Best-in-Class Resins for Process Chromatography of Antibody

Tosoh Corporation

December, 2019

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- Downstream purification process for mAb
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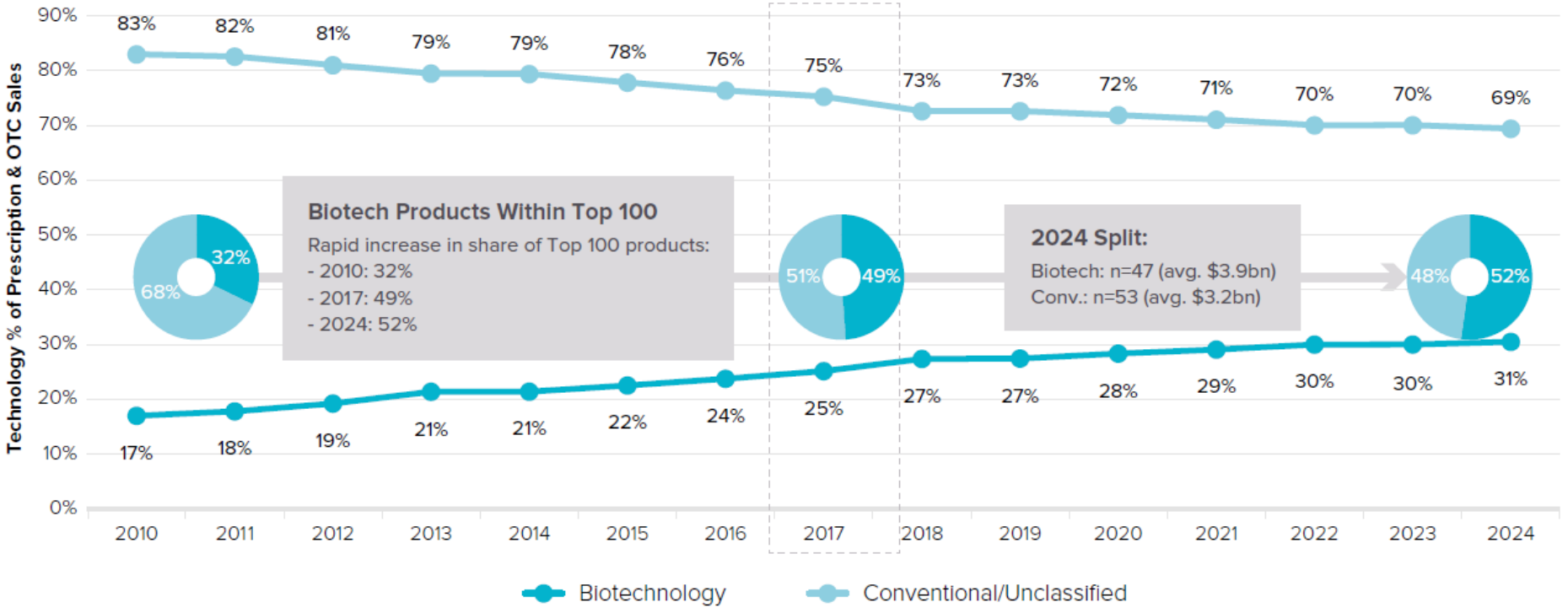
- Summary

