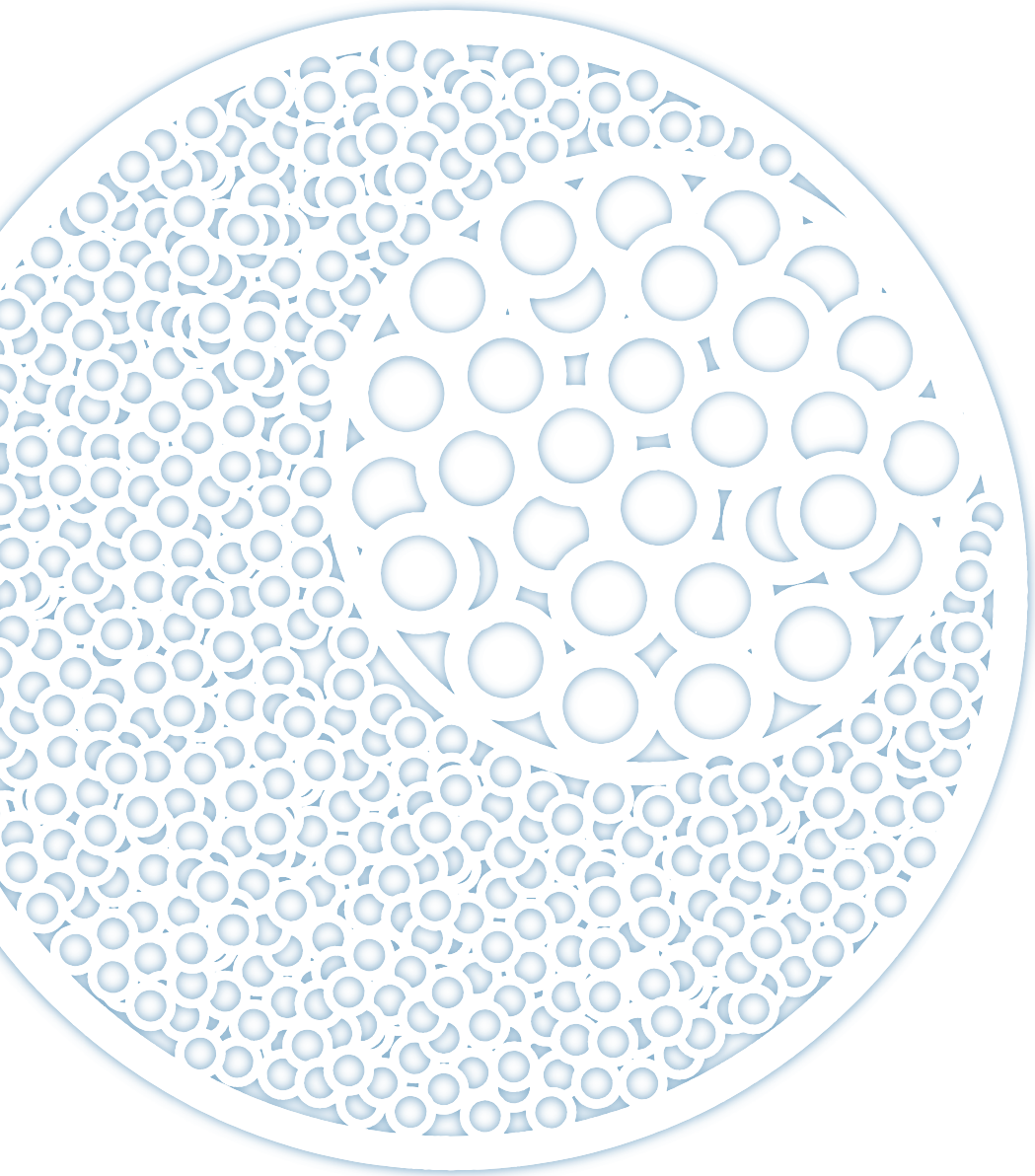
TOSOH

Principles of Hydrophobic Interaction Chromatography



TOSOH BIOSCIENCE

Table of Contents

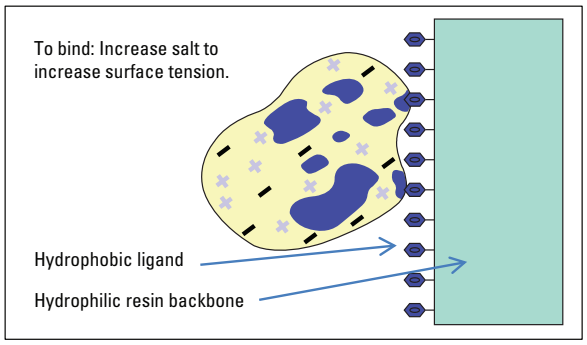
Introduction

Hydrophobic interaction chromatography (HIC) separates biomolecules, under comparatively mild conditions, according to differences in hydrophobicity on the exposed surface of the molecule. HIC is a very versatile chromatographic method and can be used as a capture, intermediate, or polishing step in a purification process. HIC chromatography media from Tosoh Bioscience is scalable and as such has been used for bench-scale separations in the development laboratory, clinical manufacturing at pilot scale, and the manufacture of commercial product in a production plant.

HIC has been widely used in protein purification as an orthogonal method to other chromatography modes that separate according to specific recognition (affinity), charge (ion exchange), or molar mass (size exclusion).

HIC is an ideal technique to use when samples have been precipitated by ammonium sulfate for initial sample purification or after being previously purified by ion exchange chromatography when the target of interest has been eluted in a high salt buffer. In both instances, the sample containing the target of interest has a high concentration of salt and may be loaded onto the HIC column directly without need for additional manipulation. In hydrophobic interaction chromatography, elevated salt levels amplify the interaction between hydrophobic patches on the surface of the target molecule and the ligand coupled to the chromatographic base resin *(Figure 1)*. While there is no universally accepted theory on the exact mechanisms that facilitate HIC separations as a group, there are a number of proposals that are available in the scientific literature.

Figure 1:



Theory

Interactions between hydrophobic regions on proteins and the ligand on HIC resins are heavily influenced by the presence of salts in the mobile phase. Large concentrations of salt boost the target-ligand interaction while a reduced salt concentration weakens it. When running a separation on HIC resins, the sample is typically loaded at a high salt concentration. To effect desorption, the ionic strength