Purification of monoclonal antibodies using new pore size optimized HIC resins

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Introduction

There are currently hundreds of monoclonal antibodies (mAbs) and monoclonal antibody derivatives in preclinical and clinical trials throughout the world. Most of the biopharmaceutical companies sponsoring this research are engineering what is called a platform technology to make the process development of these mAbs more efficient. This platform technology is designed to include two to three chromatography steps that start with a Protein A affinity chromatography step followed by some combination of ion exchange and/or hydrophobic interaction chromatography (HIC). Methacrylate based HIC resins can be utilized in a variety of mAb applications. These resins are ideal for the removal of residual Protein A, mAb aggregate and other impurities. In order to meet the growing demands of upstream technological advances (mammalian expression levels now reported to encroach 5-10g/L). We embarked on a program to improve the binding capacity of HIC resins. Pore size optimization using model immunoglobulins resulted in a 10 -15% increase in binding capacity. These new HIC resins are excellent choices for the development of methods amenable to a variety of mAb needs. This poster will describe the latest developments in the HIC resin optimization program and demonstrate the usefulness of the small screening columns in purifying mAbs.

Experimental Conditions

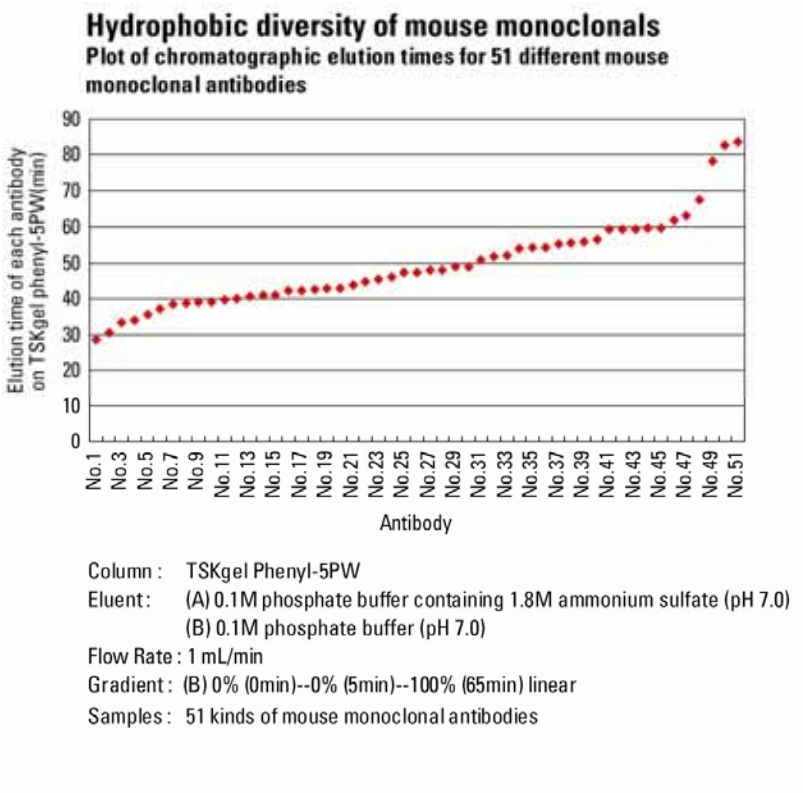
LC system: HPLC equipment (autosampler, pump, UV-detector) was supplied from Tosoh Corporation.

Columns: The columns used were 7.8mmID x 20cm stainless steel or 2.2cmID glass column; and ToyoScreen HIC pack process development columns.

MAbs: Several monoclonal antibodies were used in the initial studies from internal sources of Tosoh Corporation. One in particular, Anti-Thyroid Stimulating Hormone (Anti-TSH) IgG was used in more detailed studies. The mAbs were diluted 4 times in a mixture of elution buffer (buffer A:buffer B 1:2) prior to injection onto the columns. Humanized mAb was obtained from Boehringer-Ingelheim through Tosoh Bioscience GmbH.

