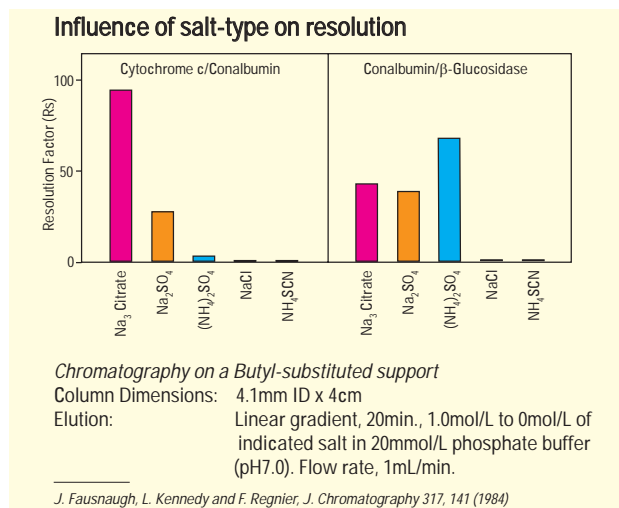


Figure 4



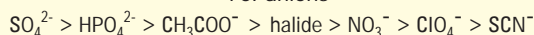
# Hydrophobic Interaction Chromatography

The Hofmeister lyotropic salt series shown in *Figure 5* ranks anions and cations by their ability to promote protein precipitation. Ions on the left are referred to as “lyotropic” while the ions on the right are called “chaotropic”. Lyotropic salts will precipitate or “salt out” proteins at high salt concentrations due to increased hydrophobic interaction, while chaotropic salts will promote protein denaturation at high salt concentrations. *Figure 5* indicates that different salt systems may generate a variety of adsorption and desorption selectivities for each resin. This feature of HIC provides an additional parameter for the optimization of a process step.

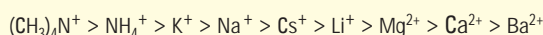
**Figure 5**

## Hofmeister lyotropic salt series

For anions



For cations



Ammonium sulfate and sodium sulfate are the most commonly used salts in HIC. NaCl is often used as well.

## Particle size optimization

Toyopearl and TSK-GEL PW-type methacrylic base beads incorporate the same polymer chemistry (see *Figure 6* or refer to the Size Exclusion section for a more detailed discussion) and are available in a variety of particle sizes:

100µm	Toyopearl C-grade	Capture
65µm	Toyopearl M-grade	Intermediate Purification
35µm	Toyopearl S-grade	Intermediate Purification/Polishing
30 and 20µm	TSK-GEL PW-type	High Resolution

In many cases analytical columns are available packed with 10 micron particles having the same selectivity as the process media.

*Figure 7* shows the variety of ligands and particle sizes available for HIC process-scale applications. This figure also depicts where each particle size is used in a chromatographic manufacturing train (i.e. for capture, intermediate purification, or polishing).

