



Maximizing IgG binding capacity of hydrophobic interaction chromatography resins using pore size optimization

J. Kevin O'Donnell¹, Scott Manz¹, Emi Sakima², Yoshimi Hashimoto², and Yasutami Mitoma².

(1) Tosoh Bioscience, 156 Keystone Drive, Montgomeryville, PA 18936, (2) Scientific Instruments Division, Tosoh Corporation



Introduction

Many of the current therapeutic proteins in clinical trials are based on human immunoglobulin (IgG) that are produced from a variety of cell culture expression system. Standard purification techniques for these molecules incorporates Protein A capture chromatography followed by a combination of cation and hydrophobic interaction chromatography steps. Chromatography theory states that under non-saturating conditions, ion exchange and hydrophobic interaction chromatography binding capacities are directly proportional to increased available surface area and ligand density. Using several monoclonal antibodies (MAbs) as a probe, the goal of these experiments is to maximizing IgG binding capacity of hydrophobic interaction chromatography resins using pore size optimization. The hydrophobic ligands used in this study were PPG (polypropylene glycol) and butyl which were attached to the well known series of Toyopearl[®] base resins. The Toyopearl base resin exhibits superior physical and chemical stability under even the harshest operating conditions.

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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.

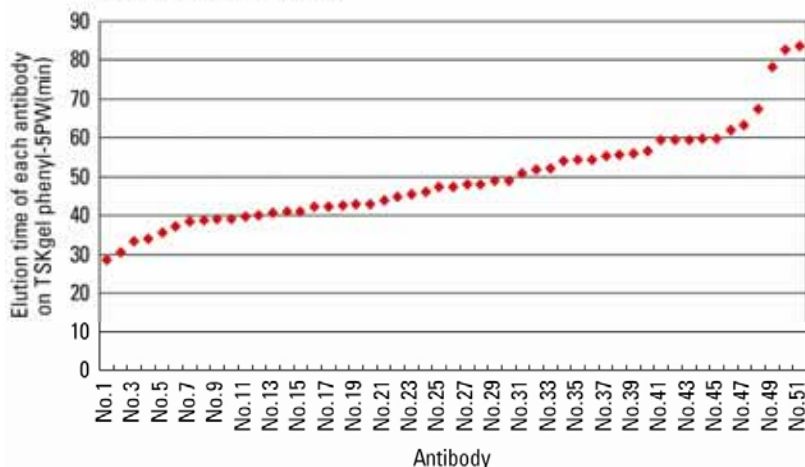
MAbs: Several monoclonal antibodies were used in the initial studies from internal sources of Tosoh Corporation. One in particular, Anti-Thyroid Stimulating Hormone (Anti-TSH) IgG was used in more detailed studies. The MAbs were diluted 4 times in a mixture of elution buffer (buffer A:buffer B 1:2) prior 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

Hydrophobic diversity of mouse monoclonals
Plot of chromatographic elution times for 51 different mouse monoclonal antibodies



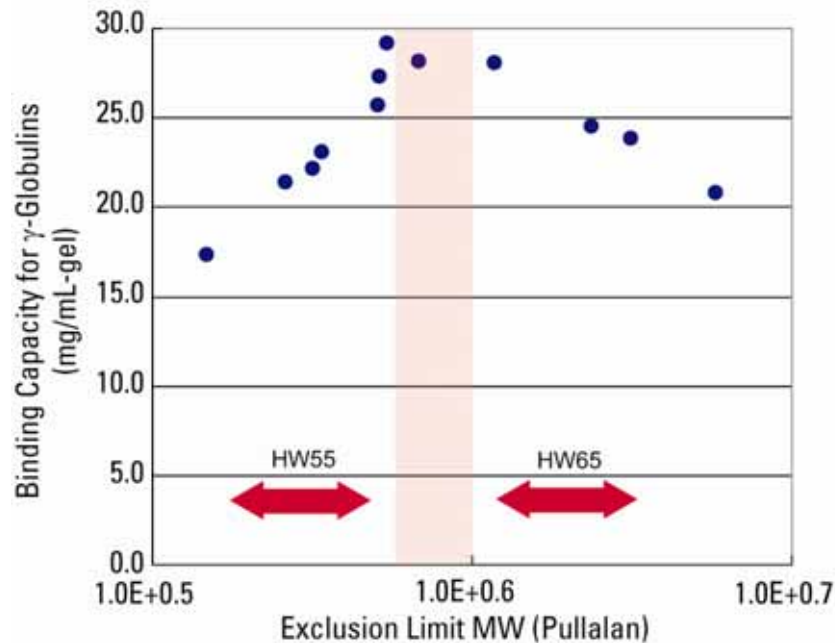
Column : TSKgel Phenyl-5PW
Eluent : (A) 0.1M phosphate buffer containing 1.8M ammonium sulfate (pH 7.0)
(B) 0.1M phosphate buffer (pH 7.0)
Flow Rate : 1 mL/min
Gradient : (B) 0% (0min)--0% (5min)--100% (65min) linear
Samples : 51 kinds of mouse monoclonal antibodies