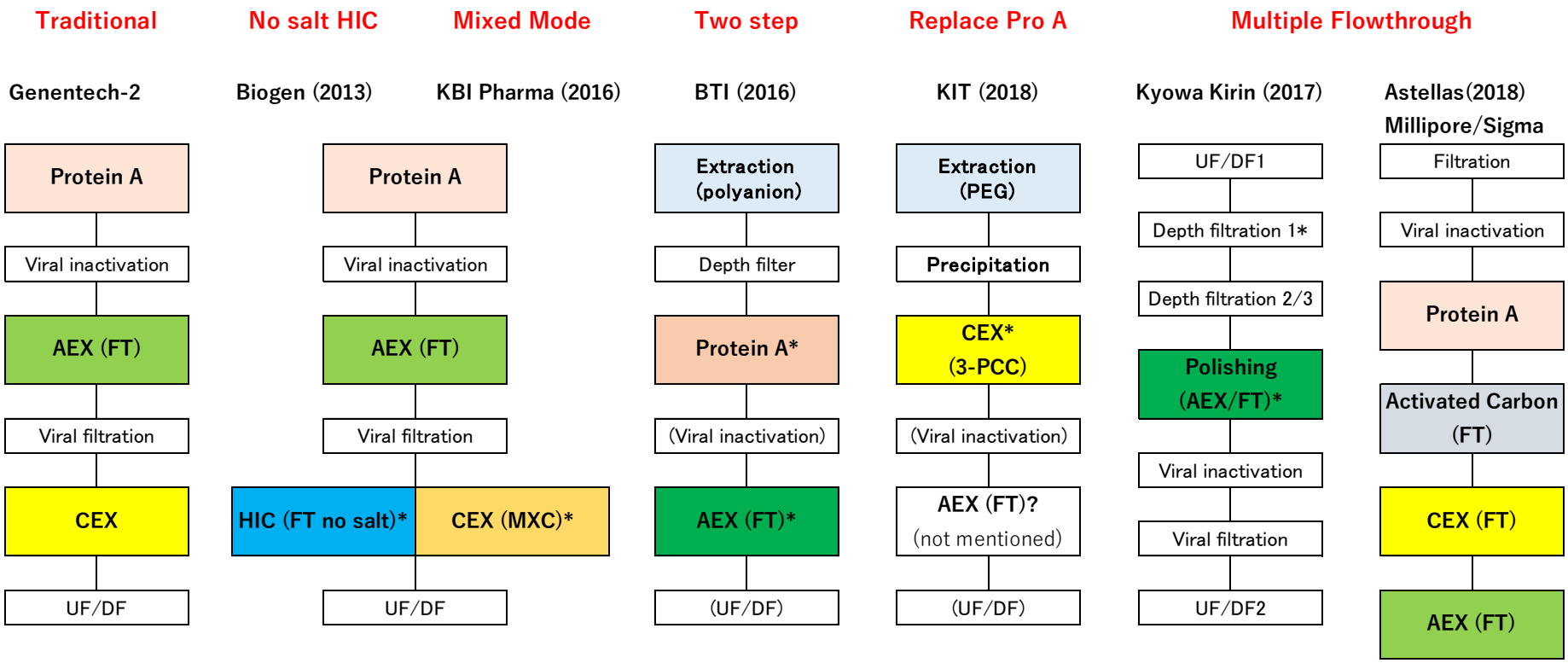
Worldwide Pharma Sales in Biotech vs. Traditional Technology in 2018-2024

Source; EvaluatePharma® May 2018



- Annual growth rate (CAGR) from 2018 to 2024 is 6.4 %.
- Pharma sales by biotech reaches 31 % in 2024.
- Pharma sales by biotech reaches 52 % in 2024 in top 100 products.
- Expected 12 mAbs become hit sales in top 20 pharma in 2024.

Examples of New mAb Purification Process



* High load HIC
TOYOPEARL®
Hexyl-650C

* Capto™ MMC
Nuvia™ cPrime
TOYOPEARAL
MX-Trp-650M
Sulfate-650F
(salt-tolerant)

* MabSelect™ SuRe LX
Capto Adhere
* TOYOPEARL
AF-rProtein A HC-650F
NH2-750F (salt-tolerant)

* TOYOPEARL
GigaCap® S-650M

* Depth Filter
Millistak® +A1HC,
Zeta Plus™ EXT, AC
* TOYOPEARL
NH2-750F (salt-tolerant)

(*Please remind any application may be patented before being public information.)