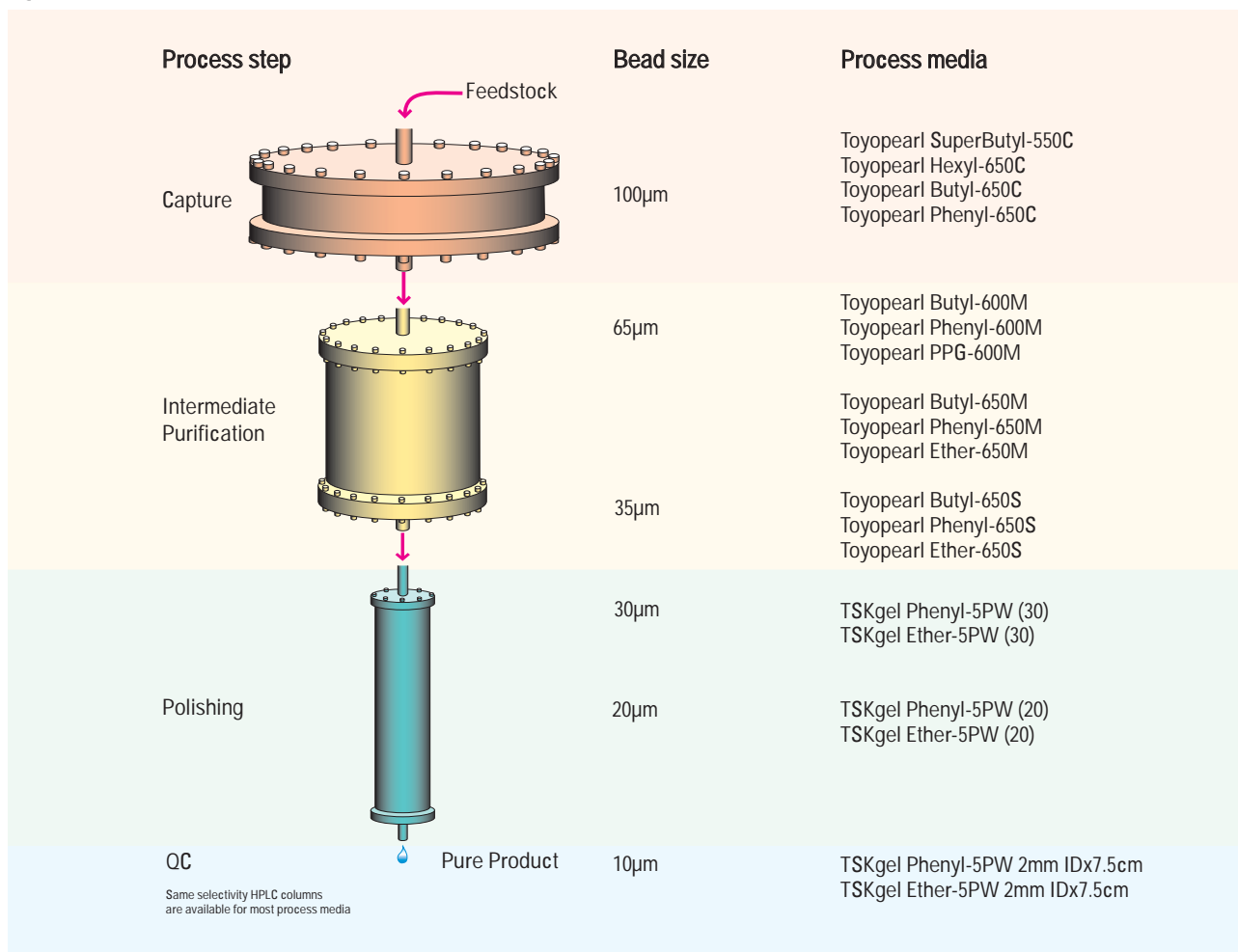
**Figure 6**

## Methacrylic base beads available for HIC

Pore size(Å)	50	125	400-500	750	1000	>1000	>1700
Product name							
Toyopearl HW-type:	40	50	55	60	65	75	80
TSK-GEL PW-type:	G1000	G2000	G4000		G5000	G6000	

← Increasing pore surface area

**Figure 7**



### Mechanical stability

The semi-rigid polymeric backbone of Toyopearl and TSK-GEL HIC resins permits high flow rates for maximum throughput and productivity. Toyopearl resins may be operated up to 3 bar and TSK-GEL PW-type resins may be operated up to 20 bar. The pressure-flow characteristics for each particle size grade of Toyopearl Phenyl-650 resins are shown in Figure 8. The beads are compatible with both fixed bed and dynamic axial compression columns.

### Chemical stability

The polymeric structure of these products also makes them resistant to a wide range of pH conditions and ionic strengths. In addition, the hydroxylated surface of the base bead reduces non-specific binding of proteins.