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

Relationship between the exclusion limit of base resin and the static binding capacities for γ -globulin



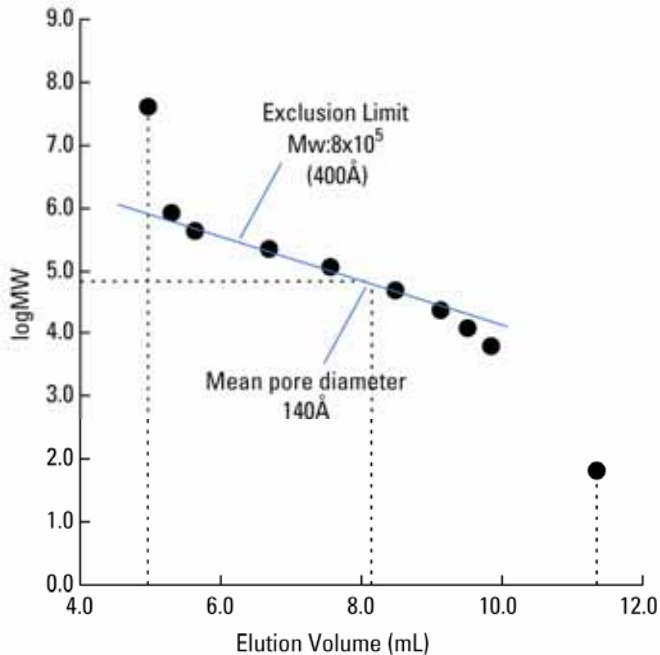
Ligand: PPG
Base resin: Toyopearl

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 γ -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.

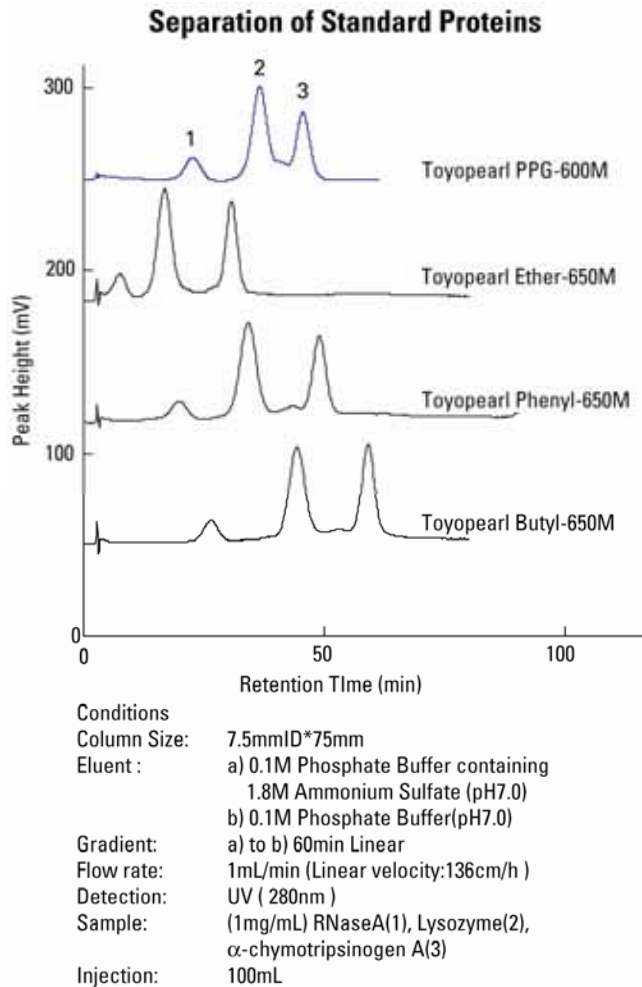


Conditions.
Column Size: 10.7mmID*150mm
Eluent: H₂O
Flow rate: 0.47mL/min (Linear velocity: 30cm/h)
Detection: RI
Injection: 40mL
Sample: Pullulan, Ethylene Glycol