Properties of TOYOPEARL Resins

- **Polymethacrylate polymer resin**
  - Pore size control ; MW range; 500 – 20,000,000
  - Particle size control; 30 – 150 um (grade S, F, M, C, EC)
  - High flow rate, large column packing
- **High capacity**
  - High capacity resin has an impact to decrease COGS
  - Grafting technology; IEC GigaCap series and other resins
  - IEC, HIC, MXC, AFC
- **Smaller particle grade**
  - TSKgel PW bulk IEC, HIC (20 um, 30 um)
  - Polishing step for protein, peptide and oligonucleotide
- **Prepacked columns for process development**
  - ToyoScreen® column (1, 5 mL), MiniChrom column (5 mL) for LC
  - RoboColumn® (200 uL, 600 uL) for Tecan microplate format
- **Supply chain**
  - Secure for large amounts supply by the 3rd manufacturing plants in 2019

The Best-In-Class Resins on TOYOPEARL
(High capacity, selectivity, purity and stability)

Affinity chromatography

- TOYOPEARL AF-rProtein A HC-650F
  - High capacity, high alkaline stability, applicable to continuous chromatography
  - Large process column packing for multiple customers
- TOYOPEARL AF-rProtein L-650F
  - High capacity, alkaline durability for antibody fragment and scFv
  - High selectivity for bi-specific antibody

Mixed-mode or salt-tolerant ion-exchange chromatography

- TOYOPEARL NH_2-750F
  - High capacity, salt tolerant anion-exchanger (as mixed-mode)
  - High efficiency for removal of aggregate, endotoxin and viral clearance
- TOYOPEARL Sulfate-650F
  - High capacity salt tolerant cation-exchanger (as mixed-mode) with different selectivity
  - High efficiency for removal of aggregate, HCP and host DNA
- Hydroxyapatite Ca^{++}Pure-HA®
  - Mixed-mode (Multi-mode) chromatography
  - Cost effective and easy transition from conventional hydroxyapatite

Best-In-Class Resin Specification

Mode	TOYOPEARL			
	Affinity		Anion-exchanger	Cation-exchanger
Product	AF-rProtein A HC-650F	AF-rProtein L-650F	NH_2-750F	Sulfate-650F
Base resin	Hydrophilic polymer			
Particle size	45 µm	45 µm	45 µm	45 µm
Ligand	Recombinant Protein A	Recombinant Protein L	Poly-amine	Sulfate
Ligand density	N/A	N/A	0.07～0.13 eq/L	≧ 0.53 eq/L
Static Binding Capacity (SBC; human IgG)	**≧ 68 g/L-resin**	**≧ 64 g/L-resin**	**≧ 70 g/L-resin**	**≧ 114 g/L-resin**
Storage	20 % Ethanol*			20 % Ethanol 0.2 mol/L sodium acetate
Storage temperature	2~8 degree C		Room temperature	

N/A; not applicable

** For TOYPEARL NH_2-750F, washing with 0.1 mol/L HCl to substitute counter ions on the resin before storage.

	HYDROXYAPATITE	TOYOPEARL
	Mixed-mode (Multi-mode)	
Mode	Ca^{++}Pure-HA	MX-Trp-650M
Base resin	Hydroxyapatite	Hydrophilic polymer
Particle size	39 µm	65 µm
Ligand	Ca^{2+}, PO_4^{3-}, OH	Tryptophan
Ligand density	N/A	N/A
Static Binding Capacity (SBC; human IgG)	**≧ 20 g/L-resin***	**≧ 75 g/L-resin**
Storage	Dry (or 1 mol/L NaOH)	20 % Ethanol
Storage temperature	Room temperature	