### Pore size optimization

Most Toyopearl HIC products are derived from the versatile size exclusion resin, Toyopearl HW-65 (1,000Å mean pore size), as the base bead for the majority of protein separations. But the pore size and accessible surface area of Toyopearl can be optimized for a given protein. More accessible surface area increases the dynamic binding capacity (DBC) of the bead for a particular therapeutic target. This has led to the development of two specialty lines of HIC materials with higher dynamic binding capacities.

### High dynamic binding capacity Toyopearl process resins

For monoclonal antibodies a pore size of 750Å is optimum. A specially made base resin, Toyopearl HW-60, has this pore size. Three ligands are available on Toyopearl HW-60: Toyopearl PPG-600M, Toyopearl Phenyl-600M and Toyopearl Butyl-600M. A comparison of their DBCs with Toyopearl Phenyl-650M is shown in Figure 9. The selectivities of Toyopearl Butyl-600M, Toyopearl PPG-600M and the new Toyopearl Phenyl-600M are shown in Figure 10.

Figure 8

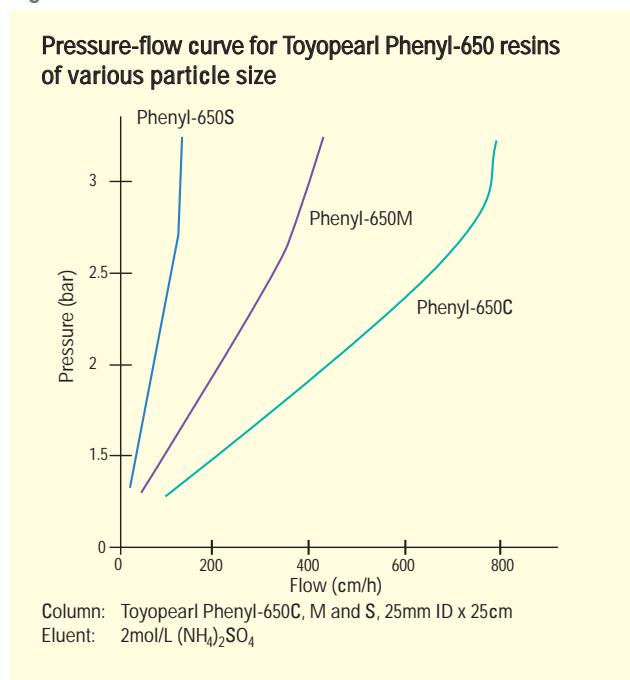


Figure 9

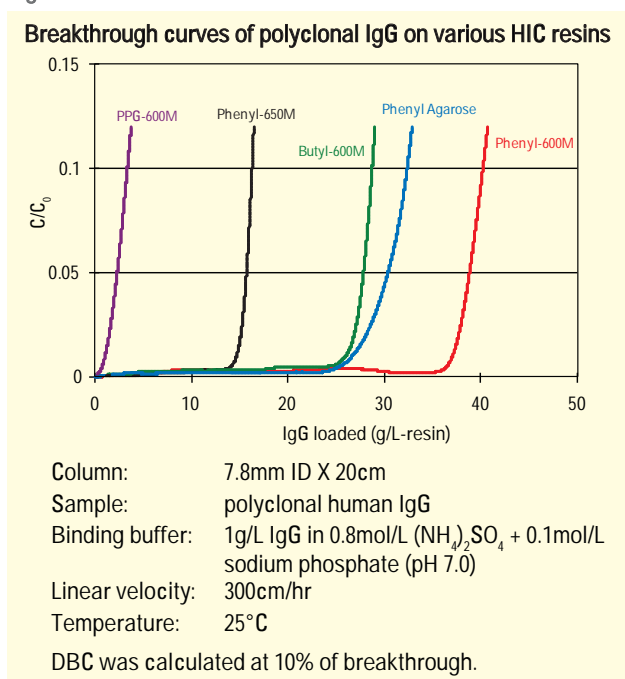


Figure 11 compares the selectivities of the new Toyopearl Phenyl-600M, Toyopearl Phenyl-650M and an agarose Phenyl resin.