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).



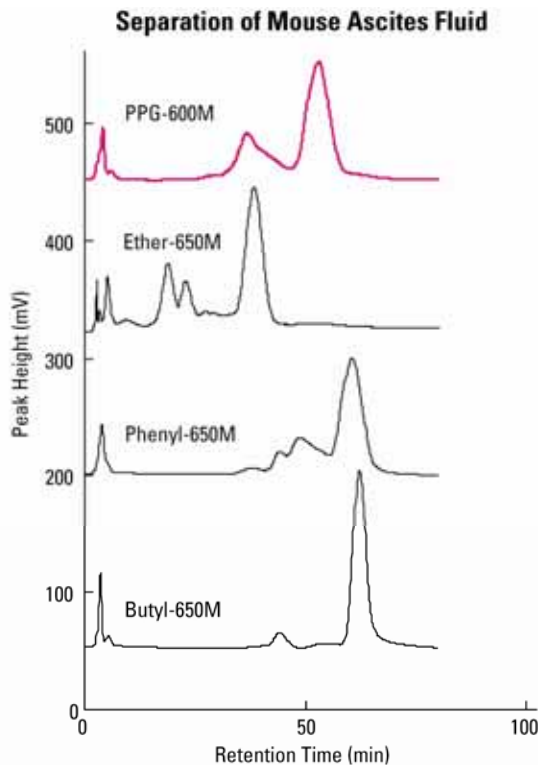
Figure 4



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 α -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 maintaining excellent selectivity at intermediate hydrophobicity.



Figure 5



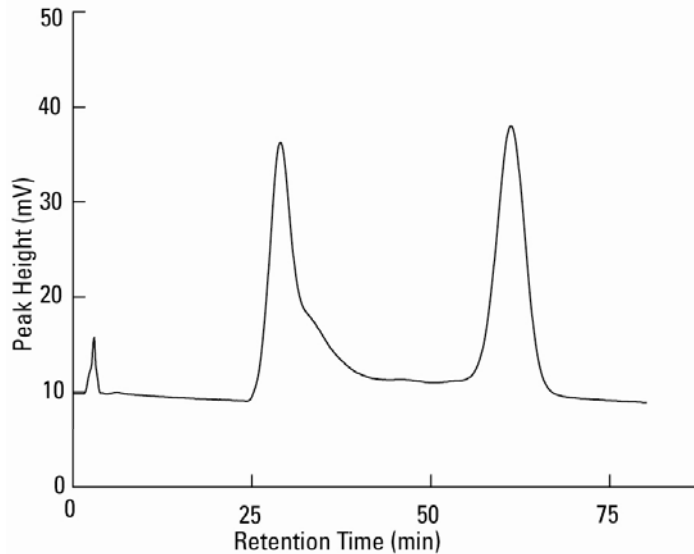
Optimizing the pore size had minimal effect on the selectivity of the resin at sub-saturating conditions. This is shown by comparing with the other HIC resins provided by Tosoh Bioscience. PPG600M shows the intermediate retentivity for IgG between current Ether-650M and Phenyl-650M and good selectivity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

