

HYDROPHOBIC INTERACTION CHROMATOGRAPHY



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➤ **TOSOH HISTORY**

- | | |
|-----------|--|
| 1935 | FOUNDING OF TOYO SODA MANUFACTURING CO., LTD. |
| 1936 | OPERATION OF NANYO MANUFACTURING COMPLEX BEGINS |
| 1971 | SCIENTIFIC INSTRUMENTS DIVISION FORMED, FIRST GPC COLUMN USING TSKgel DEVELOPED BY TOSOH |
| 1974 | HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COLUMN PLANT IS COMPLETED |
| 1979 | TOSOH DEVELOPS TOYOPEARL MEDIA |
| 1983 | TOSOH DEVELOPS HYDROPHOBIC INTERACTION MEDIA |
| 1987 | TOSOHAS US OPERATIONS FORMED IN MONTGOMERYVILLE |
| 1989 | TOSOHAS GMBH OPERATIONS FORMED IN STUTTGART |
| 1995 | TOSOH NANYO GEL FACILITY RECEIVES ISO 9001 |
| 2002/2003 | ALL TOSOH AFFILIATED SCIENTIFIC & DIAGNOSTIC SYSTEM RELATED COMPANIES IN EUROPE ARE UNIFIED UNDER THE NAME TOSOH BIOSCIENCE. |
| 2008 | EcoSEC, THE 7TH GENERATION GPC SYSTEM IS INTRODUCED GLOBALLY |
| 2010 | TOSOH CELEBRATES ITS 75TH YEAR IN BUSINESS WITH THE OPENING OF FIVE NEW PLANTS, AND CONTINUED RAPID EXPANSION IN CHINA |
| 2011 | TOSOH BIOSCIENCE CELEBRATES 40 YEARS OF OPERATION |
| 2012 | TOSOH RELEASES FIRST TOYOPEARL MIXED-MODE RESIN TOYOPEARL MX-Trp-650M |
| 2013 | TOSOH RELEASES A HIGH CAPACITY PROTEIN A CHROMATOGRAPHY RESIN |
| 2014 | TOSOH BIOSCIENCE GMBH CELEBRATES ITS 25 TH ANNIVERSARY IN STUTTGART |
| 2015 | TOSOH BIOSCIENCE SUCCESSFULLY MOVES ITS SALES & MARKETING OFFICES TO GRIESHEIM, DARMSTADT |

HYDROPHOBIC INTERACTION CHROMATOGRAPHY

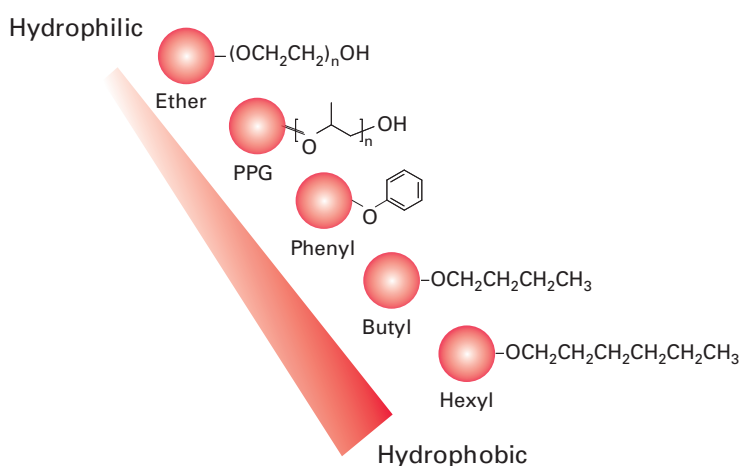


Hydrophobic Interaction Chromatography (HIC) is a widely-used technique for separation and purification of proteins and peptides. HIC sorts biomolecules by degree of their surface hydrophobicity. Samples are adsorbed to the resin at relatively high salt concentrations and eluted by applying a decreasing salt gradient. The mild conditions used in HIC separation of peptides and proteins typically maintain protein structure and biologic activity. This makes HIC a powerful tool for the process purification of biomolecules.

An optimum HIC process step will balance high dynamic binding capacity (DBC), adequate selectivity, good mass recovery and retention of biological activity. The key parameter is selecting the best resin for the given separation problem. Proteins show varying degrees of hydrophobicity depending on their amino acid composition, structure and size. Separation can therefore be optimized either by varying the mobile phase or by using different HIC packings. Matching the hydrophobicity of the target compound to the resin hydrophobicity is critical for the best overall purification performance. This is the reason why Tosoh Bioscience offers seven product lines of TOYOPEARL HIC resins using five different ligands. The different degrees of hydrophobicity and selectivity support the user in selecting the best solution for a given target.

The hydrophobicity increases through the ligand series: Ether, Polypropylenglycol (PPG), Phenyl, Butyl, Hexyl. TOYOPEARL HIC resins are available in three different average particle sizes (35 μm (S), 65 μm (M) & 100 μm (C)) for intermediate purification or capture chromatography. For high resolution HIC Tosoh Bioscience offers TSKgel resins with particle sizes of 20 and 30 μm .