of the buffer is reduced, typically using a gradient. By decreasing the salt concentration of the mobile phase, the interaction between the target and the ligand is reversed and molecules with a lower degree of hydrophobicity are eluted first while very hydrophobic molecules elute last as the salt concentration in the mobile phase is reduced along the gradient.

For applications involving HIC, selectivity is largely dependent on the ligand and the amount (density) attached onto the base resin, the nature of the base resin itself (polymeric), the physiochemical properties of the target protein/ molecule and its impurities, and the type and concentration of salt(s) used in the mobile phase. A properly developed HIC step will incorporate a balance among these several properties leading to a highly selective and robust HIC separation.

Another aspect to be considered when developing a separation involving HIC is the amount of protein that can bind to the resin under distinct operating conditions. The dynamic binding capacity (DBC) of a HIC resin is largely dependent on the hydrophobic properties of the individual resin, the target being purified and the operating conditions such as mobile phase composition, linear velocity (residence time), temperature and, to a minor extent, pH.

Unlike chromatographic methods such as ion exchange or affinity chromatography, where standard proteins such as lysozyme, BSA, and cytochrome C can be used as models to predict selectivity and capacity, hydrophobic interaction chromatography is so dependent on the roles played by such variables as ligand, target, impurities, and mobile phase composition, that capacity and selectivity must be determined and optimized experimentally.

Though ligand-target interactions, mediated by mobile phase composition, constitute the majority of the influences on the selectivity for a HIC separation, the base resin may also play a part in determining the final selectivity. Chromatography media for HIC, like all other Tosoh resins, are made from a porous polymethacrylic polymer matrix. The base resin exhibits a high level of physical and chemical stability and the hydrophilic nature of the polymer reduces non-specific interactions between the base bead and the target of interest.