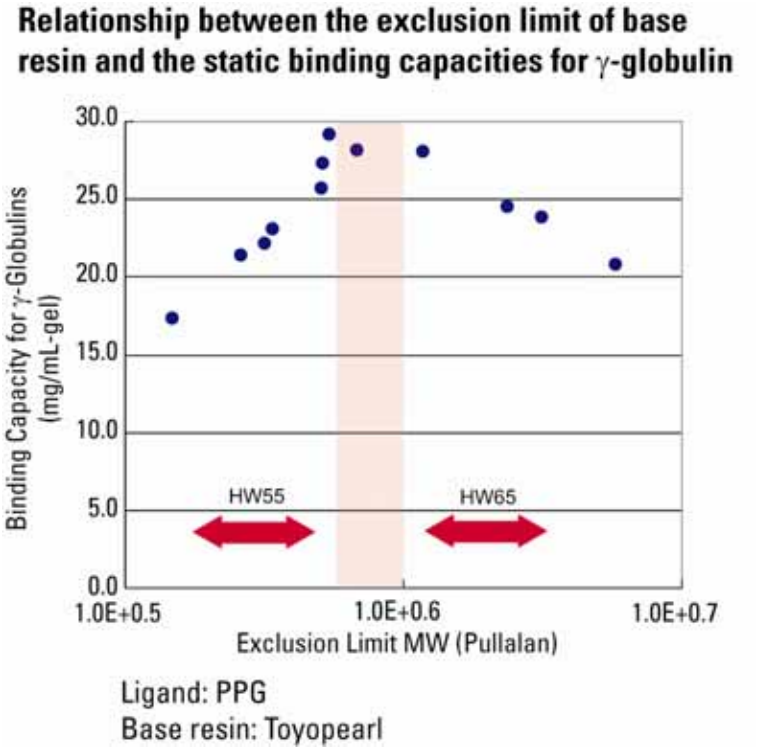
Conditions: The conditions for each experiment are described with each of the figures.

Figure 1



Monoclonal antibodies (mAbs) are commonly used as therapeutic and diagnostic proteins. Initial experiments investigated the hydrophobic diversity of monoclonal antibodies. This information can be utilized to determine an appropriate “hydrophobicity” for most mAbs. The hydrophobic spectrum of mAbs was determined on a TSK-Gel Phenyl 5PW analytical column. A total of 51 kinds different mouse monoclonal antibodies were organized in order of increasing elution time (increasing mAb hydrophobicity). The mAbs have wide hydrophobic diversity but tend to be hydrophobic proteins as compared with other serum protein (data not shown). Based on these results, a new ligand for antibodies should exhibit intermediate hydrophobicity to allow for the most versatility for a maximum number of mAbs. Two mAbs were utilized for further studies on the development of a new HIC ligand. One has moderate hydrophobicity and the other is very hydrophobic.