FIGURE 1
HIC Ligand Candidates



HIC

HOW IT WORKS

Many theories and models have been proposed to describe the HIC retention mechanism but none of them has gained universal acceptance. HIC is based upon interactions between hydrophobic patches on the surface of biomolecules and the hydrophobic ligands of the stationary phase. It is commonly believed that the driving force of interaction is the entropy gain arising from changes in the order of the water molecules surrounding the interacting hydrophobic groups. Protein binding to HIC adsorbents is promoted by moderately high concentrations of anti-chaotropic salts. Elution is achieved by a linear or stepwise decrease of salt in the mobile phase.

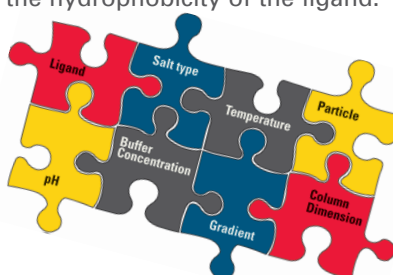
Selectivity

The hydrophobicity of a target with known structure can be roughly estimated as it often increases with the size of the protein surface. Nevertheless, practical screening experiments under standard buffer conditions are essential to select the optimum resin. The hydrophobicity of the resin determines the salt concentration necessary to adsorb the target. With low-hydrophobic ligands the difference between adsorption and precipitation might be so small that certain proteins may partially precipitate under binding conditions. On the other hand a high-hydrophobic stationary phase might cause irreversible binding of hydrophobic proteins.

HIC Method Development

The goal in purification method development is optimizing conditions for maximum capacity and recovery of the target molecules. There are several parameters which affect HIC separations in addition to the hydrophobicity of the ligand:

- Salt type
- pH
- Buffer concentration
- Temperature
- Gradient type, slope
- Particle and pore size
- Column dimensions



Optimizing Salt Type and Concentration

Besides the hydrophobicity of the resin, the eluent salts make a major impact on a HIC separation. Ammonium sulfate and sodium chloride are most commonly used for HIC applications. Sometimes citrate-buffers or dual salt systems are used to improve resolution. While the type of salt affects retention and selectivity the initial salt concentration is the key to maximize binding capacity for the target. The salt concentration required for binding is related to the size of the surface area of the protein. Small, hydrophilic proteins will need high salt, e.g. up to 3 M ammonium sulfate, for efficient binding but it can decrease below 1 M for very large proteins. Figure 2 shows the influence of salt concentration on binding capacity of TSKgel Phenyl-5PW for various proteins.

Other Parameters

pH can be used for fine tuning. A good starting pH is 7.0, irrespective of the component's isoelectric point. The pH can influence not only retention but also DBC (Figure 3).

Most HIC applications are performed at room temperature or at 4°C. A higher temperature might be used to influence binding strength and selectivity.

Elution is typically performed by gradient elution. The sample is applied at a salt concentration high enough for adsorption of the targets. As the salt concentration is lowered, proteins become increasingly desorbed and move down the column. Resolution can be increased by decreasing gradient slope. In manufacturing scale processes step gradients are more common than linear gradients.

Resolution in HIC can be improved by increasing the column lengths, since the full length of the column bed interacts continuously with sample components.

Organic modifiers can speed up a HIC separation or alter the selectivity. For purification of small molecules up to 20% ethanol might be used.

FIGURE 2
Binding Capacities of TSKgel Phenyl-5PW

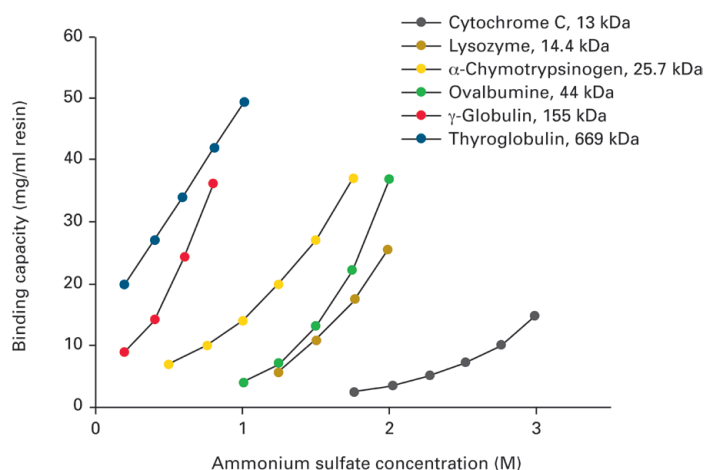
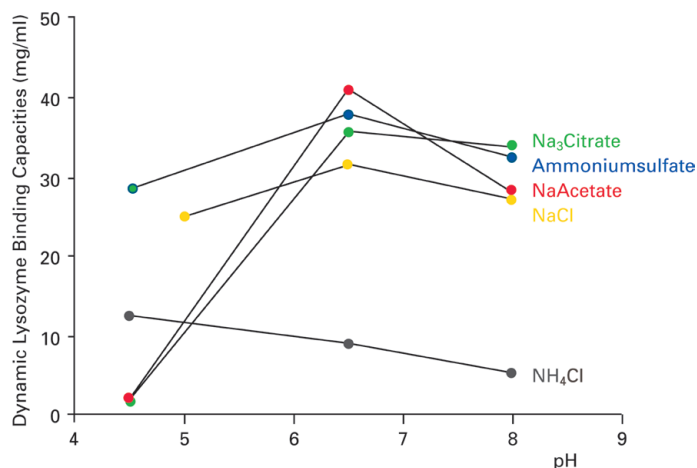


FIGURE 3
Influence of pH





HIC

TSKgel AND TOYOPEARL HIC RESINS

The particle size depends on the sample and the required resolution. Capturing steps from a crude feedstock are usually performed with coarse particles (TOYOPEARL C). In intermediate purification steps medium size particles (TOYOPEARL S or M) are used, whereas for polishing the even smaller TSKgel materials with 20 µm or 30 µm particles are ideal. TSKgel columns with 10 µm beads are best suited for analytical purposes or for small scale purifications (Figure4).