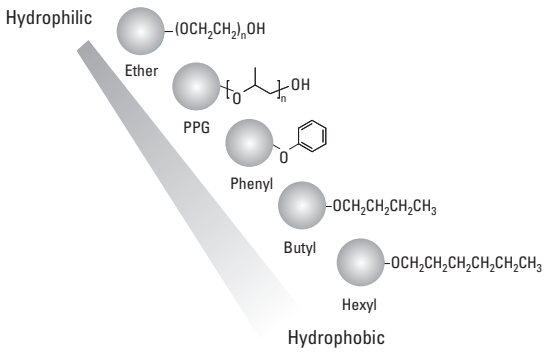
Table 1: Properties of TOYOPEARL HIC resins

TOYOPEARL resin	Hydrophobicity	Base bead	Pore size (nm)	Bead diameter (µm)	Ligand type	DBC (g/L)	Pressure rating
Ether-650S	+	HW-65	100	20 - 50	Ether	10-30	0.3 MPa
Ether-650M	+	HW-65	100	40 - 90	Ether	10-30	0.3 MPa
PPG-600M	++	HW-60	75	40 - 90	Polypropylene glycol	45 - 55	0.3 MPa
Phenyl-600M	+++	HW-60	75	40 - 90	Phenyl	45 - 65	0.3 MPa
Phenyl-650S	+++	HW-65	100	20 - 50	Phenyl	30 - 50	0.3 MPa
Phenyl-650M	+++	HW-65	100	40 - 90	Phenyl	30 - 50	0.3 MPa
Phenyl-650C	+++	HW-65	100	50 - 150	Phenyl	30 - 50	0.3 MPa
Butyl-650S	++++	HW-65	100	20 - 50	Butyl	30 - 50	0.3 MPa
Butyl-650M	++++	HW-65	100	40 - 90	Butyl	30 - 50	0.3 MPa
Butyl-650C	++++	HW-65	100	50 - 150	Butyl	30 - 50	0.3 MPa
Butyl-600M	++++	HW-60	75	40 - 90	Butyl	40 - 60	0.3 MPa
SuperButyl-550C	++++	HW-55	50	50 - 150	Butyl	52 - 70	0.3 MPa
Hexyl-650C	+++++	HW-65	100	50 - 150	Hexyl	30 - 50	0.3 MPa

TOYOPEARL and TSKgel HIC Resins from Tosoh Bioscience

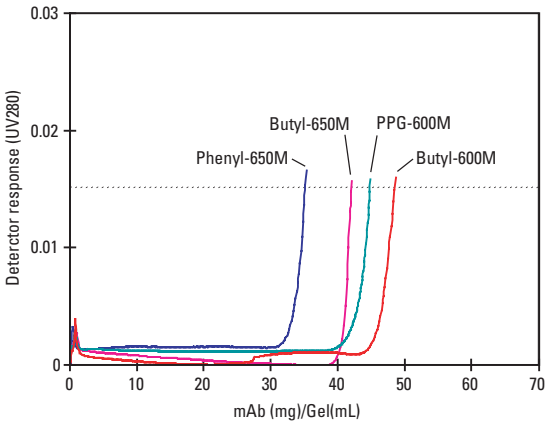
Tosoh Bioscience offers HIC resins in both the TOYOPEARL and TSKgel product lines for process scale chromatography applications. TOYOPEARL HIC resins are functionalized versions of the TOYOPEARL HW size exclusion resins and are therefore based on hydroxylated polymethacrylic polymer beads. Tosoh Bioscience offers five HIC ligands featuring different degrees of hydrophobicity and selectivity. *Table 1* lists the properties of these TOYOPEARL HIC resins. The hydrophobicity of TOYOPEARL HIC resins increases through the ligand series: ether, PPG (polypropylene glycol), phenyl, butyl, and hexyl *(Figure 2)*.

Figure 2: Available HIC ligands



TOYOPEARL HIC resins are offered in multiple grades, or particle sizes, to maximize productivity and resolution depending on where in the purification process the HIC step is located. Larger particle sizes allow higher linear velocities for capture and intermediate process steps, while smaller particle sizes promote better resolution for polishing steps. In addition, some TOYOPEARL resins have optimized pore sizes for specific applications *(Figure 3)*.