For smaller molecules such as lysozyme (12,000 Da) the even narrower pore diameter Toyopearl SuperButyl-550C (derived from the 500Å pore diameter Toyopearl HW-55) is recommended. A comparison of the DBC of Toyopearl SuperButyl-550C with other Toyopearl HIC resins is shown in Figure 12.

Figure 10

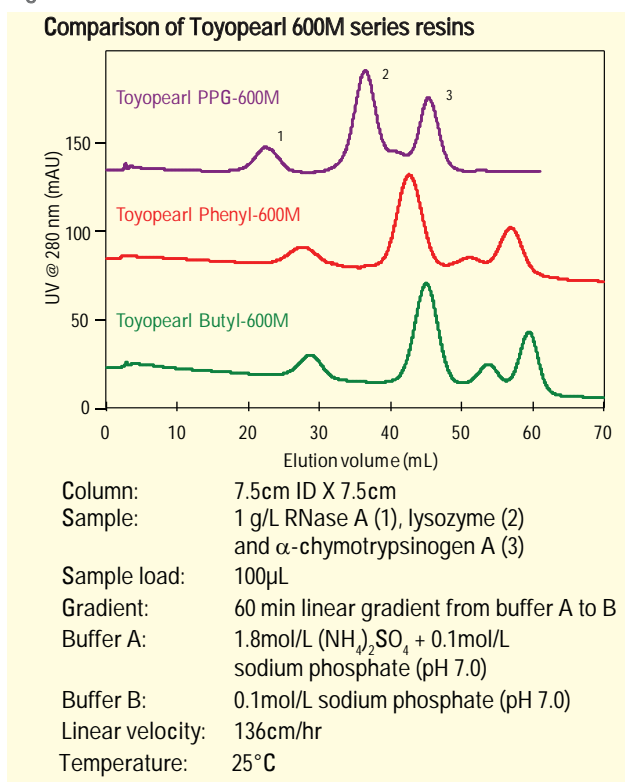


Figure 11

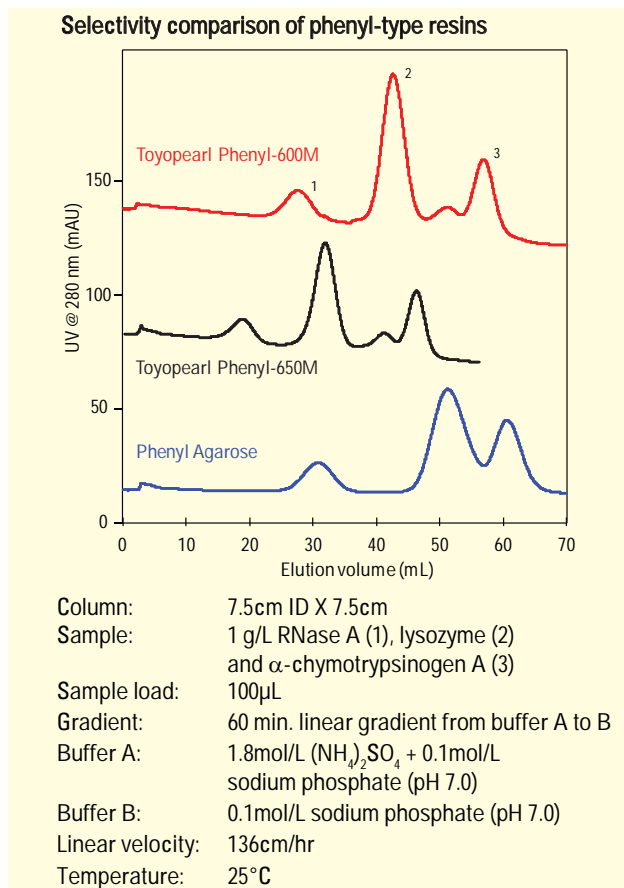


Figure 12

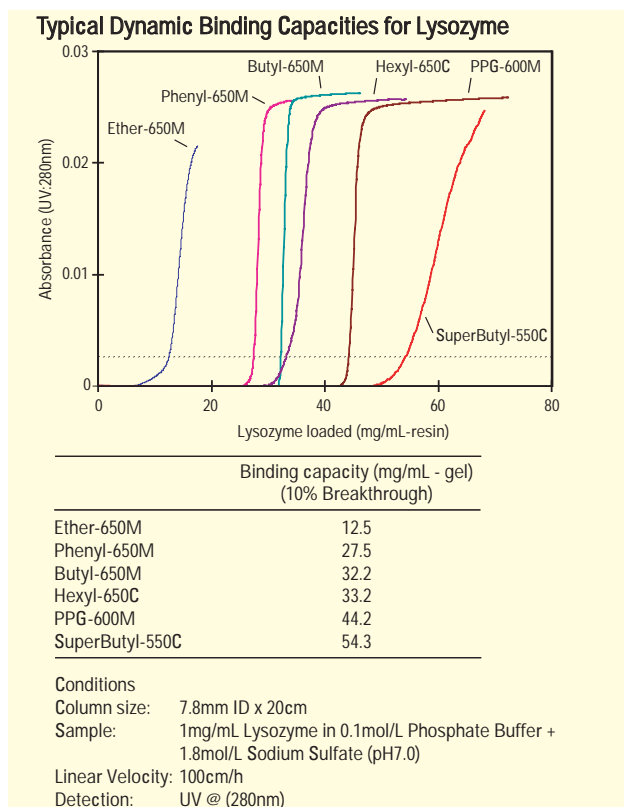
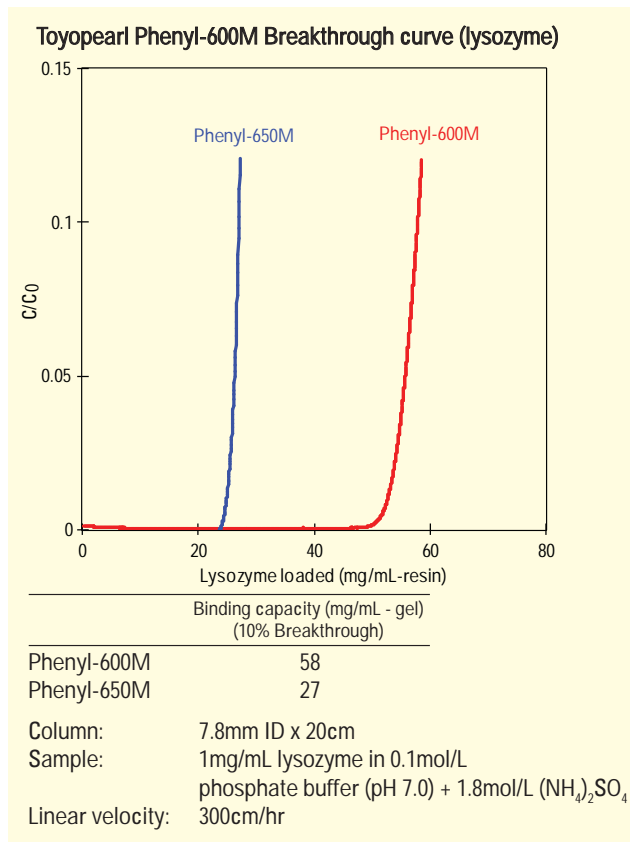


Figure 13



The new Toyopearl Phenyl-600M also has a high DBC for lysozyme. (Figure 13).