TOYOPEARL HIC Material

TOYOPEARL and TSKgel HIC resins are specifically designed for use in biopharmaceutical production. Their rigid methacrylic polymer structure shows excellent pressure/flow properties enabling high process throughput. Large pore diameters and narrow particle size distribution allow rapid adsorption kinetics and exceptional resolution. For seamless scale-up Tosoh Bioscience offers a complete HIC toolbox, ranging from analytical TSKgel HPLC columns up to bulk media used for pilot and production scale.

HIC Ligands

The wide range of TOYOPEARL and TSKgel HIC selectivities enables a developer to optimize protein separations at the extremes of the hydrophobic spectrum. The hydrophobicity of the resins increases through the series:

Ether < PPG < Phenyl < Butyl < Hexyl

Highly retentive Hexyl and Butyl resins are used to separate hydrophilic proteins and should be considered for separations requiring a low ionic strength. TOYOPEARL Ether resin is used for the purification of very hydrophobic targets such as certain monoclonal antibodies and membrane proteins. PPG and Phenyl complement the other HIC ligands and offer alternatives for mid-range hydrophobic proteins.

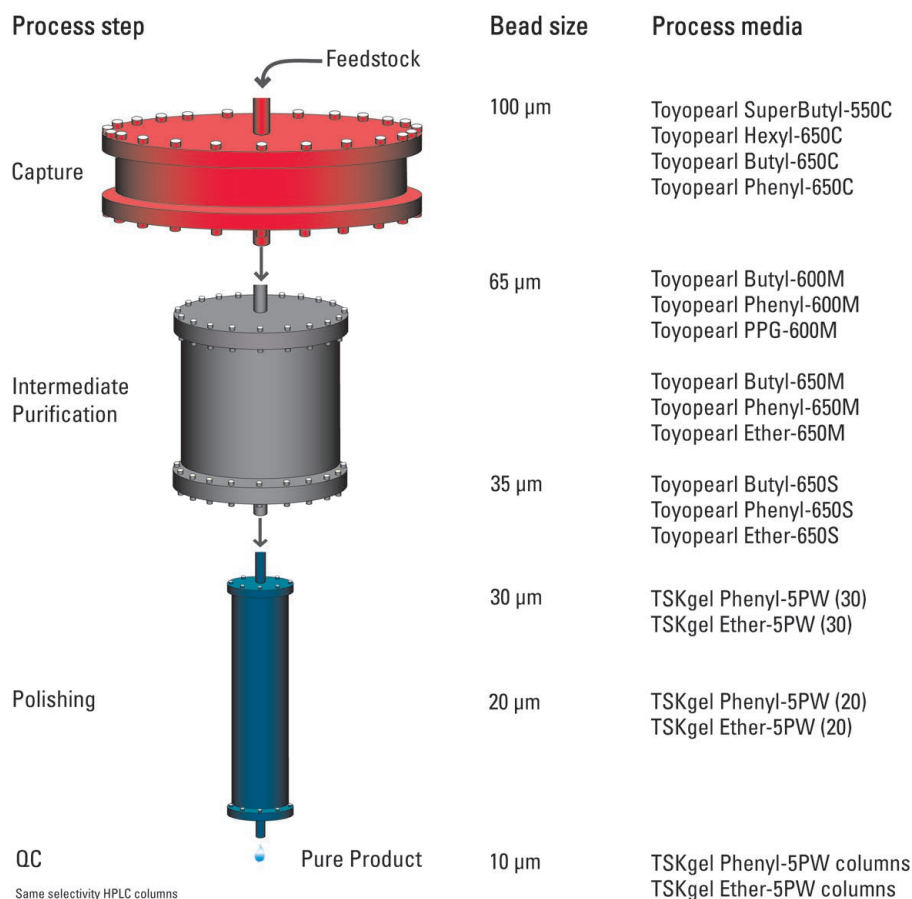
Today high-resolution HIC applications gain more and more interest. TSKgel 5PW media with small particle sizes are ideally suited if high resolution is an issue. TSKgel 5PW bulk material is available with the ligands Ether and Phenyl. TSKgel columns in various dimensions are available with Ether, Phenyl and Butyl chemistry.

Regulatory Support

Pharmaceutical industry all over the world successfully uses TOYOPEARL HIC resins in the downstream processing of a variety of biologically active proteins, including several FDA-approved therapeutic drugs. For TOYOPEARL HIC resins 'Regulatory Support Files', describing the specifications, the manufacturing and the QA/QC of the product are registered at the FDA. In addition, Tosoh Bioscience's application specialists are available for discussion of your specific separation challenge or process validation issues.

