TOSOH

interchim®

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 TSK-GEL DEVELOPED BY TOSOH
1974	HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COLUMN PLANT IS COMPLETED
1979	TOSOH DEVELOPS TOYOPEARL MEDIA
1983	TOSOH DEVELOPS HYDROPHOBIC INTERACTION MEDIA
1987	TOSOHAAS US OPERATIONS FORMED IN MONTGOMERYVILLE
1989	TOSOHAAS GMBH OPERATIONS FORMED IN STUTTGART
1995	TOSOH NANYO GEL FACILITY RECEIVES ISO 9001
2000	IN NOVEMBER FORMER TOSOHAAS US OPERATIONS BECOMES TOSOH BIOSEP LLC, A 100% SUBSIDIARY OF TOSOH CORPORATION
2001	IN JANUARY FORMER TOSOHAAS GMBH EUROPEAN OPERATIONS BECOMES TOSOH BIOSEP GMBH, A 100% SUBSIDIARY OF TOSOH CORPORATION
2002/ 2003	TOSOH CORPORATION ANNOUNCES THAT ALL TOSOH AFFILIATED SCIENTIFIC AND DIAGNOSTIC SYSTEM RELATED COMPANIES IN EUROPE, WILL BE UNIFIED UNDER THE NEW NAME TOSOH BIOSCIENCE.
2008	ECOSEC , THE 7TH GENERATION GPC SYSTEM IS INTRODUCED GLOBALLY
2009	20TH ANNIVERSARY OF TOSOH BIOSCIENCE GMBH, STUTTGART

HYDROPHOBIC INTERACTION CHROMATOGRAPHY

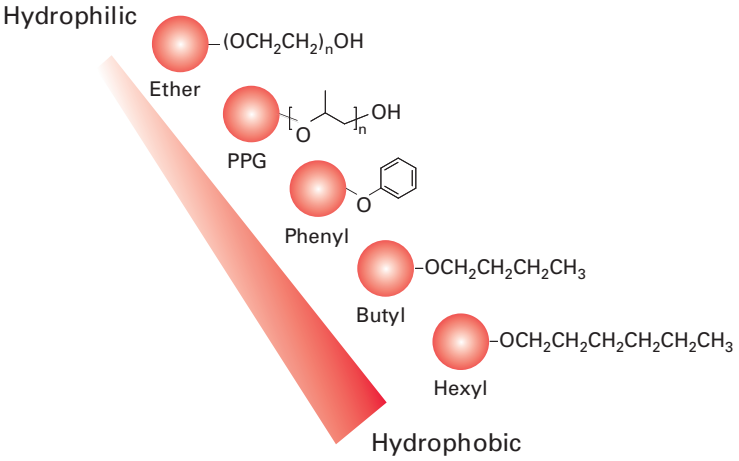
Hydrophobic Interaction Chromatography (HIC) is a widely-used technique for separation and purification of proteins and peptides. HIC sorts biomolecules by degree of their surface hydrophobicity. Samples are adsorbed to the resin at relatively high salt concentrations and eluted by applying a decreasing 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 TSK-GEL 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.