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



Figure 6

Separation of IgG (Anti-LH) and BSA



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

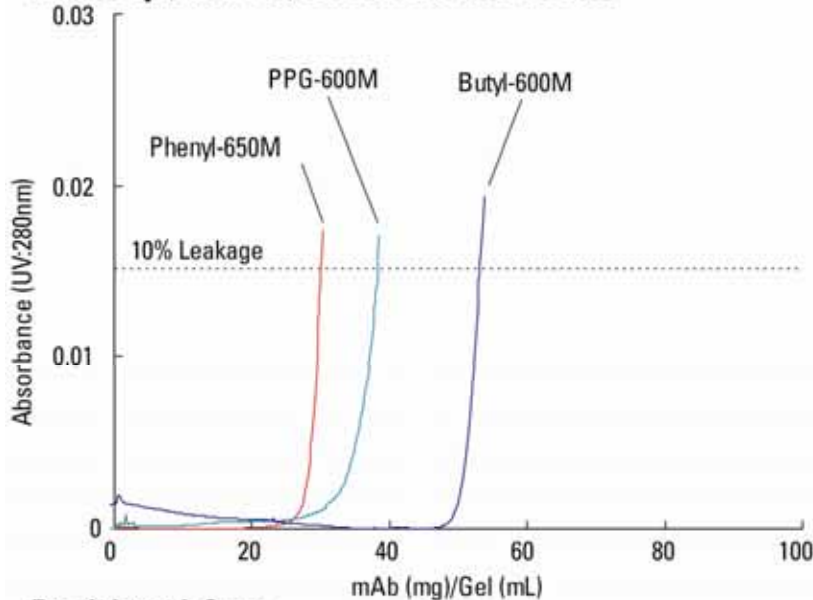
LH: Luteinizing Hormone

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

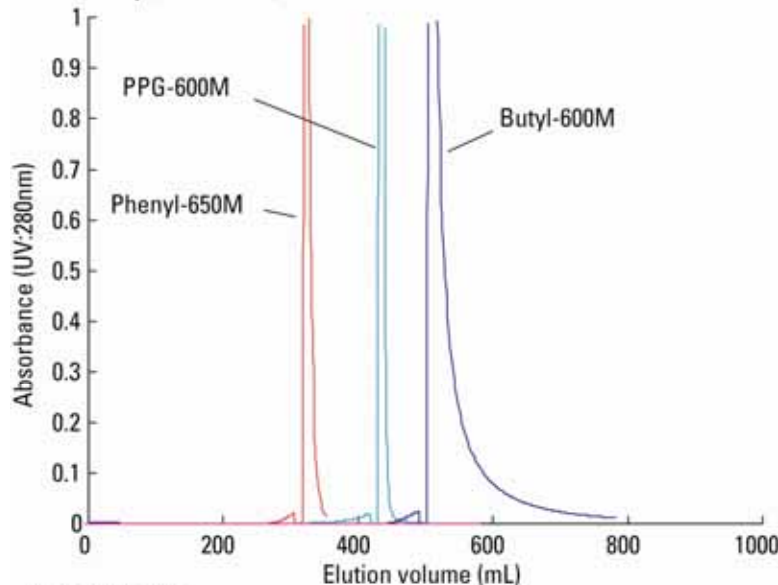
Conditions
Column size: 7.8mmID*20cm
Feed : Mouse monoclonal antibody (Anti-LH) 1mg/mL in 0.1mol/L Phosphate buffer + 0.8mol/L Ammonium sulfate (pH7.0)
Linear velocity: 300cm/h
Temperature: 25°C

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.



Figure 8

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



Elution Profile

Procedure: Feed (until 10%leak, above) --> Wash (3CV) --> Elution

Conditions

Wash: 0.1mol/L Phosphate buffer + 1.1mol/L Ammonium sulfate (pH7.0)

Elution: 0.1mol/L Phosphate buffer (pH7.0)

Linear velocity: 300cm/h

Temperature: 25C

Product recovery is critical to the successful separation on HIC resins. This is the elution pattern of anti LH antibody from Phenyl-650M, PPG600M and Butyl-600M. Both Phenyl-650M and PPG600M show sharp elution. However, PPG-600M shows sharper elution compared with Phenyl-650M. This would seem to indicate a better selectivity and a more effective separation of impurities from the target protein. The sharper peak is an added advantage to subsequent unit operations in that the peak elutes at higher concentration.