➤ **FIGURE 4**

HIC Resins



HIC

TSKgel AND TOYOPEARL HIC RESINS

Dynamic Binding Capacity

In downstream processing steps, the dynamic binding capacity (DBC) of the resin for the target is even more important than selectivity. Selecting media with a different pore size is an option, if DBCs are not satisfying. Tosoh Bioscience provides resins designed for maximum dynamic binding capacity for dedicated proteins. The standard TOYOPEARL resins have an average pore size of 1000 Å, suitable for most targets.

Smaller Pore Size HIC Resins

The accessible surface area of a porous bead increases by decreasing the mean pore diameter and so does the dynamic binding capacity. This lead to the development of two specialty lines of HIC materials with smaller pores. For monoclonal antibodies a pore size of 750 Å is sometimes favorable. TOYOPEARL resins exhibiting this pore size are available with three ligands: PPG-600, Phenyl-600 and Butyl-600. For smaller molecules such as peptides TOYOPEARL resins with even narrower pore diameter (500 Å) are used to create the SuperButyl-550C resin.

The variety of HIC phases increases the probability of matching a resin best to the given target, at the same time making the screening procedure more complex. Figure 6 shows all available TOYOPEARL resins sorted according to their pore size and relative hydrophobicities.

The TOYOPEARL Phenyl-600M resin was designed as a high-sub type. The higher ligand density results in a higher hydrophobicity than TOYOPEARL Phenyl-650 resins.

FIGURE 6
Hydrophobicity and Average Pore Size of TOYOPEARL HIC

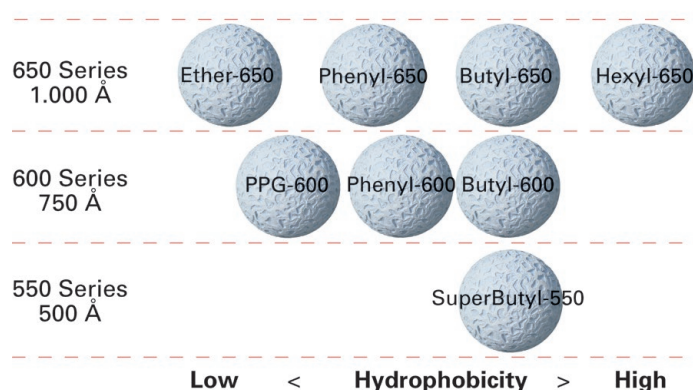
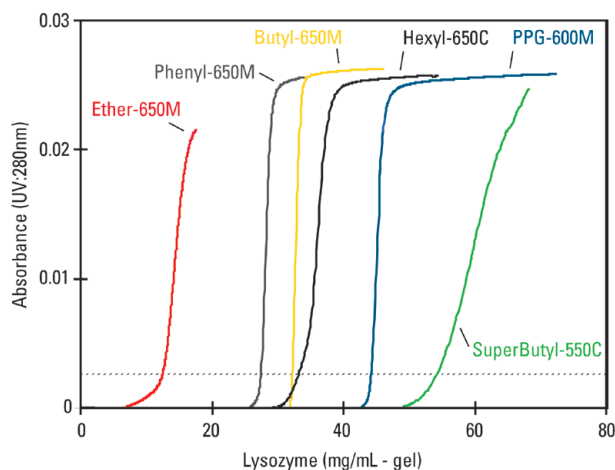


Figure 5 and 7 show the dynamic binding capacities of TOYOPEARL resins for Lysozyme and a monoclonal antibody. For a small protein such as Lysozyme the SuperButyl-550C is the best choice (Figure 5). Figure 7 demonstrates the superior DBC of the Butyl-600M and Phenyl-600M resins for large proteins.

FIGURE 5
Typical Dynamic Binding Capacities of Lysozyme

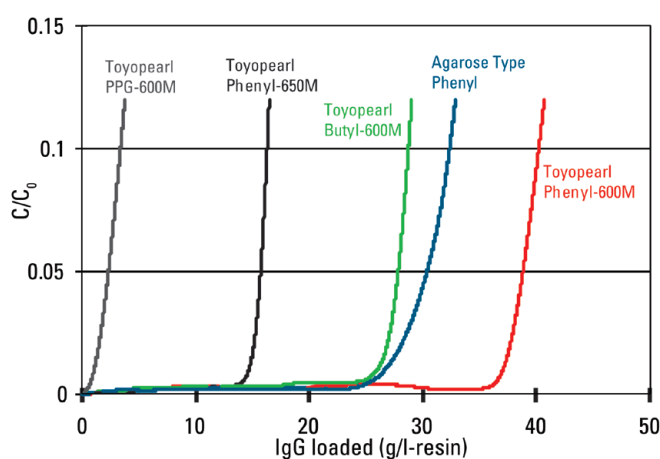


Binding capacity (mg/mL - gel) (10% Breakthrough)	
Ether-650M	12.5
Phenyl-650M	27.5
Butyl-650M	32.2
Hexyl-650C	33.2
PPG-600M	44.2
SuperButyl-550C	54.3

Conditions

Column size: 7.8 mm ID x 20 cm L
 Samples: 1 mg lysozyme in 0.1 M phosphate buffer +
 1.8 M sodium sulfate (pH 7.0)
 Linear velocity: 100 cm/h
 Detection: UV @280 nm

FIGURE 7
Breakthrough Curves of a Polyclonal IgG on Various HIC Resins



Binding capacity (mg/mL - gel) (10% breakthrough)	
Toyopearl Phenyl-600M	40.0
Agarose Type Phenyl	32.0
Toyopearl Butyl-600M	29.0
Toyopearl Phenyl-650M	16.0
Toyopearl PPG-600M	3.0