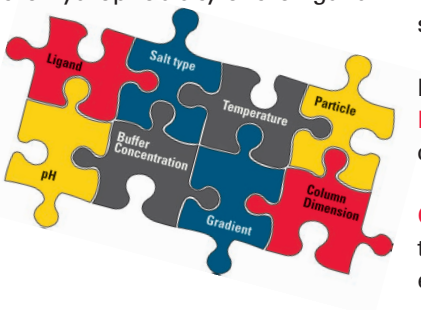
Selectivity

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

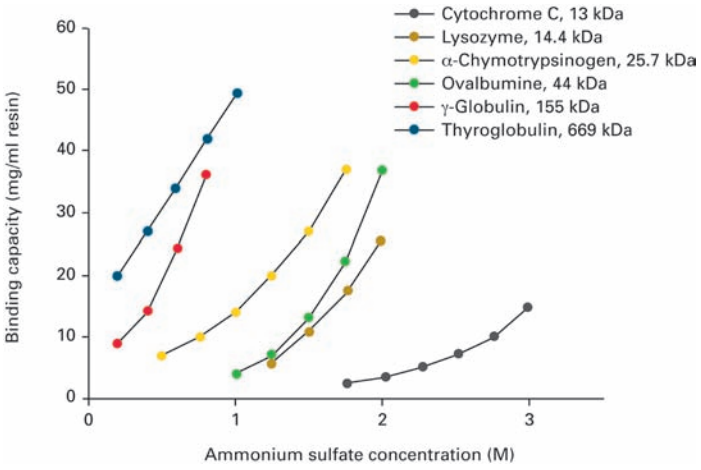
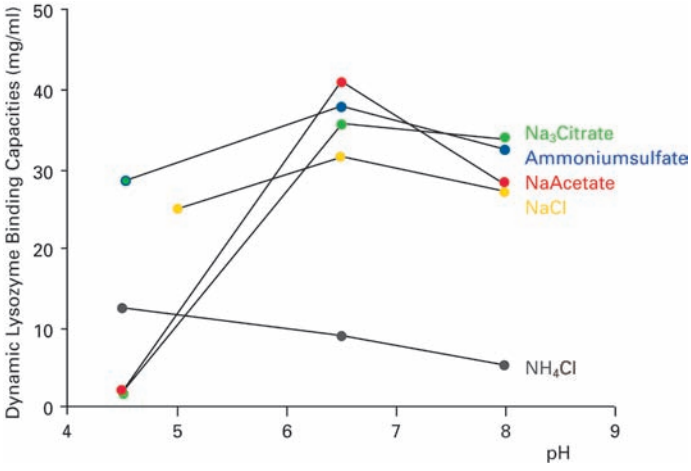


FIGURE 3

Influence of pH



HIC
TSK-GEL 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 TSK-GEL materials with 20 µm or 30 µm particles are ideal. TSK-GEL columns with 10 µm beads are best suited for analytical purposes or for small scale purifications (Figure4).

Toyopearl HIC Material

Toyopearl and TSK-GEL HIC resins are specifically designed for use in biopharmaceutical production. Their rigid methacrylic polymer structure shows excellent pressure/flow properties enabeling 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 TSK-GEL HPLC columns up to bulk media used for pilot and production scale.

HIC Ligands

The wide range of Toyopearl and TSK-GEL 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.

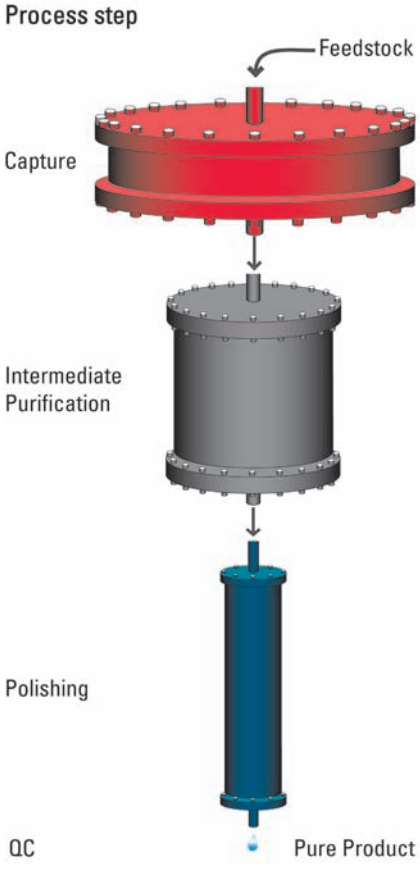
Today high-resolution HIC applications gain more and more interest. TSK-GEL 5PW media with small particle sizes are ideally suited if high resolution is an issue. TSK-GEL 5PW bulk material is available with the ligands Ether and Phenyl. TSK-GEL columns in various dimensions are available with Ether,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