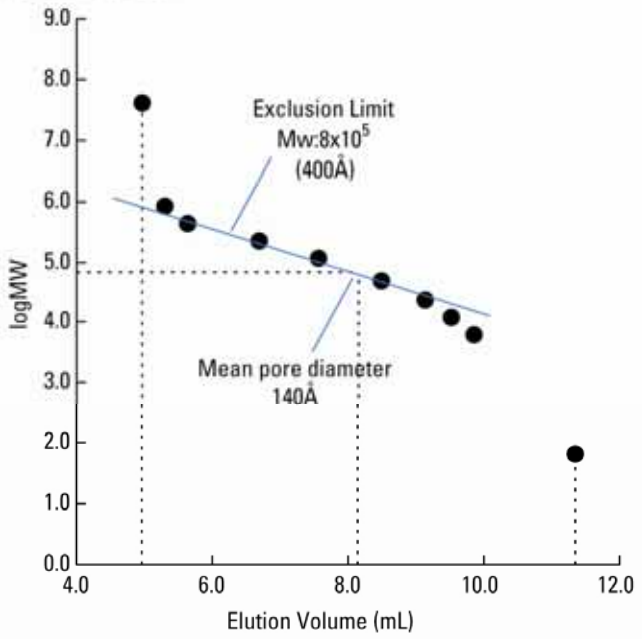
Figure 2



According to existing information on HIC ligands, increased binding capacity is correlated with increased ligand hydrophobicity. Therefore, if PPG exhibits intermediate hydrophobicity (compared to Phenyl and Butyl), the overall binding capacity will be less. To resolve this problem, the pore size of the PPG resin was optimized using g-globulin as a model protein. Several resins were prepared having different pore sizes and then PPG was attached at a constant ligand density. There is a maximum capacity around 800,000 Dalton exclusion limit.

Figure 3

Calibration curve of base resin having optimized pore for Antibodies.

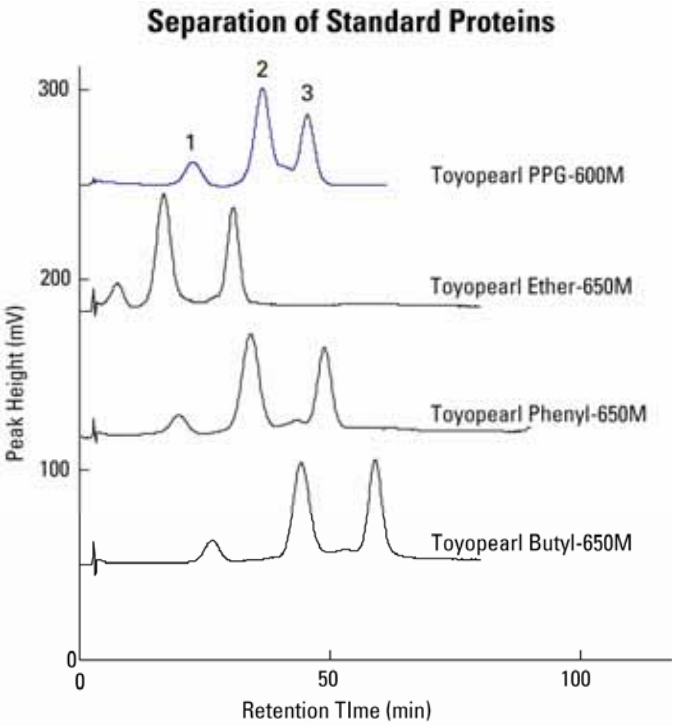


Conditions.

Column Size:	10.7mmID*150mm
Eluent :	H_2O
Flow rate :	0.47mL/min (Linear velocity: 30cm/h)
Detection:	RI
Injection:	40mL
Sample:	Pullulan, Ethylene Glycol

The optimal resin from the previous figure was further characterized and the exclusion limit was determined with Pullulan and Ethylene-glycol standards. The exclusion limit Mw was calculated to be 800,000 Daltons. The calculated pore diameter from the exclusion limit Mw is 400 Angstroms The calculated mean pore diameter from this calibration curve is about 140 Angstroms. It is interesting to note that this is approximately the same diameter of the crystal structure of an IgG molecule (data not shown).