Column size: 7.8 mm ID x 20 cm
 Sample: polyclonal human IgG
 Binding buffer: 1 g/l IgG in 0.8 mol/l (NH₄)₂SO₄ + 0.1 mol/l
 sodium phosphate (pH 7.0)
 Linear velocity: 300 cm/h
 Temperature: 25°C

HIC SCREENING



ToyoScreen® for Easy Resin Scouting

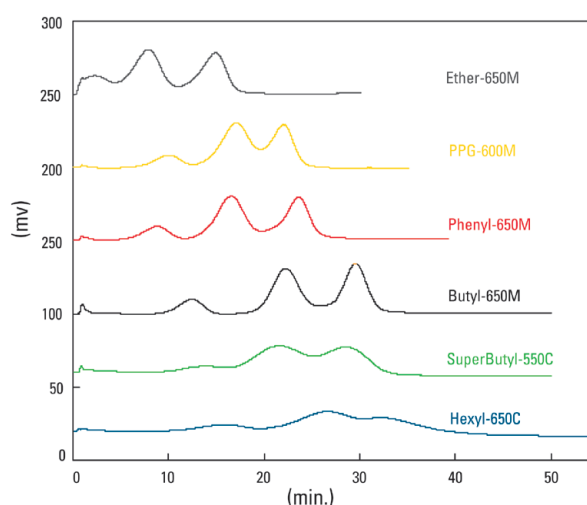
In order to simplify the screening process, Tosoh Bioscience offers sets of prepacked columns with different resins. They provide a convenient way to screen different resins effectively for both, target retention and recovery. ToyoScreen is available with 1 and 5 ml bed volumes for most TOYOPEARL resins and can be connected to common laboratory liquid chromatography instruments. If the LC system is equipped with automated solvent and column switching valves, screening of resins at various buffer conditions can be easily performed in overnight runs.

The effect of the different hydrophobicities of TOYOPEARL resins on retention and resolution of standard proteins are illustrated in Figure 8. A standard mixture of proteins was separated using ToyoScreen columns. Fast screening of a larger number of resins under various conditions can be realized by applying robotic fluid handling systems and high throughput screening tools in 96 well plate formats.

Comparison of HIC Resins

Non-specific binding effects from the base material of the resin can alter resolution and selectivity. The matrix of TOYOPEARL and TSKgel HIC resins is a uniform, hydrophilic polymer. HIC resins from other manufacturers, based on different base resins, might exhibit different properties regarding hydrophobicity, selectivity and resolution even if they are functionalized with the same ligand. This is important to consider when screening resins of various manufacturers.

FIGURE 8
Screening of TOYOPEARL HIC Resins - Standard Proteins



Column: ToyoScreen (1 ml)
 Eluent A: 0.1 M Phosphate Buffer + 1.8 M Sodium Sulfate (pH 7.0)
 Eluent B: 0.1 M Phosphate Buffer (pH 7.0)
 Flow Rate: 1 ml/min
 Gradient: 30 min linear
 Inj.Vol.: 50 l
 Samples: Ribonuclease A, Lysozyme, α -Chymotrypsinogen, 1 mg/ml



HIC SCALE UP

Seamless scale up

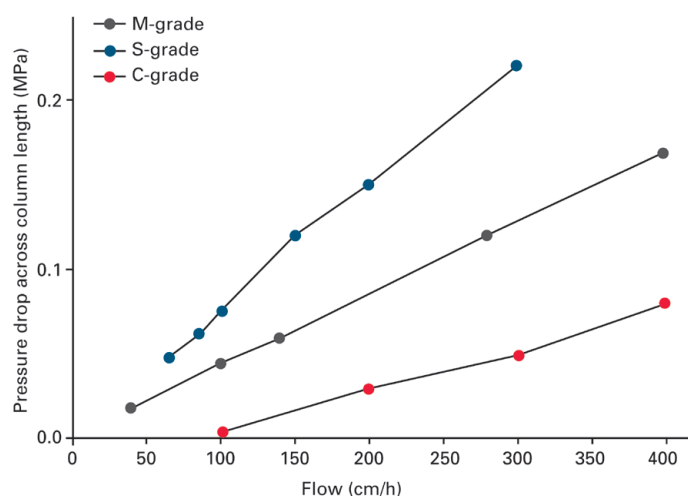
In terms of cost efficiency a production step should deliver maximum yield of the active product in short time. It will always be a compromise between throughput, resolution and recovery. The capacity of the column must fit to the yield of the upstream process or of the previous purification steps respectively. The target capacity determines the column dimensions, while the nature of the sample and the approached resolution determine the particle size.

The chemistry of the resins is very similar from the pre-packed TSKgel PW HPLC columns to the TSKgel-5PW and TOYOPEARL bulk resins. This offers the opportunity to find the ideal particle size for the intended use regardless of whether it is laboratory scale purification, a process polishing, intermediate or capture step. Figure 10 shows the separation of four standard proteins on various Phenyl media. Increasing the bead size from 10 µm (TSKgel Phenyl-5PW) over 35 µm and 65 µm up to 100 µm only reduces resolution but does not impair selectivity.