Bead size	Process media
100 µm	Toyopearl SuperButyl-550C Toyopearl Hexyl-650C Toyopearl Butyl-650C Toyopearl Phenyl-650C
65 µm	Toyopearl Butyl-600M Toyopearl Phenyl-600M Toyopearl PPG-600M
	Toyopearl Butyl-650M Toyopearl Phenyl-650M Toyopearl Ether-650M
35 µm	Toyopearl Butyl-650S Toyopearl Phenyl-650S Toyopearl Ether-650S
30 µm	TSKgel Phenyl-5PW (30) TSKgel Ether-5PW (30)
20 µm	TSKgel Phenyl-5PW (20) TSKgel Ether-5PW (20)
10 µm	TSKgel Phenyl-5PW columns TSKgel Ether-5PW columns

HIC
TSK-GEL 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 Resins

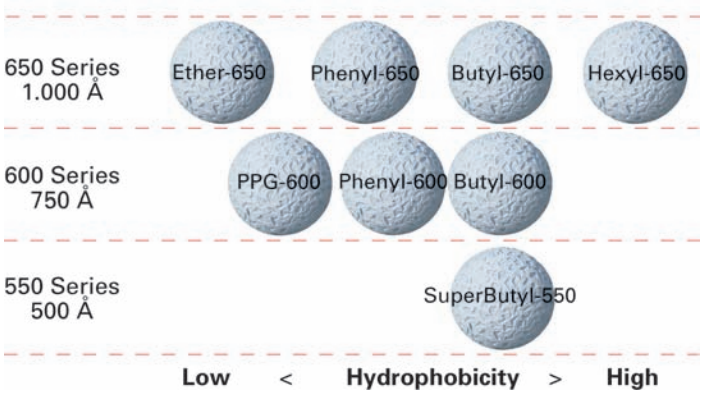
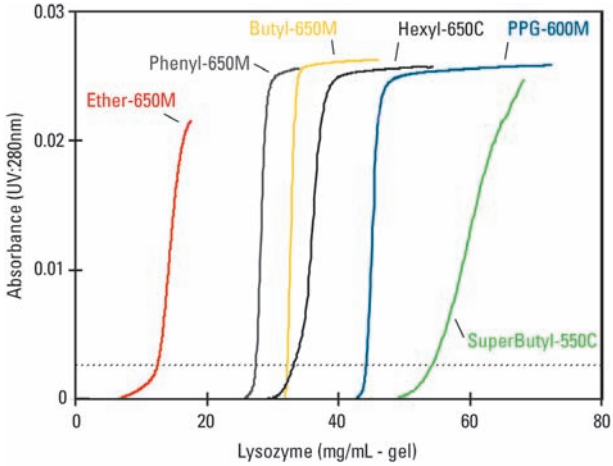


Figure 5 and 7 show the dynamic binding capacities of Toyopearl resins for Lysozyme and a monoclonal antibody. For a small protein such as Lysozyme the SuperButyl-550C is the best choice (Figure 5). Figure 7 demonstrates the superior DBC of the Butyl-600M and Phenyl-600M resins for large proteins.