Figure 4

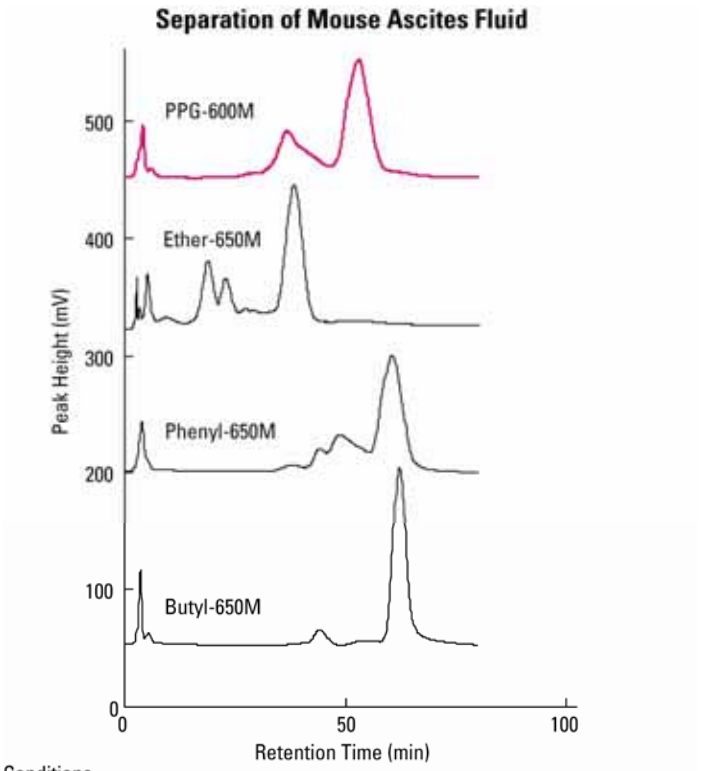


Conditions

Column Size:	7.5mmID*75mm
Eluent :	a) 0.1M Phosphate Buffer containing 1.8M Ammonium Sulfate (pH7.0)
	b) 0.1M Phosphate Buffer(pH7.0)
Gradient:	a) to b) 60min Linear
Flow rate:	1mL/min (Linear velocity:136cm/h)
Detection:	UV (280nm)
Sample:	(1mg/mL) RNaseA(1), Lysozyme(2), α-chymotripsinogen A(3)
Injection:	100mL

PPG was selected as the ligand and the pore size was optimized for IgG. That pore size is between the commercially available Toyopearl HW55 and HW65 and, therefore, the new HIC resin is named PPG-600M. Standard protein (RNase A, Lysozyme, and a-chymotripsinogen A) separation was tested by using current Toyopearl HIC resins and the new PPG-600M resin. The retentivity of Toyopearl PPG-600M is between Ether-650M and Phenyl-650M for standard proteins while maintainig excellent selectivity at intermediate hydrophobicity.

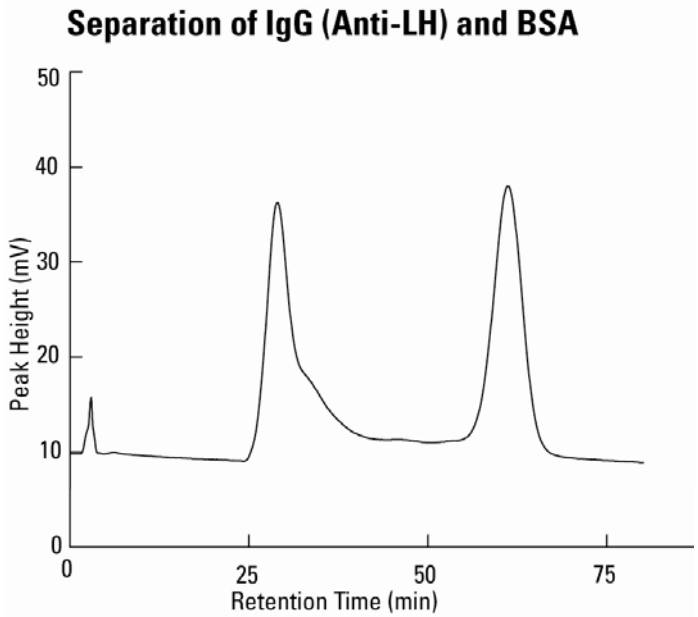
Figure 5



Conditions
Column Size: 7.5mmID*75mm
Eluent : a) 0.1M Phosphate Buffer containing 1.8M Ammonium Sulfate (pH7.0)
b) 0.1M Phosphate Buffer(pH7.0)
Gradient: a) to b) 60min Linear
Flow rate: 1mL/min
(Linear velocity:136cm/h)
Detection: UV (280nm)
Sample: Monoclonal IgG1(Anti human IgE) in mouse ascites fluid (diluted 4x)
Injection: 100mL

Optimizing the pore size had minimal effect on the selectivity of the resin at sub-saturating conditions. This is shown by comparing with the other HIC resins provided by Tosoh Bioscience. PPG600M shows the intermediate retentivity for IgG between current Ether-650M and Phenyl-650M and good selectivity for albumin and IgG.