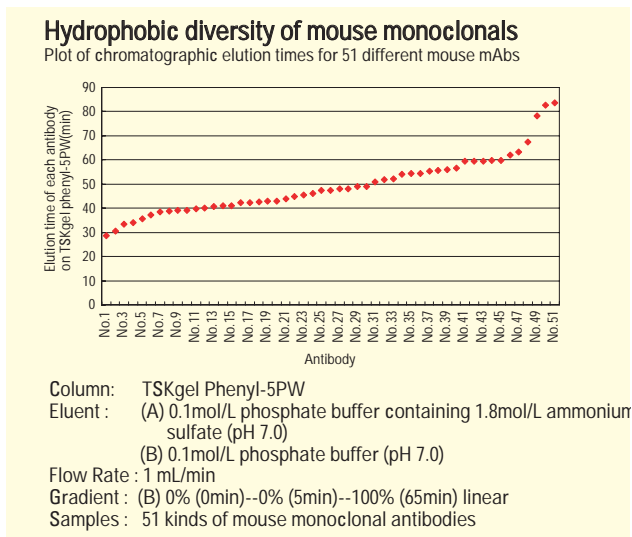
The engineered higher dynamic binding capacity of the 600 and 550 series HIC products for their specific targets and the selectivity differences induced by the smaller mean pore size of the respective beads, can have a dramatic impact on process economics. Improving target feedstock concentration and purity for downstream steps can greatly enhance throughput and reduce process costs.

### Monoclonal antibodies

Hydrophobic interaction is a very useful technique for the purification of monoclonal antibodies. The diverse hydrophobic nature of mAbs is seen in Figure 14. This figure measures the hydrophobicity (using elution time as a surrogate measurement) of 51 different mouse IgGs on a TSK-GEL Phenyl-5PW analytical column. Some of the IgGs have elution times 2-3 times longer than others indicating greater hydrophobicity. The Toyopearl series of HIC ligands (Figure 2) with their different hydrophobicities gives chromatographic developers a range of options for finding the right ligand for their target molecule.

For a very hydrophobic mAb, such as mouse anti-chicken 14kDa lectin, the less hydrophobic Toyopearl Ether ligand works quite well. The purification from ascites fluid (Figure 15) was performed with a 10 $\mu$ m TSKgel Ether-5PW semi-preparative column. Identical selectivity for scale-up was found with corresponding 65 $\mu$ m Toyopearl Ether-650M.

Figure 14



### Protein aggregate removal

The larger pore Toyopearl products such as Butyl-650 and Phenyl-650 are very useful for protein aggregate separation and removal. Early work by Karger et al<sup>1</sup> in 1989 involving proteins and aggregates larger than 200 kDa demonstrated the effectiveness of HIC for this application.

### Glycoproteins

Toyopearl HIC resins can purify glycoproteins, which often bind irreversibly to saccharide-based chromatographic media. Figure 16 shows the purification of a large glycoprotein on Toyopearl Butyl-650S resin.

### DNA plasmid purification and endotoxin removal

Toyopearl Hexyl-650C resin was successfully used for plasmid DNA purification by Cambrex, Baltimore, MD (US patent 6,953,686). Hexyl-650C was shown to be the most effective among HIC resins for endotoxin removal with capacities exceeding 2 million EU/mL of resin. Additionally, RNA and protein impurities were effectively eliminated. Hexyl-650C was also effective in separating the supercoiled and open circular forms of plasmid DNA.

### Removal of misfolded proteins

Because misfolded proteins will generally be more hydrophobic than the native protein, Toyopearl Butyl-650M resin is used frequently for the removal of misfolded proteins. In many cases flow through chromatography can be accomplished under eluent conditions binding the misfolds while allowing the target to flow through the column.

Please check the database on our website, [www.separations.us.tosohbioscience.com](http://www.separations.us.tosohbioscience.com), for additional applications.