FIGURE 5

Typical Dynamic Binding Capacities of Lysozyme

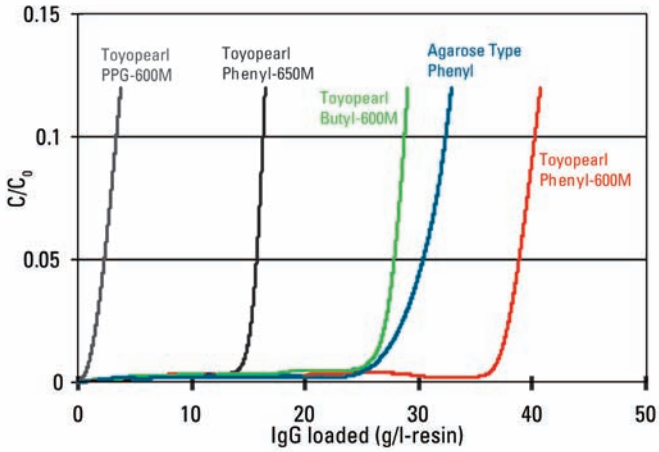


	Binding capacity (mg/mL - gel) (10% Breakthrough)
Ether-650M	12.5
Phenyl-650M	27.5
Butyl-650M	32.2
Hexyl-650C	33.2
PPG-600M	44.2
SuperButyl-550C	54.3

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 $(NH_4)_2SO_4$ + 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.

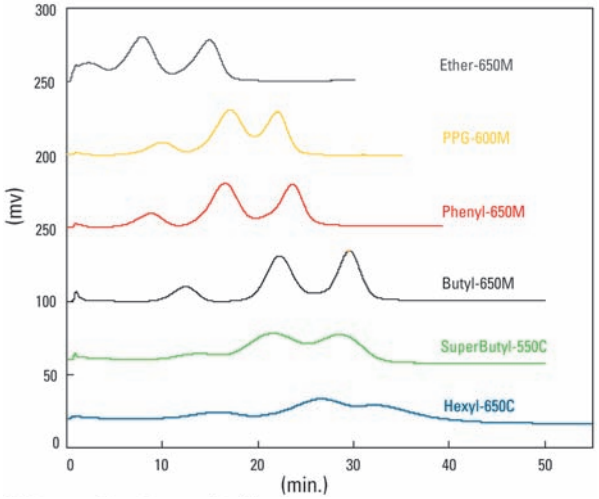
The effect of the different hydrophobicities of Toyopearl resins on retention and resolution of standard proteins are illustrated in Figure 8. A standard mixture of proteins was separated using ToyoScreen columns. Fast screening of a larger number of resins under various conditions can be realized by 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 TSK-GEL 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.

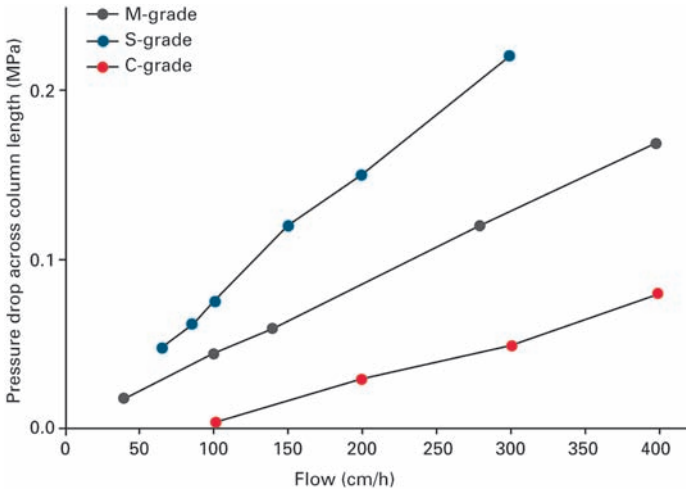
The chemistry of the resins is very similar from the pre-packed TSK-GEL PW HPLC columns to the TSK-GEL-5PW and Toyopearl bulk resins. This offers the opportunity to find the ideal particle size for the intended use regardless of whether it is laboratory scale purification, a process polishing, intermediate 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 TSK-GEL 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