Figure 6



Conditions

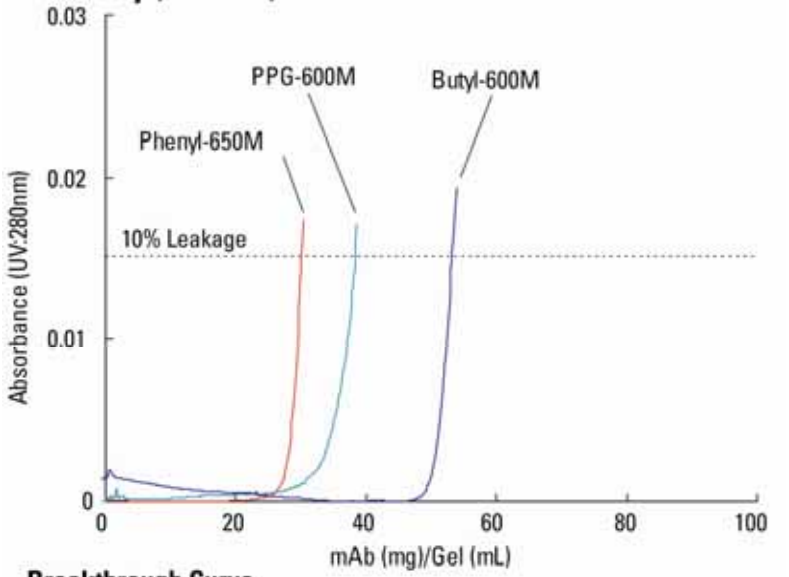
Column Size:	7.5mmID*75mm
Eluent :	a) 0.1M Phosphate Buffer containing 1.8M Ammonium Sulfate (pH7.0)
	b) 0.1M Phosphate Buffer(pH7.0)
Gradient:	a) to b) 60min Linear
Flow rate :	1mL/min (Linear velocity:136cm/h)
Detection :	UV (280nm)
Sample :	BSA 3mg/mL, IgG1(Anti-LH MoAb) 1mg/mL
Injection :	100mL

LH: Luteinizing Hormone

When a very hydrophobic mAb (from Figure 1) is applied to existing HIC resins the sample generally elutes with significant tailing. This phenomena was tested with the new PPG-600M resin to see if the intermediate hydrophobicity and optimized pore size resin would look similar. In this figure, Anti LH and BSA were effectively separated with minimal tailing even though the Anti LH mAb is quite hydrophobic.

Figure 7

Comparison of adsorption profile of mouse monoclonal antibody (Anti-LH) on various HIC resins



Breakthrough Curve

Conditions

Column size:	7.8mmID*20cm
Feed :	Mouse monoclonal antibody (Anti-LH) 1mg/mL in 0.1mol/L Phosphate buffer + 0.8mol/L Ammonium sulfate (pH7.0)
Linear velocity:	300cm/h
Temperature:	25°C

The dynamic binding capacities of Toyopearl Phenyl-650M, PPG-600M and Butyl-600M for the Anti-LH mAb were compared. At 300cm/hr, the less hydrophobic PPG resin showed a higher dynamic binding capacity than the more hydrophobic Toyopearl Phenyl 650M resin. This raises the question whether the capacity of Toyopearl Phenyl 650M can be increased through a similar pore size optimization program. These studies are currently underway.