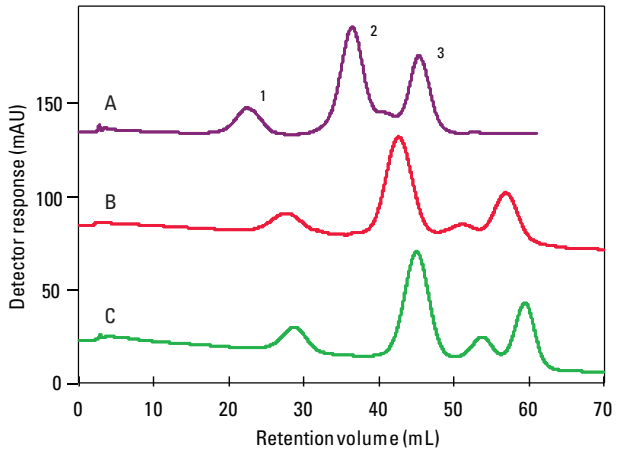
Figure 3: Typical Dynamic Binding Capacities for mAb on TOYOPEARL HIC resins with different pore sizes



	Binding capacity (mg/mL - gel) (10% Breakthrough)
Phenyl-650M	35.2
Butyl-650M	42.1
PPG-600M	44.8
Butyl-600M	48.5

Three HIC resins are available in the TOYOPEARL -600 resin format, PPG, phenyl, and butyl. These resins are based on the TOYOPEARL HW-60 base resin which has a 75 nm pore size that has been optimized for the purification of monoclonal antibodies (mAbs). The selectivities of TOYOPEARL Butyl-600M, TOYOPEARL PPG-600M and the TOYOPEARL Phenyl-600M resins are shown in *Figure 6*. Available in the TOYOPEARL -650 series (100 nm pore size) are the following four HIC ligands: hexyl, butyl, phenyl, and ether. The remaining resin available in the TOYOPEARL HIC line is SuperButyl-550C (50 nm pore size).

Figure 6: Comparison of TOYOPEARL -600M resins



Resins:	A. TOYOPEARL PPG-600M B. TOYOPEARL Phenyl-600M C. TOYOPEARL Butyl-600M
Column size:	7.5 mm ID × 7.5 cm
Mobile phase:	Buffer A: 1.8 mol/L $(NH_4)_2SO_4$ + 0.1 mol/L sodium phosphate, pH 7.0 Buffer B: 0.1 mol/L sodium phosphate, pH 7.0
Gradient:	60 min linear gradient from buffer A to B
Flow rate:	136 cm/hr (1.0 mL/min)
Detection:	UV @ 280 nm
Temperature:	ambient
Samples:	1 g/L of: 1. ribonuclease A 2.lysozyme 3. α-chymotrypsinogen
Load volume:	100 µL

The larger pore TOYOPEARL products such as TOYOPEARL Butyl-650 and TOYOPEARL Phenyl-650 resins are very useful for protein aggregate separation and removal. In addition, Tosoh Bioscience HIC resins are very effective in separating misfolded proteins from the native protein form. 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 misfolded protein while allowing the native target protein to flow through the column.

The same ether and phenyl ligands that are used for the TOYOPEARL resins are also available within the TSKgel HIC resin product line. Properties of TSKgel HIC resins are listed in *Table 2*. The TSKgel HIC resins use the same methacrylic polymer chemistry as the TOYOPEARL resins *(Table 3)* but have a higher degree of crosslinking, making for a more rigid bead.

Table 3: Methacrylic base beads available for HIC

Pore size (nm)	5	12.5	40-50	75	100	>100	>170
Resin							
TOYOPEARL HW-type:	40	50	55	60	65	75	80
TSKgel PW-type:	G1000	G2000	G4000		G5000	G6000	

Increasing pore surface area

Table 2: Properties of TSKgel HIC resins

TSKgel resin	Hydrophobicity	Base bead	Pore size (nm)	Bead diameter (µm)	Ligand type	DBC (g/L)	Pressure rating
Ether-5PW (20)	+	PW5000	100	15 - 25	Ether	10 - 30	2.0 MPa
Ether-5PW (30)	+	PW5000	100	20 - 40	Ether	10 - 30	2.0 MPa
Phenyl-5PW (20)	++	PW5000	100	15 - 25	Phenyl	10 - 30	2.0 MPa
Phenyl-5PW (30)	++	PW5000	100	20 - 40	Phenyl	10 - 30	2.0 MPa