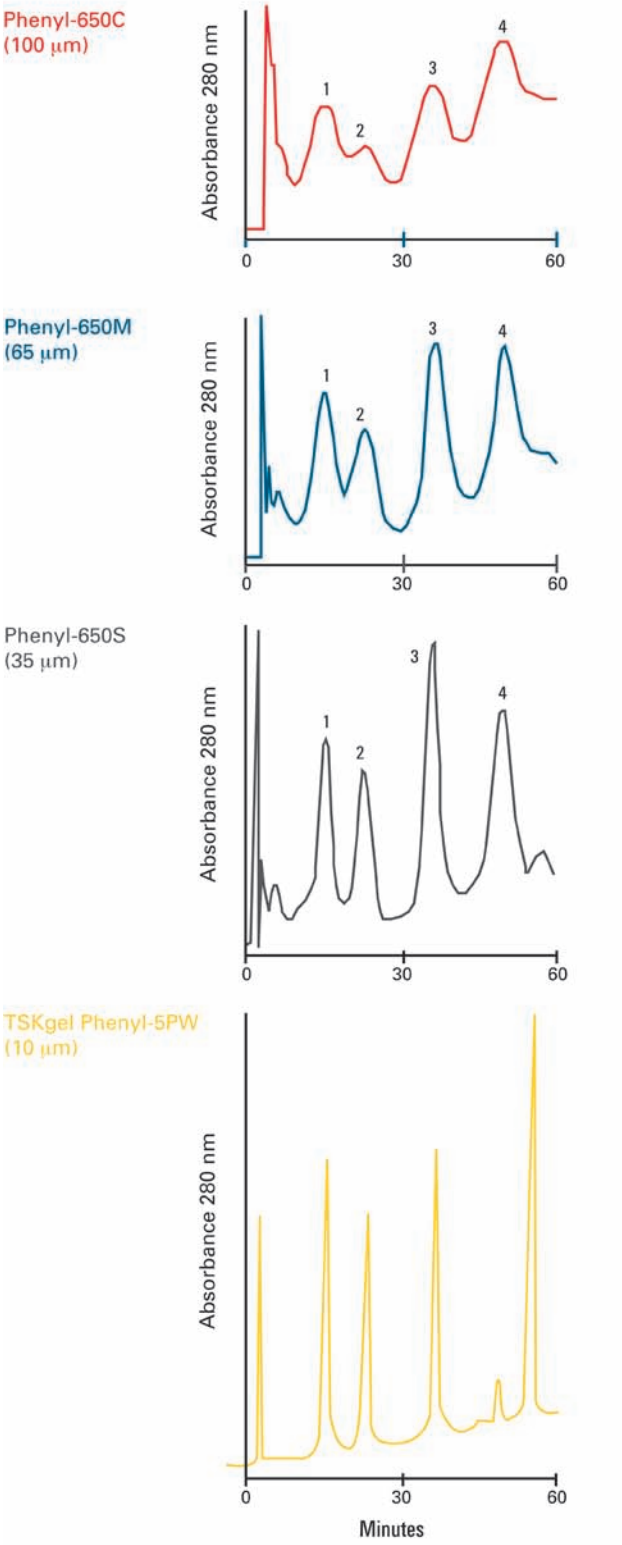
Pressure Flow Curve



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 $(NH_4)_2SO_4$ in 0.1 M phosphate buffer, pH 7.0
Flow rate:	1.0 ml/min.
Detection:	UV @ 280 nm

HIC APPLICATIONS

Applications

Toyopearl and TSK-GEL 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.