<sup>1</sup> Grinberg, N. Blanco, R. Yarmush, D. Karger, B. L. *Protein Aggregation in High Performance Liquid Chromatography: Hydrophobic Interaction Chromatography of  $\beta$ -Lactoglobulin*. Anal. Chem. 1989, 61, 514-520

Figure 15

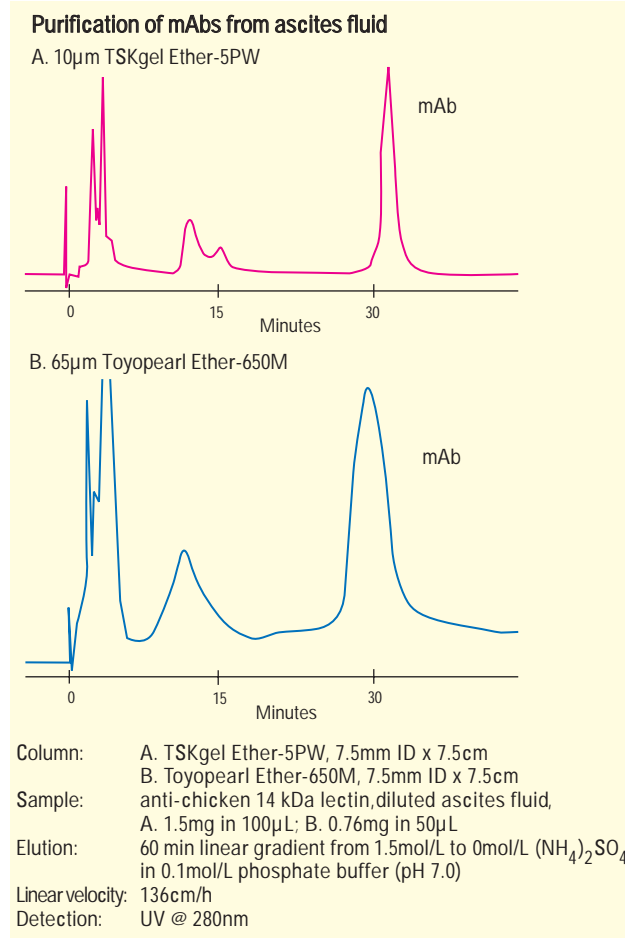
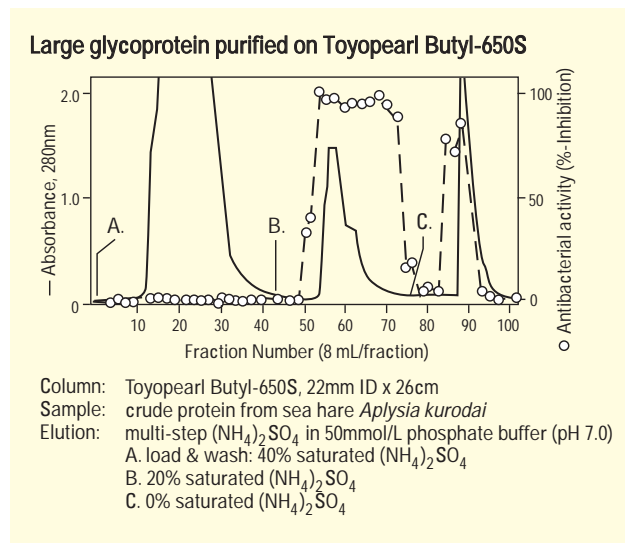


Figure 16



## Ordering Information

### ToyoScreen process development columns for HIC

<i>Part #</i>	<i>Product description</i>	<i>Package</i>
21372	ToyoScreen Ether-650M, 1mL	1mL x 6 each
21373	ToyoScreen Ether-650M, 5mL	5mL x 6 each
21374	ToyoScreen Phenyl-650M, 1mL	1mL x 6 each
21375	ToyoScreen Phenyl-650M, 5mL	5mL x 6 each
21376	ToyoScreen Butyl-650M, 1mL	1mL x 6 each
21377	ToyoScreen Butyl-650M, 5mL	5mL x 6 each
21378	ToyoScreen Hexyl-650C, 1mL	1mL x 6 each
21379	ToyoScreen Hexyl-650C, 5mL	5mL x 6 each
21380	ToyoScreen PPG-600M, 1mL	1mL x 6 each
21381	ToyoScreen PPG-600M, 5mL	5mL x 6 each
21892	ToyoScreen Phenyl-600M, 1mL	1mL x 6 each
21893	ToyoScreen Phenyl-600M, 5mL	5mL x 6 each
21382	ToyoScreen SuperButyl-550C, 1mL	1mL x 6 each
21383	ToyoScreen SuperButyl-550C, 5mL	5mL x 6 each
21398	ToyoScreen HIC Mix Pack, 1mL	1mL x 6 Grades x 1 each
21399	ToyoScreen HIC Mix Pack, 5mL	5mL x 6 Grades x 1 each