* 55 g/L at 5 min residence time

Protein A & Protein L Affinity Chromatography

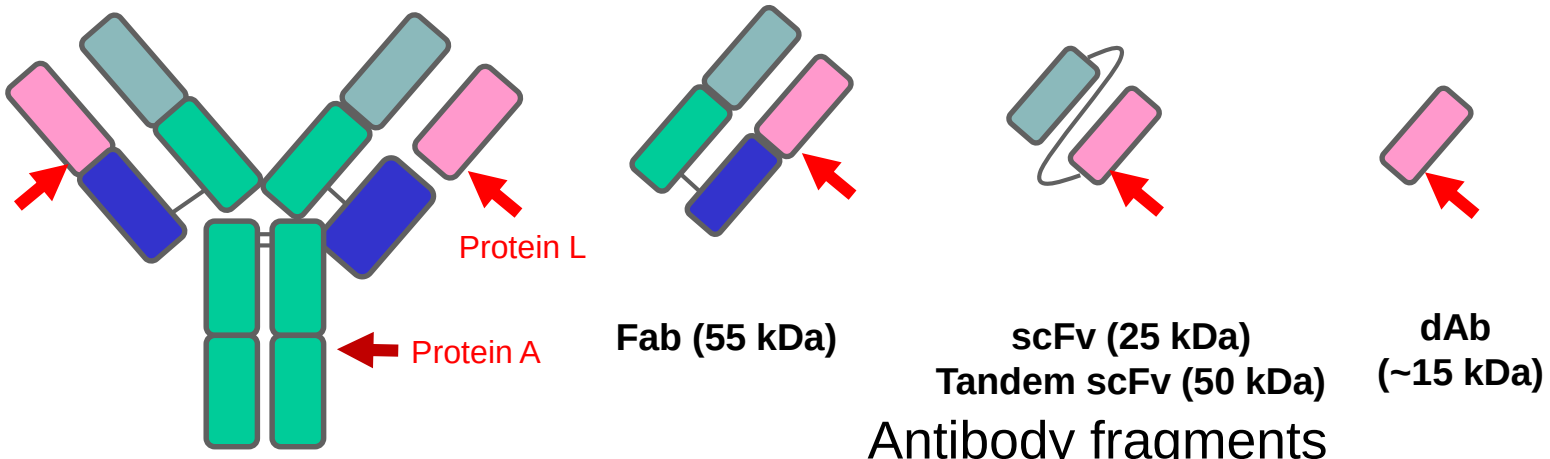
Binding of Protein A/Protein L to Antibody

Protein A; Binding to Fc region

- Intact antibody (Tosoh protein A resin can also weakly bind to Fab region)
- Fc-fusion protein

Protein L; Binding to kappa type light chain

- Antigen-binding fragment (Fab)
- Single-chain variable fragment (scFv)
- Single-domain antibody fragment (dAb)



TOYOPEARL AF-rProtein A HC-650F

TOYOPEARL AF-rProtein A HC-650F

- High capacity type
  - The highest binding capacity (similar to that on GE PrismA)
  - Higher (double) binding capacity at low temperature in a cold room against commercial Protein A resin
  - Applicable to weak binding antibody (e.g. IgM, Fab, Fc-fusion protein, mouse IgG)
- Purity
  - Improvement of removal of impurities with intermediate wash
  - Intermediate wash buffer at higher pH, addition of arginine or alcohol
- Alkaline stability
  - CIP over 300 cycles with 0.2 mol/L NaOH
- Installment of large process columns
  - 60 cm I.D. column (over 50 L resin), secure supply chain
  - 15 cm height column gives better productivity

Protein A Resin;