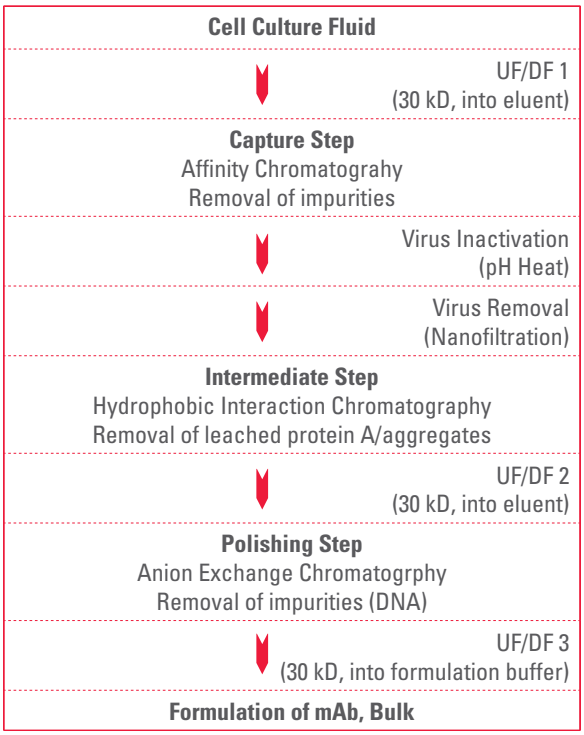
Aggregate Removal

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 sucessfully for a variety of other applications such as plasmid purification and endotoxin removal.

FIGURE 12

Example of Industrial mAb Purification



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

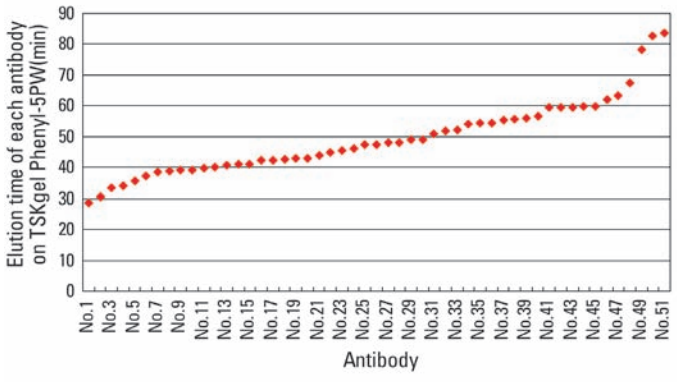
Regeneration of the Column

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 TSK-GEL 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 11

Hydrophobic Diversity of Mouse mAbs

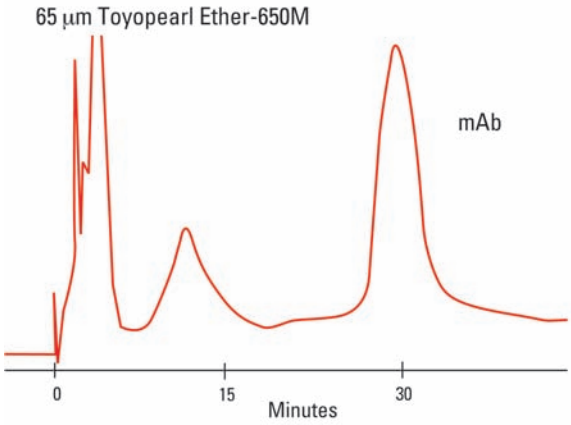
Plot of chromatographic elution times for 51 different mouse mAbs



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
Samples: 51 kinds of mouse monoclonal antibodies

FIGURE 13

Purification of mAbs from Ascites Fluid



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 $(NH_4)_2SO_4$ in 0.1 M phosphate buffer (pH 7.0)
Flow rate: 136 cm/h
Detection: UV @ 280 nm

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	43151	20-50	1000
			100	16172		
			1,000	16174		
			5,000	16176		
		Ether-650M	25	19805	40-90	1000
			100	16173		
			1,000	16175		
			5,000	16177		
medium	HW60-(OCH(CH$_3$)-CH$_2$)n-OH	PPG-600M	25	21301	40-90	750
			100	21302		
			1,000	21303		
			5,000	21304		
	HW65-OC$_6$H$_5$	Phenyl-650S	25	43152	20-50	1000
			100	14477		
			1,000	14784		
			5,000	14935		
		Phenyl-650M	25	19818	40-90	1000
			100	14478		
			1,000	14783		
			5,000	14943		
		Phenyl-650C	25	43126	50-150	1000
			100	14479		
			1,000	14785		
	HW60-OC$_6$H$_5$	Phenyl-600M	25	21887	40-90	750
			100	21888		
			1,000	21889		
			5,000	21890		
strong	HW65-O-(CH$_2$)$_3$-CH$_3$	Butyl-650S	25	43153	20-50	1000
			100	07476		
			1,000	14701		
			5,000	07975		
		Butyl-650M	25	19802	40-90	1000
			100	07477		
			1,000	14702		
			5,000	07976		
		Butyl-650C	25	43127	50-150	1000
			100	07478		
			1,000	14703		
			5,000	07977		
	HW60-O-(CH$_2$)$_3$-CH$_3$	Butyl-600M	25	21448	40-90	750
			100	21449		
			1,000	21450		
			5,000	21451		
	HW55-O-(CH$_2$)$_3$-CH$_3$	SuperButyl-550C	25	19955	50-150	500
			100	19956		
			1,000	19957		
			5,000	19958		
	HW65-O-(CH$_2$)$_5$-CH$_3$	Hexyl-650C	25	44465	50-150	1000
			100	19026		
			1,000	19027		
			5,000	19028		