Superior pressure/flow characteristics

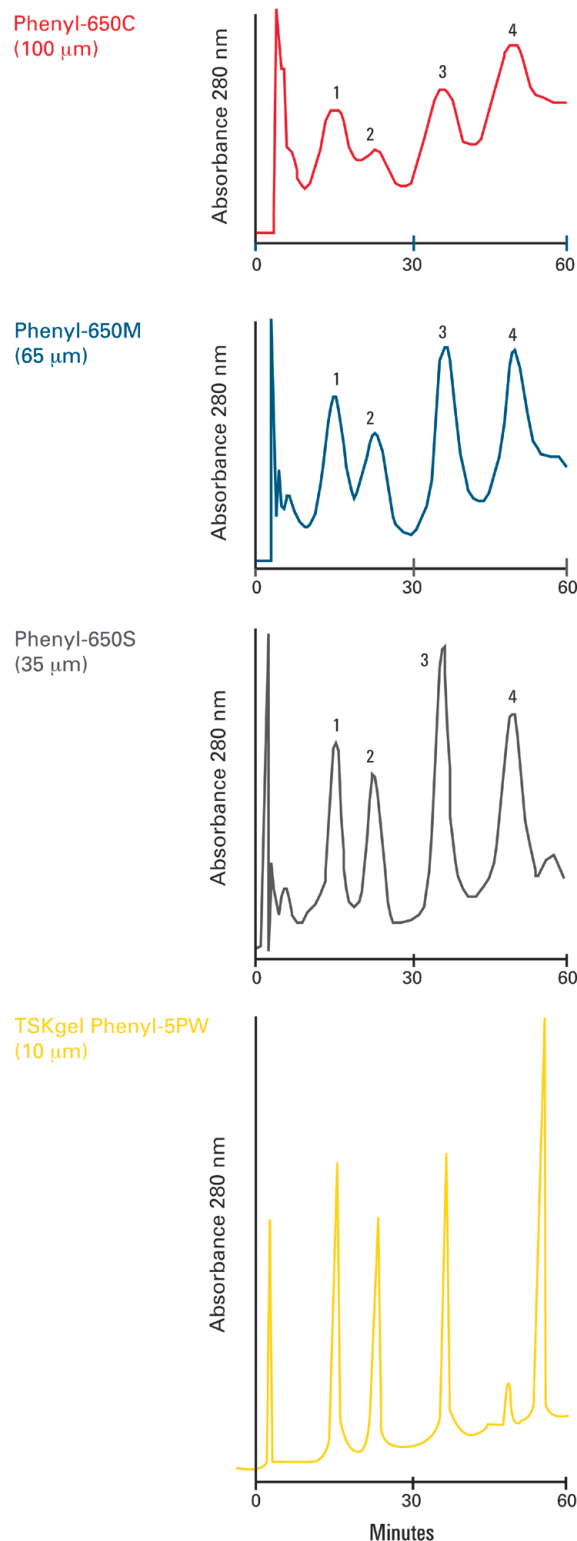
High flow rates reduce process cycle time and increase productivity. The rigid polymeric backbone of TOYOPEARL and TSKgel HIC resins assures superior pressure/flow characteristics over a wide range of flow rates. Figure 9 shows the excellent pressure flow/curves for all grades of TOYOPEARL Butyl-650, determined on a production size column with 40 cm ID and 20 cm length.

FIGURE 9
Pressure Flow Curve



Resin: TOYOPEARL Butyl-650
Column Size: 40 cm ID x 20 cm L
Eluent: Water
Temp.: Room Temperature

FIGURE 10
Improvement of Performance by Reducing Particle Size



Column: 7.5 mm ID x 7.5 cm L
Sample: 1. Myoglobin, 2. Ribonuclease A, 3. Lysozyme, 4. α -Chymotrypsinogen
Injection: 200 µl
Elution: 60 min. linear gradient from 2.0 M to 0 M of $(\text{NH}_4)_2\text{SO}_4$ in 0.1 M phosphate buffer, pH 7.0
Flow rate: 1.0 ml/min.
Detection: UV @ 280 nm

HIC APPLICATIONS

Applications

TOYOPEARL and TSKgel HIC resins are used in downstream purification of a variety of biopharmaceuticals. HIC is often used in capture steps following an ammonium sulphate precipitation. It is decreasing the salt concentration at the same time as conducting a purification step. HIC is a common intermediate process step for the purification of monoclonal antibodies. It is typically used to remove leached Protein A and aggregates subsequent to an affinity step. A typical industrial purification scheme for the isolation of mAbs from a cell culture supernatant is shown in Figure 12.

Monoclonal Antibodies

The diverse hydrophobic nature of mAbs is shown in Figure 11. The retention time as an indicator of hydrophobicity was measured for 51 different mouse IgGs on a TSKgel Phenyl-5PW analytical column. The elution time differs by a factor of 2-3 indicating very different hydrophobicities. The TOYOPEARL series of HIC ligands with different hydrophobicities offers a range of options for finding the right resin for the target molecule. For the highly hydrophobic mouse anti-chicken 14 kDa lectin the hydrophilic Ether ligand works well. Figure 13 shows the purification of this antibody from ascites fluid with TOYOPEARL Ether-650M material.