Table 1

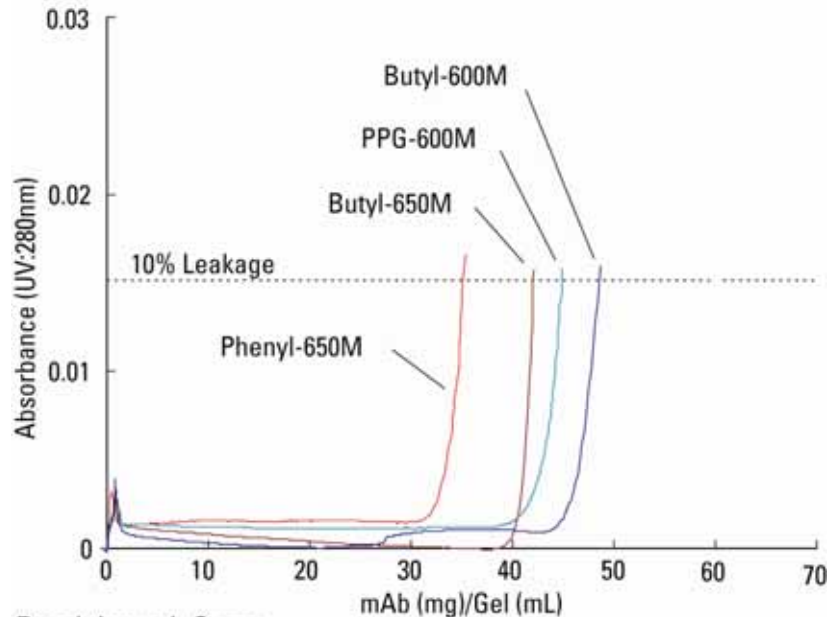
	Binding capacity (10% Leakage) (mg/mL - gel)	Recovered capacity (mg/mL - gel)	Recovery (%)
Phenyl-650M (10D)	30.1	29.3	97
PPG-600M (501F)	38.3	39.0	102
Butyl-600M (502G)	53.9	48.8	91

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



Figure 9

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

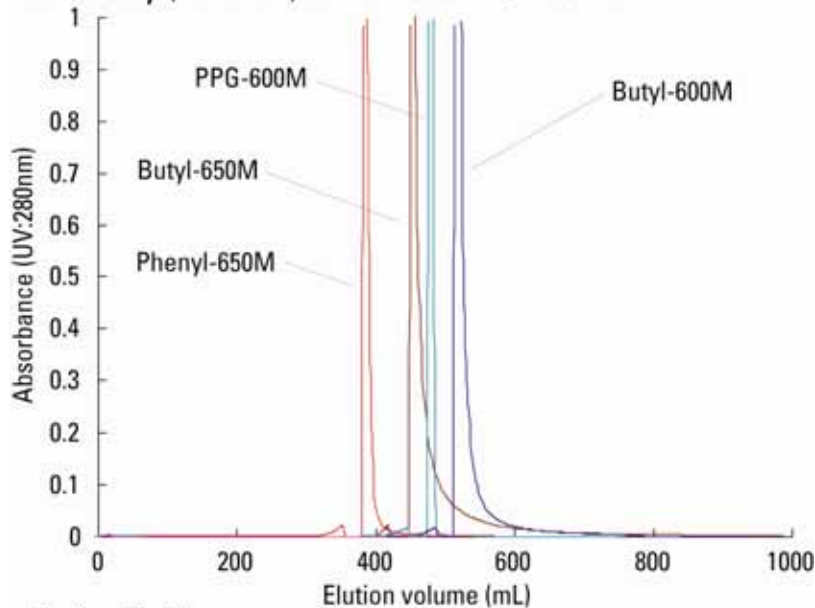
Temperature: 25°C

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



Figure 10

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



Elution Profile

Procedure: Feed (until 10%leak, above) --> Wash (3CV) --> Elution

Conditions

Wash : 31mmol/L Citrate, 69mmol/L Phosphate buffer
+ 1.0mol/L Ammonium sulfate (pH6.6)

Elution : 0.1mol/L Phosphate buffer (pH7.0)

Linear velocity: 300cm/h

Temperature: 25C

The recoveries of the “humanized” MAb were also evaluated. Again the product was eluted with minimal peak tailing.