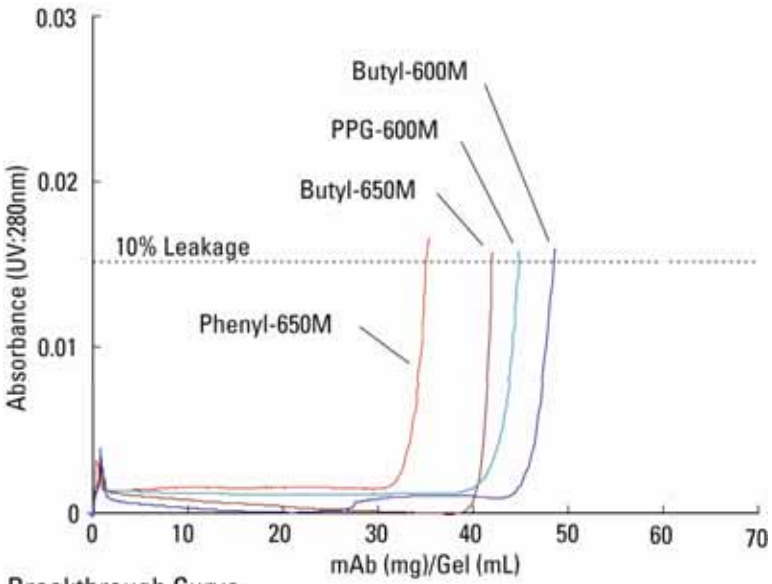
Table 1

	Binding capacity (10% Leakage) (mg/mL - gel)	Recovered capacity (mg/mL - gel)	Recovery (%)
Phenyl-650M (10D)	30.1	29.3	97
PPG-600M (501F)	38.3	39.0	102
Butyl-600M (502G)	53.9	48.8	91

The values determined from the breakthrough and recovery chromatograms of the Anti LH MAb are listed.

Figure 8

Comparison of adsorption profile of humanized monoclonal antibody on various HIC resins



Breakthrough Curve

Conditions

Column size:	7.8mmID*20cm
Feed:	Humanized monoclonal antibody 1mg/mL in 31mmol/L Citrate, 69mmol/L Phosphate buffer + 1.0mol/L Ammonium sulfate (pH6.6) 0.1mol/L Phosphate buffer + 1.0mol/L Ammonium sulfate (pH7.0)
Linear velocity:	300cm/h
Temperature:	25°C

A similar breakthrough study was conducted on a “humanized” mAb (kindly provided by Boerhinger Ingelheim, Germany through Tosoh Bioscience GmbH). Capacities similar to the anti-LH MAb were obtained.

Table 2

	Binding capacity (10% Leakage) (mg/mL - gel)	Recovered capacity (mg/mL - gel)	Recovery (%)
Phenyl-650M (04F)	35.2	34.5	98
Butyl-650M (08E)	42.1	36.6	87
PPG-600M (501F)	44.8	46.1	103
Butyl-600M (502G)	48.5	44.6	92

The values determined from the breakthrough and recovery chromatograms of the “humanized” mAb are listed.

Table 3

Bind and elution scout on ToyoScreen HIC pack for monoclonal antibodies

pmAb01 pH 6.0	Ammonium Sulfate					Sodium Chloride				
	100mM	250mM	500mM	750mM	1000mM	300mM	750mM	1500mM	2250mM	3000mM
Ether 650M										
PPG 600M										
Phenyl 650M										
Butyl 650M										
Super Butyl 550C										
Hexyl 650C										
pmAb01 pH 7.0	100mM	250mM	500mM	750mM	1000mM	300mM	750mM	1500mM	2250mM	3000mM
Ether 650M										
PPG 600M										
Phenyl 650M										
Butyl 650M										
Super Butyl 550C										
Hexyl 650C										