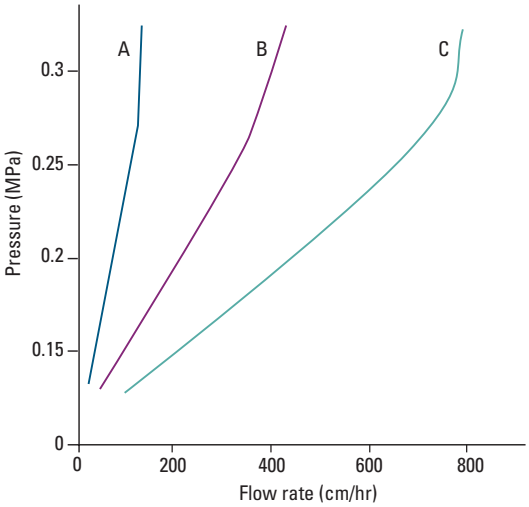
This is necessitated by the higher pressures generated when using smaller particles for chromatography. Greater crosslinking decreases the number of sites available for ligand attachment and thus a TSKgel resin will have a lower dynamic binding capacity than the corresponding TOYOPEARL resin.

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

Because TOYOPEARL and TSKgel HIC resins have the same backbone polymer chemistry, the selectivity for proteins and impurities will be unchanged. *Table 4* shows the ligands and particle sizes available for TOYOPEARL and TSKgel HIC resins and is arranged in increasing levels of resolution by bead size (i.e. low, medium, and high resolution). The semi rigid polymeric backbone of TOYOPEARL and TSKgel HIC resins permits high flow rates for maximum throughput and productivity. TOYOPEARL HIC resins may be operated at pressures up to 0.3 MPa and TSKgel -5PW HIC resins may be operated up to 2.0 MPa. The pressure-flow characteristics for each particle size grade of TOYOPEARL Phenyl-650 resins are shown in *Figure 7*. Resolution increases with decreasing particle size. Resin particle size is proportional to HETP and inversely proportional to the column efficiency and resolution of two peaks. TOYOPEARL HIC resins are available in three particle sizes, though not all ligands are available in each grade:

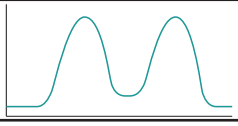
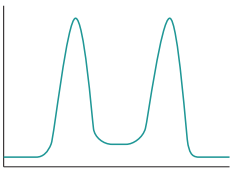
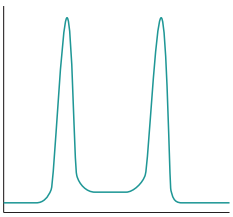
- S-grade = 35 µm (Superfine)
- M-grade = 65 µm (Fine)
- C-grade = 100 µm (Coarse)

Figure 7: Pressure-flow curve for Toyopearl Phenyl-650 resins of various particle sizes



Resins:	A. TOYOPEARL Phenyl-650S B. TOYOPEARL Phenyl-650M C. TOYOPEARL Phenyl-650C
Column size:	25 mm ID × 25 cm
Mobile phase:	2.0 mol/L (NH4)2SO4
Flow rate:	as indicated in figure

Table 4: Resolution of TOYOPEARL and TSKgel HIC resins

Resolution		Bead diameter (µm)	Pore size (nm)	HIC resin
Low		100	50 100 100 100	TOYOPEARL SuperButyl-550C TOYOPEARL Hexyl-650C TOYOPEARL Butyl-650C TOYOPEARL Phenyl-650C
Medium		65	75 75 75	TOYOPEARL Butyl-600M TOYOPEARL Phenyl-600M TOYOPEARL PPG-600M
		65	100 100 100	TOYOPEARL Butyl-650M TOYOPEARL Phenyl-650M TOYOPEARL Ether-650M
High		35	100 100 100	TOYOPEARL Butyl-650S TOYOPEARL Phenyl-650S TOYOPEARL Ether-650S
		30	100 100	TSKgel Phenyl-5PW (30) TSKgel Ether-5PW (30)
		20	100 100	TSKgel Phenyl-5PW (20) TSKgel Ether-5PW (20)