### ToyoScreen column accessories

<i>Part #</i>	<i>Product description</i>	<i>Comment</i>
21400	ToyoScreen Column Holder	
42194	ToyoScreen Holder w/ Fittings	Includes 21400, 42195 and 42196 (qty. 2)
42195	Column to Column Connector	
42196	Adaptor, M6 female to 10-32 male, PEEK	
42197	Adaptor, 1/4 - 28 female to 10-32 male, PEEK	

### TSK-GEL LabPak

<i>Part #</i>	<i>Product description</i>	<i>Container size (mL)</i>	<i>Particle size (µm)</i>
43278	HICPAK PW (20) (Ether-5PW, Phenyl-5PW)	2 x 25mL	10-30
43175	HICPAK PW (30) (Ether-5PW, Phenyl-5PW)	2 x 25mL	20-40

## Toyopearl HIC resins

Part #	Product description	Container size (mL)	Particle size ( $\mu\text{m}$ )	Typical capacity (mg lysozyme/mL resin)
43151	Toyopearl Ether-650S	25	20-50	10-30
16172		100		
16174		1,000		
16176		5,000		
19805		25		
16173	100			
16175	1,000			
16177	5,000			
21301	Toyopearl PPG-600M	25	40-90	45-55
21302		100		
21303		1,000		
21304		5,000		
21305		50,000		
21887	Toyopearl Phenyl-600M	25	40-90	45-65
21888		100		
21889		1,000		
21890		5,000		
20891		50,000		
43152	Toyopearl Phenyl-650S	25	20-50	30-50
14477		100		
14784		1,000		
14935		5,000		
19818		25		
14478	100			
14783	1,000			
14943	5,000			
18364	50,000			
43126	Toyopearl Phenyl-650C	25	50-150	30-50
14479		100		
14785		1,000		
14944		5,000		
43153		Toyopearl Butyl-650S		
07476	100			
14701	1,000			
07975	5,000			
18826	50,000			
19802	Toyopearl Butyl-650M	25	40-90	30-50
07477		100		
14702		1,000		
07976		5,000		
18355		50,000		
43127	Toyopearl Butyl-650C	25	50-150	30-50
07478		100		
14703		1,000		
07977		5,000		
21448		Toyopearl Butyl-600M		
21449	100			
21450	1,000			
21451	5,000			
21452	50,000			
19955	Toyopearl SuperButyl-550C	25	50-150	52-70
19956		100		
19957		1,000		
19958		5,000		
19959		50,000		
44465	Toyopearl Hexyl-650C	25	50-150	30-50
19026		100		
19027		1,000		
19028		5,000		



# Hydrophobic Interaction Chromatography

<i>Part #</i>	<i>Product description</i>	<i>Container size (mL)</i>	<i>Particle size (μm)</i>	<i>Adsorption capacity (mg lysozyme/mL resin)</i>
<b>TSK-GEL 5PW HIC resins for high resolution</b>				
43276	TSKgel Ether-5PW (20)	25	10-30	10-30
16052		250		
16053		1,000		
18437		5,000		
43176	TSKgel Ether-5PW (30)	25	20-40	10-30
16050		250		
16051		1,000		
18439		5,000		
43277	TSKgel Phenyl-5PW (20)	25	10-30	10-30
14718		250		
14719		1,000		
18438		5,000		
43177	TSKgel Phenyl-5PW (30)	25	20-40	10-30
14720		250		
14721		1000		
17210		5,000		