Aggregate Removal

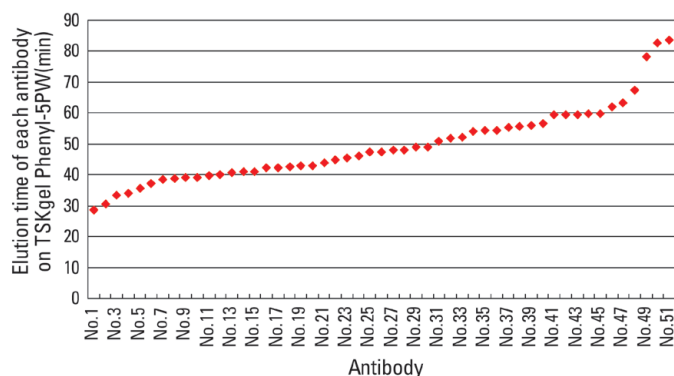
HIC in flow through mode is often used to remove aggregates generated in Protein A purification steps for mAbs. These impurities have chemical properties very similar to the target but they will generally be more hydrophobic than the native protein. Therefore they bind at relatively low salt concentrations to Butyl or Phenyl resins allowing the target to flow through the column.

In addition to the mentioned examples HIC is used successfully for a variety of other applications such as plasmid purification and endotoxin removal.

FIGURE 11

Hydrophobic Diversity of Mouse mAbs

Plot of chromatographic elution times for 51 different mouse mAbs

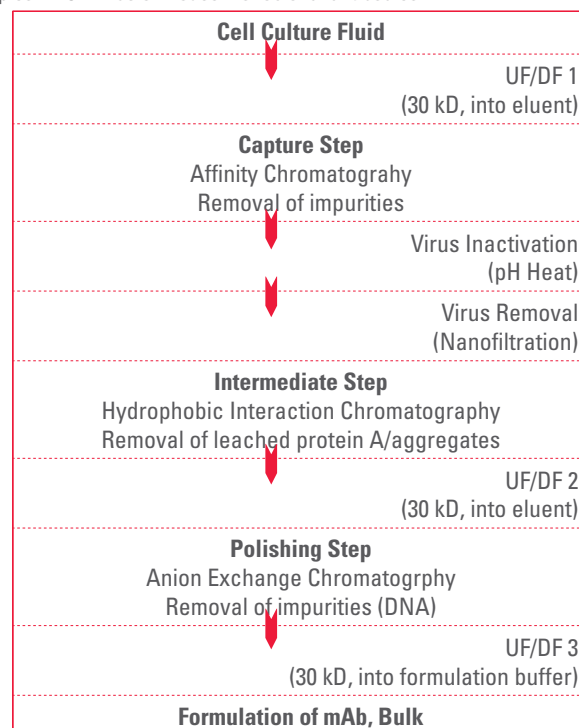


Column: TSKgel Phenyl-5PW
 Eluent: (A) 0.1 M phosphate buffer containing 1.8 M ammonium sulfate (pH 7.0)
 (B) 0.1 M phosphate buffer (pH 7.0)
 Flow rate: 1 mL/min
 Gradient: (B) 0% (0 min)--0% (5 min)--100% (65 min) linear

FIGURE 12

Example of Industrial mAb Purification

Samples: 51 kinds of mouse monoclonal antibodies



Even glycoproteins, which often bind irreversibly to saccharide based media, can be purified by HIC on polymer based resins.

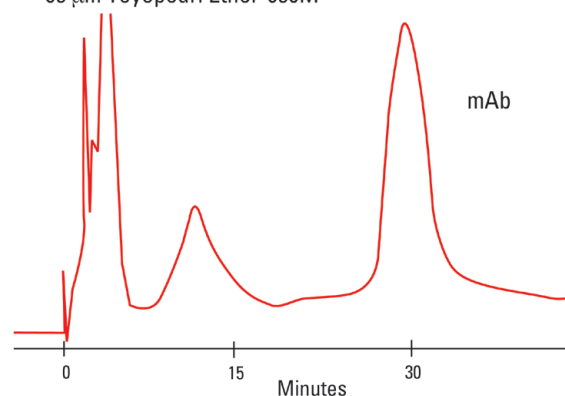
Regeneration of the Column

The type and frequency of regeneration of a column naturally depends on the samples applied. Standard cleaning procedures involve washing with high pH (e.g. 0.5 N NaOH). TOYOPEARL and TSKgel HIC resins are recommended for use from pH 2.0 to 12.0, although short exposures to higher pH for cleaning in place are possible.

FIGURE 13

Purification of mAbs from Ascites Fluid

65 µm Toyopearl Ether-650M



Column: TOYOPEARL Ether-650M, 7.5 mm ID x 7.5 cm L
 Sample: anti-chicken 14 kDa lectin, diluted ascites fluid, 0.76 mg in 50 µl
 Elution: 60 min. linear gradient from 1.5 M to 0 M (NH₄)₂SO₄ in 0.1 M phosphate buffer (pH 7.0)
 Flow rate: 136 cm/h



ORDERING INFORMATION

ORDERING INFORMATION

TOYOPEARL HIC resins:

Hydrophobicity	Chemical Structure	Product description	Container size (mL)	Part #	Particle size (μm)	Pore Size(Å)
weak	HW65-(OCH ₂ CH ₂) _n -OH	Ether-650S	25	0043151	20-50	1000
			100	0016172		
			1,000	0016174		
			5,000	0016176		
			25	0019805	40-90	1000
	Ether-650M		100	0016173		
			1,000	0016175		
			5,000	0016177		
medium	HW60-(OCH(CH ₃)-CH ₂) _n -OH	PPG-600M	25	0021301	40-90	750
			100	0021302		
			1,000	0021303		
			5,000	0021304		
	HW65-OC ₆ H ₅	Phenyl-650S	25	0043152	20-50	1000
			100	0014477		
			1,000	0014784		
			5,000	0014935		
		Phenyl-650M	25	0019818	40-90	1000
			100	0014478		
			1,000	0014783		
			5,000	0014943		
		Phenyl-650C	25	0043126	50-150	1000
			100	0014479		
			1,000	0014785		
	HW60-OC ₆ H ₅	Phenyl-600M	25	0021887	40-90	750
			100	0021888		
			1,000	0021889		
			5,000	0021890		
strong	HW65-O-(CH ₂) ₃ -CH ₃	Butyl-650S	25	0043153	20-50	1000
			100	0007476		
			1,000	0014701		
			5,000	0007975		
		Butyl-650M	25	0019802	40-90	1000
			100	0007477		
			1,000	0014702		
			5,000	0007976		
		Butyl-650C	25	0043127	50-150	1000
			100	0007478		
			1,000	0014703		
			5,000	0007977		
	HW60-O-(CH ₂) ₃ -CH ₃	Butyl-600M	25	0021448	40-90	750
			100	0021449		
			1,000	0021450		
			5,000	0021451		
	HW55-O-(CH ₂) ₃ -CH ₃	SuperButyl-550C	25	0019955	50-150	500
			100	0019956		
			1,000	0019957		
			5,000	0019958		
	HW65-O-(CH ₂) ₅ -CH ₃	Hexyl-650C	25	0044465	50-150	1000
			100	0019026		
			1,000	0019027		
			5,000	0019028		

TOYOPEARL LABPAK:

Part #	Product description	Container size (mL)	Particle size (μm)
0043150	HICPAK HP (Ether, Phenyl, Butyl-650S)	3 x 25 mL	35
0019806	HICPAK (Ether, Phenyl, Butyl-650M)	3 x 25 mL	65
0043125	HICPAK-C (Phenyl, Butyl, Hexyl-650C)	3 x 25 mL	100

ORDERING INFORMATION



ORDERING INFORMATION

TSKgel 5PW HIC resins for high resolution:

Hydrophobicity	Chemical Structure	Product description	Container size (mL)	Part #	Particle size (μm)	Pore Size (Å)
weak	5PW-(OCH ₂ CH ₂) _n -OH	Ether-5PW (20)	25	0043276	10-30	1000
			250	0016052		
			1,000	0016053		
			5,000	0018437		
		Ether-5PW (30)	25	0043176	20-40	1000
			250	0016050		
			1,000	0016051		
			5,000	0018439		
		Phenyl-5PW (20)	25	0043277	10-30	1000
			250	0014718		
medium	5PW-OC ₆ H ₅		1,000	0014719		
			5,000	0018438		
		Phenyl-5PW (30)	25	0043177	20-40	1000
			250	0014720		
			1,000	0014721		
			5,000	0017210		

ToyoScreen process development columns for HIC:

Part #	Product description	Package
0021372	ToyoScreen Ether-650M, 1 mL	1 mL x 6 each
0021373	ToyoScreen Ether-650M, 5 mL	5 mL x 6 each
0021374	ToyoScreen Phenyl-650M, 1 mL	1 mL x 6 each
0021375	ToyoScreen Phenyl-650M, 5 mL	5 mL x 6 each
0021376	ToyoScreen Butyl-650M, 1 mL	1 mL x 6 each
0021377	ToyoScreen Butyl-650M, 5 mL	5 mL x 6 each
0021378	ToyoScreen Hexyl-650C, 1 mL	1 mL x 6 each
0021379	ToyoScreen Hexyl-650C, 5 mL	5 mL x 6 each
0021380	ToyoScreen PPG-600M, 1 mL	1 mL x 6 each
0021381	ToyoScreen PPG-600M, 5 mL	5 mL x 6 each
0021495	ToyoScreen Butyl-600M, 1 mL	1 mL x 6 each
0021494	ToyoScreen Butyl-600M, 5 mL	5 mL x 6 each
0021892	ToyoScreen Phenyl-600M, 1 mL	1 mL x 6 each
0021893	ToyoScreen Phenyl-600M, 5 mL	5 mL x 6 each
0021382	ToyoScreen SuperButyl-550C, 1 mL	1 mL x 6 each
0021383	ToyoScreen SuperButyl-550C, 5 mL	5 mL x 6 each
0021398	ToyoScreen HIC Mix Pack, 1 mL	1 mL x 6 Grades x 1 each
0021399	ToyoScreen HIC Mix Pack, 5 mL	5 mL x 6 Grades x 1 each

ToyoScreen column accessories

0021400	ToyoScreen Column Holder
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TSKgel LABPAK:

Part #	Product description	Container size (mL)	Particle size (μm)
0043278	HICPAK PW (20) (Ether-5PW, Phenyl-5PW)	2 x 25 mL	10-30
0043175	HICPAK PW (30) (Ether-5PW, Phenyl-5PW)	2 x 25 mL	20-40



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