Some processes, such as the purification of antibody-drug conjugates, require resins that are capable of higher resolution separations. For these separations, smaller diameter TOYOPEARL S-grade or TSKgel resins are preferred. TSKgel HIC resins are currently available in two ligands and two bead sizes:

- TSKgel Ether-5PW (30) = 30 µm
- TSKgel Ether-5PW (20) = 20 µm
- TSKgel Phenyl-5PW (30) = 30 µm
- TSKgel Phenyl-5PW (20) = 20 µm

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 biological activity. TOYOPEARL and TSKgel Ether resins are 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 and TOYOPEARL and TSKgel Phenyl resins complement the other HIC ligands available in the HIC series and offer alternatives for moderately hydrophobic proteins.

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 selectivities for TOYOPEARL and TSKgel resins enables a developer to optimize protein separations at the extremes of the hydrophobic spectrum. The more hydrophobic ligands on 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.

Getting Started with HIC

In the early stages of process development, it is advisable to screen multiple combinations of resins and mobile phases by using small, pre-packed columns such as ToyoScreen® or RoboColumns®. High throughput screening can also be accomplished by using Resin Seeker 96-well plates packed with TOYOPEARL HIC resins *(Figure 4)*. By taking advantage of the various screening formats, resins can be quickly screened for selectivity and capacity. High throughput screening, often in conjunction with a Design of Experiments protocol, is advantageous at this stage of development to rapidly evaluate multiple combinations of variables to determine the optimum selectivity, binding capacity, and recovery for the intended separation.

Figure 4: Resin Seeker 96-well plates

Prior to beginning any resin screening process to evaluate HIC resins, it is recommended that you determine the salt tolerance limit for the sample you wish to purify. As an example of how to accomplish this, try adding increasing amounts of salt to the sample in order to verify the concentration at which precipitation occurs. This process should be done for all salts you wish to evaluate in your mobile phase. When you begin resin screening, make sure that the concentration of salts in your sample is below this level in order to avoid precipitation on the resin or in the column. If feasible, test for activity of the target protein post evaluation to establish the concentration range over which activity can be maintained.

Where mobile phase salts are concerned, sulfates of sodium, potassium or ammonium are most effective at promoting ligand-protein interactions and are known to have little destructive effect on protein structure. However, ammonium acetate, potassium chloride and sodium chloride are also popular choices for mobile phase salts. As with the selection of the proper resin, the choice of mobile phase salts for a HIC separation can be a matter of trial and error since each salt differs in its ability to promote hydrophobic interactions. There is also evidence that a combination of two different salts can increase binding capacity and selectivity. The Hofmeister series of lyotropic and chaotropic ions *(Figure 5)* provides a template for salt selection.

Figure 5: Hofmeister Series

"Salting-Out" (Lyotropic)

SCN^-, I^-, ClO_4^-, NO_3^-, Br^-, Cl^-, COO^-, SO_4^{2-}, PO_4^{3-}

Ba^{2+}, Ca^{2+}, Mg^{2+}, Li^+, Cs^+, Na^+, K^+, Rb^+, NH_4^+

"Salting-In" (Chaotropic)

Using HIC as a capture step allows the chromatographer to quickly bind the target of interest from the crude feedstock while removing process impurities that may damage the target such as glycosidases and proteases. With capture steps, it may be necessary and advisable to trade resolution for speed and capacity as further downstream steps will be used to complete the purification process.

When HIC is employed as an intermediate purification step, it is most often used to remove significant impurities such as host cell proteins, endotoxins, viruses, and nucleic acids. For intermediate purification, the emphasis is on resolution and capacity. Selectivity and capacity need to be maximized to keep operating costs down and increase productivity. At this point, speed is less critical than it is in a capture step as damaging contaminants have been removed during capture and the sample volume has been greatly reduced.

The polishing step of a purification process is used to remove trace amounts of impurities or structural deviations of the target protein as well as aggregates and bring them down to acceptable levels.

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