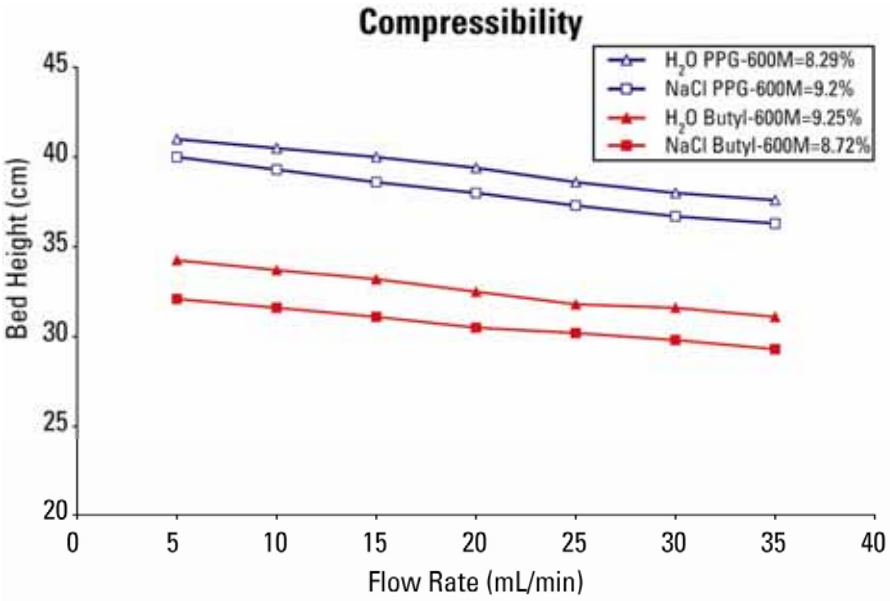
pmAb02 pH 6.0	Ammonium Sulfate					Sodium Chloride				
	100mM	250mM	500mM	750mM	1000mM	300mM	750mM	1500mM	2250mM	3000mM
Ether 650M										
PPG 600M										
Phenyl 650M										
Butyl 650M										
Super Butyl 550C										
Hexyl 650C										
pmAb02 pH 7.0	100mM	250mM	500mM	750mM	1000mM	300mM	750mM	1500mM	2250mM	3000mM
Ether 650M										
PPG 600M										
Phenyl 650M										
Butyl 650M										
Super Butyl 550C										
Hexyl 650C										

Protein Present in Gradient Elution
Protein Present in Flow Through and Gradient Elution
Protein Present in Flow Through
Protein Bound irreversibly
— To Be Determined

Conditions
Feed: Humanized monoclonal antibodies @ ~4mg/mL (in given salt condition for each run)
Equilibration/Wash: a) 20mmol/L Sodium Phosphate/ 1mol/L Ammonium Sulfate
b) 20mmol/L Sodium Phosphate/ 3mol/L Sodium Chloride
Elution: 20mmol/L Sodium Phosphate
Flow rate: 3mL/min
Temp: 25°C

The 1mL ToyoScreen HIC pack was tested against two different salt types at two different pH's for two monoclonal antibodies to look at how effective the ToyoScreen columns are at creating purification profiles for establishing purification conditions and resin selection for scale up. The table displays two columns, each column consisting of five different salt concentrations tested for each salt type. The subsequent results are displayed in each column based on a color coded system.