Effective Intermediate Wash for Purification for mAb IgG_2

Intermediate wash conditions	Sample Loading (mg/mL)	Yield (%)	HCP (ppm)	HMW (%)	Productivity (%)
Target (similar level to MabSelect SuRe LX)	35 - 49	> 85	< 1,400	< 3.0	-
Original; 25 mM Tris-HCl (pH 7.4) + NaCl (conductivity 12-14 mS/cm)	54	94	3,600	2.3	100
25 mM Tris-HCl (pH 9.0) + 10 % IPA	54	85	1,199	2.4	119
25 mM Tris-HCl (pH 9.0) + 0.5 mol/L arginine	54	89	951	2.5	125
25 mM Tris-HCl (pH 9.0)	54	93	1,725	2.5	131
25 mM Tris-HCl (pH 9.0) + 2 mol/L urea	54	81	1,118	2.5	114
25 mM Tris-HCl (pH 9.0) + 2 mol/L urea + 10 % IPA	54	65	439	2.2	91
25 mM Tris-HCl (pH 9.0) + 1 mol/L NaCl	54	84	1,953	2.5	119
25 mM Tris-HCl (pH 5.0) + 1 mol/L NaCl	54	81	2,415	2.4	113
25 mM Tris-HCl (pH 5.0) + 2 mol/L urea	54	74	1,429	2.4	104
25 mM Tris-HCl (pH 7.5) + 0.5 mol/L arginine	54	86	873	2.5	120

* Ref.; K. Mehta et al., American Pharmaceutical Review, posted on Feb. 16, 2018
https://www.americanpharmaceuticalreview.com/Featured-Articles/347357-Comparing-Performance-of-New-Protein-A-Resins-for-Monoclonal-Antibody-Purification/
Data was modified from the original source.

Dynamic Binding Capacity of mAb IgG_1 at Low Temperature on Protein A Affinity Chromatography

Temperature	Dynamic Binding Capacity (IgG_1, g/L)			
	TOYOPEARL AF-rProtein A HC-650F		MabSelect SuRe	
	125 cm/hr	250 cm/hr	125 cm/hr	250 cm/hr
4 ℃	33.2	27.8	18.6	11.0
22 ℃	40.8	33.2	25.6	17.9
40 ℃	40.2	34.2	30.8	24.7

Column size; 5 mm I.D. x 5 cm
Residence time; 2.4 min and 1.2 min, respectively
Data modified from the reference of W. Krepper et al., J. Chromatogr., A 1551 (2018) 59-68

- TOYOPEARL AF-rProtein A HC-650F showed the highest binding capacity at lower temperature in operation.

TOYOPEARL AF-rProtein A HC-650F

Chromatographic Conditions (1) Regular IgG

- **Binding and intermediate washing**
  - 0.02 mol/L sodium phosphate buffer (pH 7.2), 0.15 mol/L NaCl
  - When other impurities like green pigment is adsorbed, additional stepwise washing with buffer at pH above 4 may be effective.
  - Intermediate washing with 50 mM Tris-HCl (pH 8.0), 1 mol/L NaCl [*1]

    Or washing with 50 mM borate buffer (pH 9.0), 0.5 mol/L NaCl, 10 % isopropanol

    Or washing with 25 mM Tris-HCl (pH 7.5 - 9.0), 0.5 mol/L arginine, or 10 % isopropanol[*2]
  - Pre-washing with 0.1 mol/L NaCl (no buffer) would be also effective prior to low pH elution.
- **Elution**
  - **Elution pH is 0.2 point lower** than other commercial Protein A resin
  - 0.1 mol/L acetate or citrate (pH 3.7-3.0) or 0.1 mol/L glycine-HCl (pH 3.7-3.0)
  - When aggregates were observed in eluted fraction, decrease concentration of acetate or citrate buffer below 0.06 mol/L.
- **Cleaning**
  - Acidic solution of pH 2.7 - 2.5
  - 0.1 mol/L NaOH, 0.5 mol/L NaOH is effective[*1] to remove accumulated DNA/chromatin , while such cleaning conditions would deteriorate Protein A resin.
  - For hydrophobic impurities, 20-30 % ethanol may be effective.