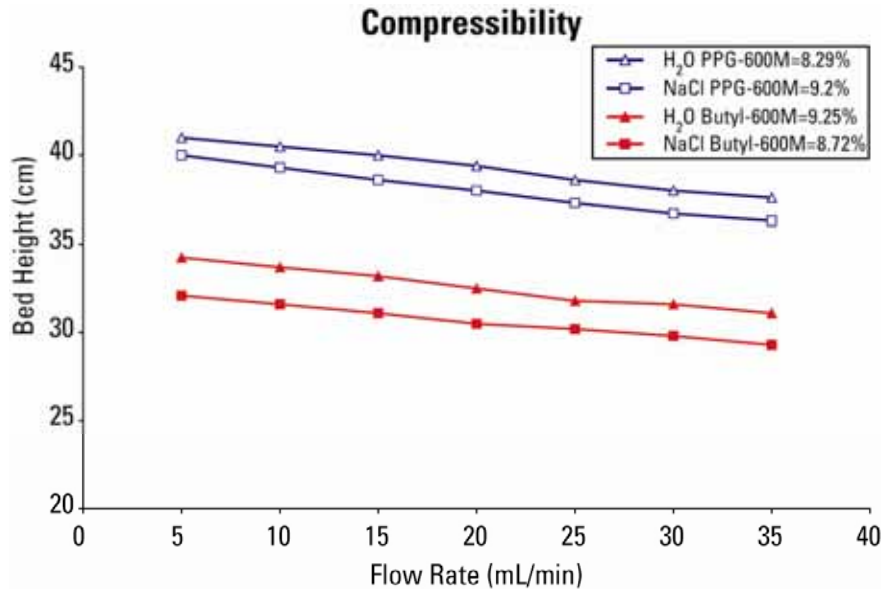
Table 2

	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.



Figure 11



Conditions

Mobile phase 1: H₂O

Mobile phase 2: 1.0M NaCl

Final column size: H₂O PPG-600M: 2.2cmID x 36.4cm

NaCl PPG-600M: 2.2cmID x 35.4cm

H₂O Butyl-600M: 2.2cmID x 30.3cm

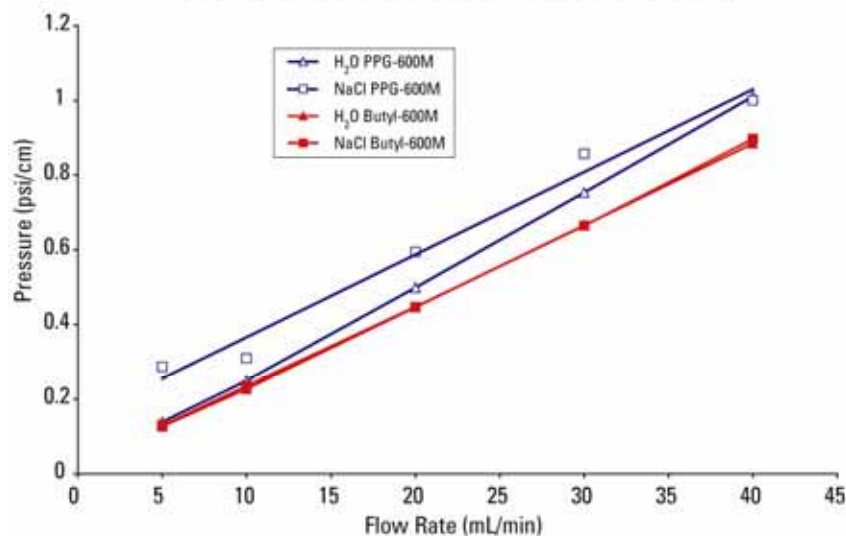
NaCl Butyl-600M: 2.2cmID x 28.6cm

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 12

Toyopearl 600 Series Pressure Flow Data



Conditions

Mobile phase 1: H₂O

Mobile phase 2: 1.0M NaCl

Final column size: H₂O PPG-600M: 2.2cmID x 36.4cm

NaCl PPG-600M: 2.2cmID x 35.4cm

H₂O Butyl-600M: 2.2cmID x 30.3cm

NaCl Butyl-600M: 2.2cmID x 28.6cm

The packed columns from figure 11 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 H₂O and 1M NaCl.



Table 3

	Packed in H ₂ O				Packed in NaCl			
	NaCl		Acetone		H ₂ O		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 H₂O 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 H₂O.