Figure 9

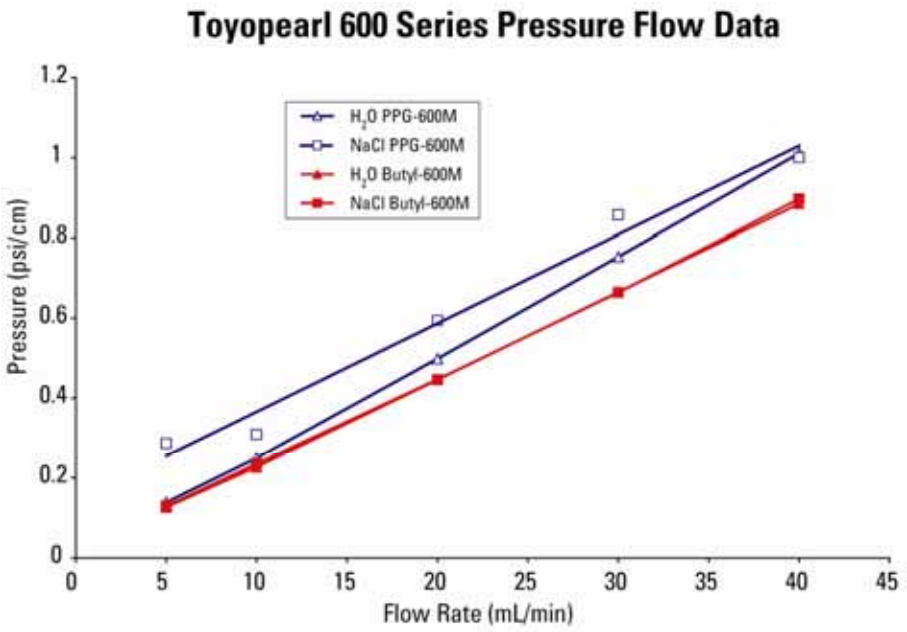


Conditions

Mobile phase 1:	H_2O
Mobile phase 2:	1.0M NaCl
Final column size:	H_2O PPG-600M: 2.2cmID x 36.4cm
	NaCl PPG-600M: 2.2cmID x 35.4cm
	H_2O Butyl-600M: 2.2cmID x 30.3cm
	NaCl Butyl-600M: 2.2cmID x 28.6cm

Toyopearl PPG-600M and Toyopearl Butyl-600M were packed in water and 1M NaCl and the amount of compression for each resin was measured. At 2 bar backing pressure the compression of both resins was 8.3 to 9.3%.

Figure 10



Conditions

Mobile phase 1:	H_2O
Mobile phase 2:	1.0M NaCl
Final column size:	H_2O PPG-600M: 2.2cmID x 36.4cm
	NaCl PPG-600M: 2.2cmID x 35.4cm
	H_2O Butyl-600M: 2.2cmID x 30.3cm
	NaCl Butyl-600M: 2.2cmID x 28.6cm

The packed columns from figure 9 were tested for pressure flow characteristics. Differences in column bed height were normalized. Toyopearl Butyl-600M showed slightly better pressure flow characteristics compared to Toyopearl PPG-600M in both H_2O and 1M NaCl.

Table 4

	Packed in H_2O				Packed in NaCl			
	NaCl		Acetone		H_2O		Acetone	
	A_s	N/m	A_s	N/m	A_s	N/m	A_s	N/m
PPG-600M	1.31	3710	2.57	2672	1.30	2716	1.37	2595
Butyl-600M	0.55	4348	1.06	4126	0.99	4603	1.12	2682

Performance testing using conductivity and absorbance on resins packed in H_2O and 1M NaCl.

Conclusions

- Optimization of pore size on HIC resins for individual proteins is an effective way to increase binding capacity.

- By applying this approach, a new resin, Toyopearl PPG-600M designed for antibody purification, was developed which has novel HIC ligand showing the intermediate hydrophobicity between Ether and Phenyl.

- The Toyopearl PPG-600M showed not only high binding capacity and efficient recovery for IgG compared with Toyopearl Phenyl-650M, but also better selectivity in removing impurities from antibodies.

- Using a similar pore size optimization strategy, a new Toyopearl Butyl-600M resin was also developed which exhibits even higher binding capacity. However, with more hydrophobic MAbs, Toyopearl Butyl-600M will have decreased recoveries (data not shown).

- Both Toyopearl PPG-600M and Butyl-600M had minimal compression and exhibited excellent pressure flow characteristics when packed to a final pressure of 2 bar.

- Testing of Toyopearl PPG-600M and Butyl-600M indicated that packing HIC resins in 1M NaCl gave better performance criteria than columns packed similarly in H_2O.

- The 1mL ToyoScreen HIC pack proved useful as a tool for establishing mobile phase conditions and resin selection for monoclonal antibody purification scale up. Loading at 6mg/mL is sufficient to minimize signal/noise ratio to yield clear chromatography profiles with measurable retentions for gradient elution.