Reference *1; P. Gagnon et al., J. Chromatogr., A, 1340 (2014)68-78

*2; K. Mehta et al, American Pharmaceutical Review, posted in web on Feb. 16, 2018

TOYOPEARL AF-rProtein A HC-650F

Chromatographic Conditions (2) Fab, IgM, Mouse IgG

- **Binding and intermediate washing**
  - Higher pH buffer without salt is recommended.
  - 0.02 mol/L Tris-HCl (pH 8.4), no salt
  - 0.02 mol/L sodium phosphate buffer (pH 7.2), no salt
  - Pre-washing with 0.1 mol/L NaCl (no buffer) would be also effective prior to low pH elution.
- **Elution**
  - Salt gradient from 0 to 0.5 mol/L NaCl in binding buffer
  - 0.1 mol/L glycine-HCl (pH 3.7-3.0) or 0.1 mol/L acetate buffer (pH 3.7-3.0)
  - When aggregates were observed in eluted fraction, decrease concentration of acetate or citrate buffer below 0.06 mol/L.
- **Cleaning**
  - Acidic solution of pH 2.7-2.5
  - 0.1 mol/L NaOH, to remove accumulated DNA/chromatin , 0.5 mol/L NaOH is effective[*1], while the cleaning conditions degradate Protein A resin.
  - For hydrophobic impurities, 20-30 % ethanol may be effective.

*1; Reference; P. Gagnon et al., J. Chromatogr., A, 1340 (2014)68-78

- Weak binding antibody and fragment would be applied to Protein L /Protein G affinity chromatography.

TOYOPEARL AF-rProtein L-650F

TOYOPEARL AF-rProtein L-650F

- High capacity type
  - The highest binding capacity
  - Higher (double) binding capacity at low temperature in a cold room against commercial Protein A resin
  - Artificial low molecule antibody (Fab, scFv, diabody)
- Purity
  - Improvement of removal of impurities by intermediate wash at higher pH, addition of arginine, alcohol, or denaturing agents
  - Intact bispecific antibody separation by both pH gradient and salt gradient at the same time
- Alkaline stability
  - Chemically stable with 0.1 mol/L NaOH for 10 hr
  - CIP over 300 cycles with 0.05 mol/L NaOH（combination with acidic pH 2.0 washing is effective.)
- Large scale purification
  - Process column for 10 L - 30 L at 15 cm height column

Protein L Resin;

Effective Intermediate Wash at pH 6.5 for Purification of Bispecific Tandem scFv from CHO Cell Culture Media

Loading amount (mg)	Wash pH	Additive for wash	Step elution pH	Recovery (%)	Monomer (relative %)	Aggregate (relative %)	Fragment (relative %)	HCP (ppm)	DNA (ppm)	Leached Protein L (ppm)*
Feed	-	-	-	-	24.6	63.2	12.3	14,782,068	2,676	-
5	6.5	-	2.85	86.9	94.5	5.1	0.4	7,341	7	<LOD
5	6.5	+ 0.25 M Arg	2.85	85.5	93.4	6.1	0.5	6,872	2	<LOD
5	6.5	+ 0.25 M Arg + 5% iPrOH	2.85	85.4	93.9	5.7	0.4	6,016	1	<LOD
20	6.5	-	2.85	83.4	83.4	16.1	0.4	10,522	3	<LOD
20	6.5	+ 0.25 M Arg	2.85	90.1	80.8	18.7	0.4	8,368	2	<LOD
20	6.5	+ 0.25 M Arg + 5% iPrOH	2.85	90.1	82.9	16.8	0.4	6,966	1	<LOD