Toyopearl LABPAK:

Part #	Product description	Container size (mL)	Particle size (µm)
43150	HICPAK HP (Ether, Phenyl, Butyl-650S)	3 x 25 mL	35
19806	HICPAK (Ether, Phenyl, Butyl-650M)	3 x 25 mL	65
43125	HICPAK-C (Phenyl, Butyl, Hexyl-650C)	3 x 25 mL	100

ORDERING INFORMATION

ORDERING INFORMATION

TSK-GEL 5PW HIC resins for high resolution:

Hydrophobicity	Chemical Structure	Product description	Container size (mL)	Part #	Particle size (µm)	Pore Size (Å)
weak	5PW-(OCH_2CH_2)n-OH	Ether-5PW (20)	25	43276	10-30	1000
			250	16052		
			1,000	16053		
			5,000	18437		
		Ether-5PW (30)	25	43176	20-40	1000
			250	16050		
			1,000	16051		
			5,000	18439		
		Phenyl-5PW (20)	25	43277	10-30	1000
medium	5PW-OC_6H_5		250	14718		
			1,000	14719		
			5,000	18438		
		Phenyl-5PW (30)	25	43177	20-40	1000
			250	14720		
			1,000	14721		
			5,000	17210		

ToyoScreen process development columns for HIC:

Part #	Product description	Package
21372	ToyoScreen Ether-650M, 1 mL	1 mL x 6 each
21373	ToyoScreen Ether-650M, 5 mL	5 mL x 6 each
21374	ToyoScreen Phenyl-650M, 1 mL	1 mL x 6 each
21375	ToyoScreen Phenyl-650M, 5 mL	5 mL x 6 each
21376	ToyoScreen Butyl-650M, 1 mL	1 mL x 6 each
21377	ToyoScreen Butyl-650M, 5 mL	5 mL x 6 each
21378	ToyoScreen Hexyl-650C, 1 mL	1 mL x 6 each
21379	ToyoScreen Hexyl-650C, 5 mL	5 mL x 6 each
21380	ToyoScreen PPG-600M, 1 mL	1 mL x 6 each
21381	ToyoScreen PPG-600M, 5 mL	5 mL x 6 each
21495	ToyoScreen Butyl-600M, 1 mL	1 mL x 6 each
21494	ToyoScreen Butyl-600M, 5 mL	5 mL x 6 each
21892	ToyoScreen Phenyl-600M, 1 mL	1 mL x 6 each
21893	ToyoScreen Phenyl-600M, 5 mL	5 mL x 6 each
21382	ToyoScreen SuperButyl-550C, 1 mL	1 mL x 6 each
21383	ToyoScreen SuperButyl-550C, 5 mL	5 mL x 6 each
21398	ToyoScreen HIC Mix Pack, 1 mL	1 mL x 6 Grades x 1 each
21399	ToyoScreen HIC Mix Pack, 5 mL	5 mL x 6 Grades x 1 each

ToyoScreen column accessories

21400	ToyoScreen Column Holder

TSK-GEL LABPAK:

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