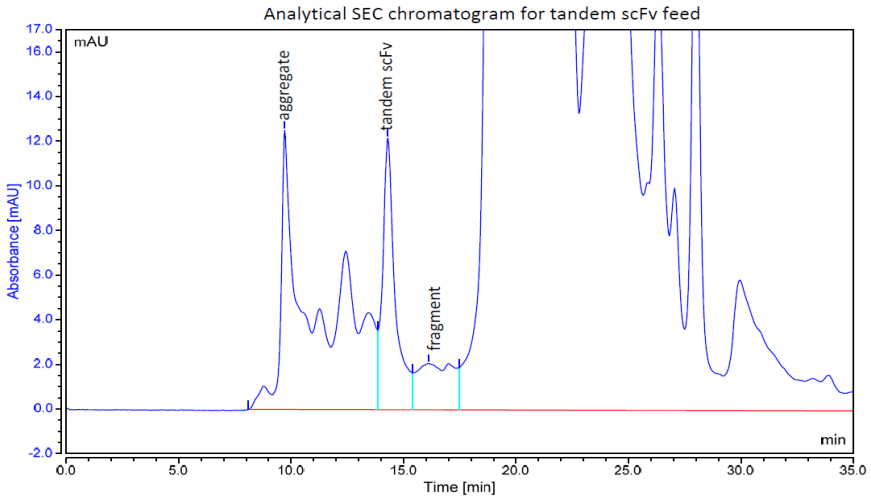
Buffer; 0.1 mol/L sodium citrate buffer (pH 6.5)
Column size; 1 mL

LOD; Limit of detection

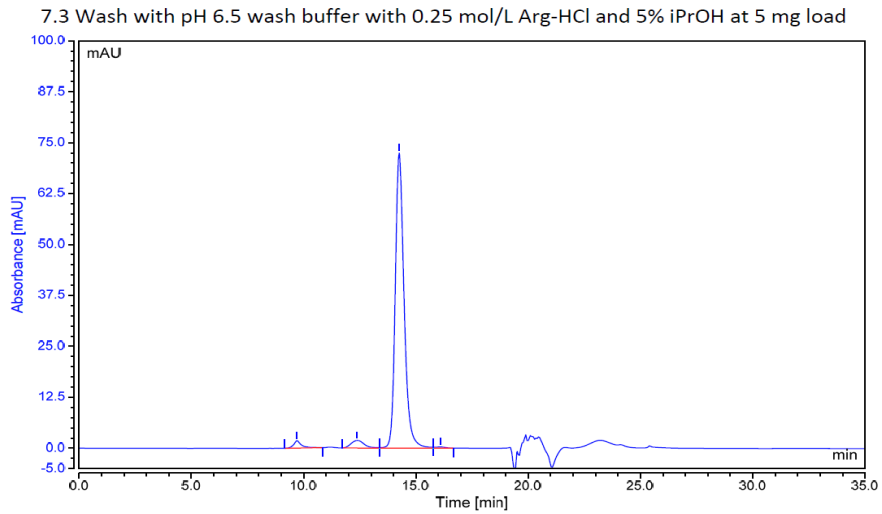
- By preliminary experiment, intermediate washing with 0.25 mol/L arginine was found effective to remove HCP from scFv fraction in CHO cell culture media.
- Additional isopropanol with arginine was found to be the most effective to remove HCP and to improve removal of other impurities.
- DBC of tandem scFv was calculated as 20 g/L by 10 % breakthrough curve at 0.5 g/L of the purified sample.
- In case of large sample loading, aggregate contents increased with decrease of monomer.

SEC analysis of Tandem scFv purification

Optimized Conditions (5 mg Load) in purification of Tandem scFv by TOYOPEARL AF-rProtein L-650F



Before purification



After AF-rProtein L-650F one step purification for 5 mg (20 mg) load

Tandem scFv monomer purity; 93.9 % (82.9%)

TOYOPEARL AF-rProtein L-650F

Chromatographic Conditions for Single Chain Fragment (scFv)

- **Adsorption and intermediate wash**

(1) scFv, tandem scFv（from CHO cell）

- 0.02 - 0.1 mol/L sodium citrate buffer (pH 6.5)
- 0.02 - 0.1 mol/L sodium citrate buffer (pH 6.5), 0.25 - 1.0 mol/L arginine + 5 - 10 % isopropanol

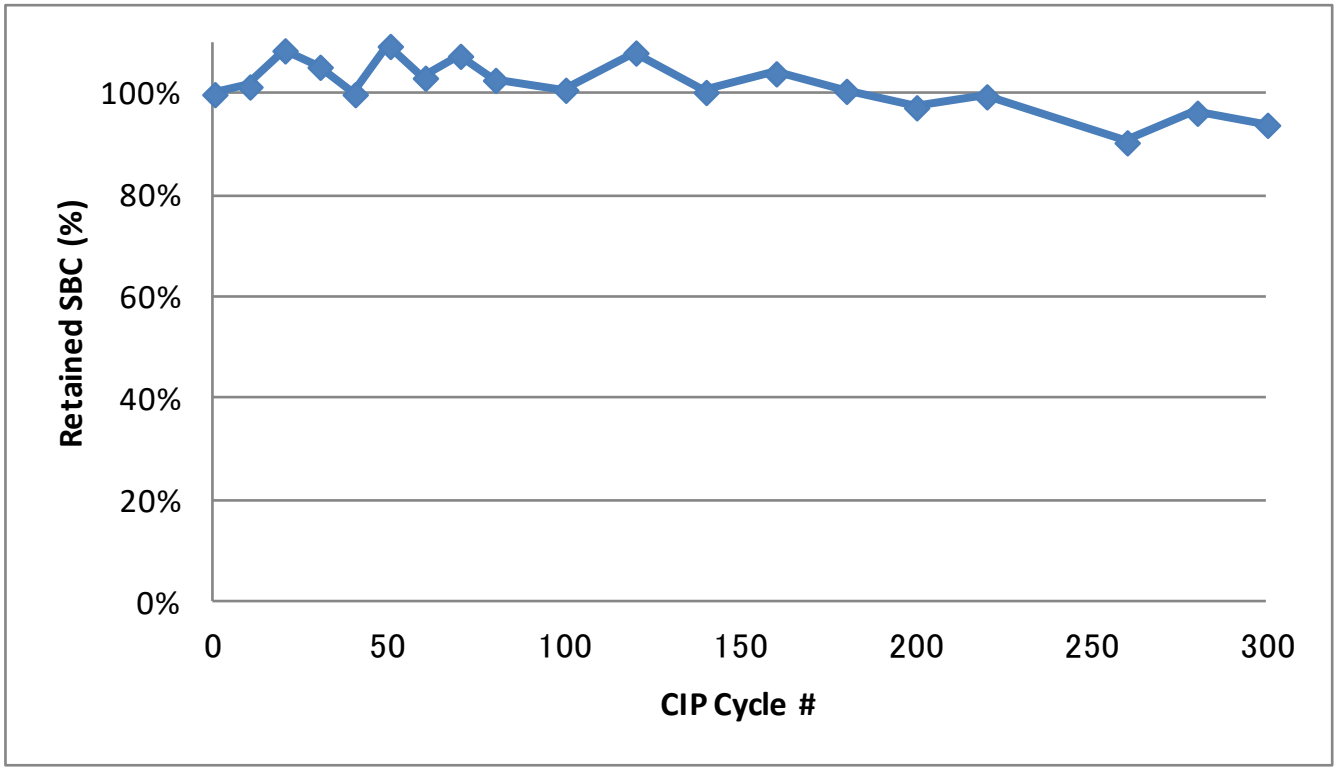
(2) scFv（inclusion body from *E. coli*）

- 0.1 mol/L sodium phosphate buffer (pH 6.5)
- 0.1 mol/L sodium phosphate buffer (pH 6.5), 2 mol/L guanidine/HCl

(Reference; J. Vajda et al., Tosoh Bioscience Technical Note)

- **Elution**
  - 0.1 mol/L citrate or glycine-HCl (pH 2.0 – pH 2.85)
- **Cleaning**
  - Acidic solution described above at pH 2.0 – pH 2.2
  - CIP; 0.05 - 0.1 mol/L NaOH (limited alkaline washing; contact time for 15 min or 1 CV)
  - For cleaning of hydrophobic impurities; 10-20% ethanol or isopropanol

CIP of TOYOPEARL AF-rProtein L-650F with 0.05 mol/L NaOH



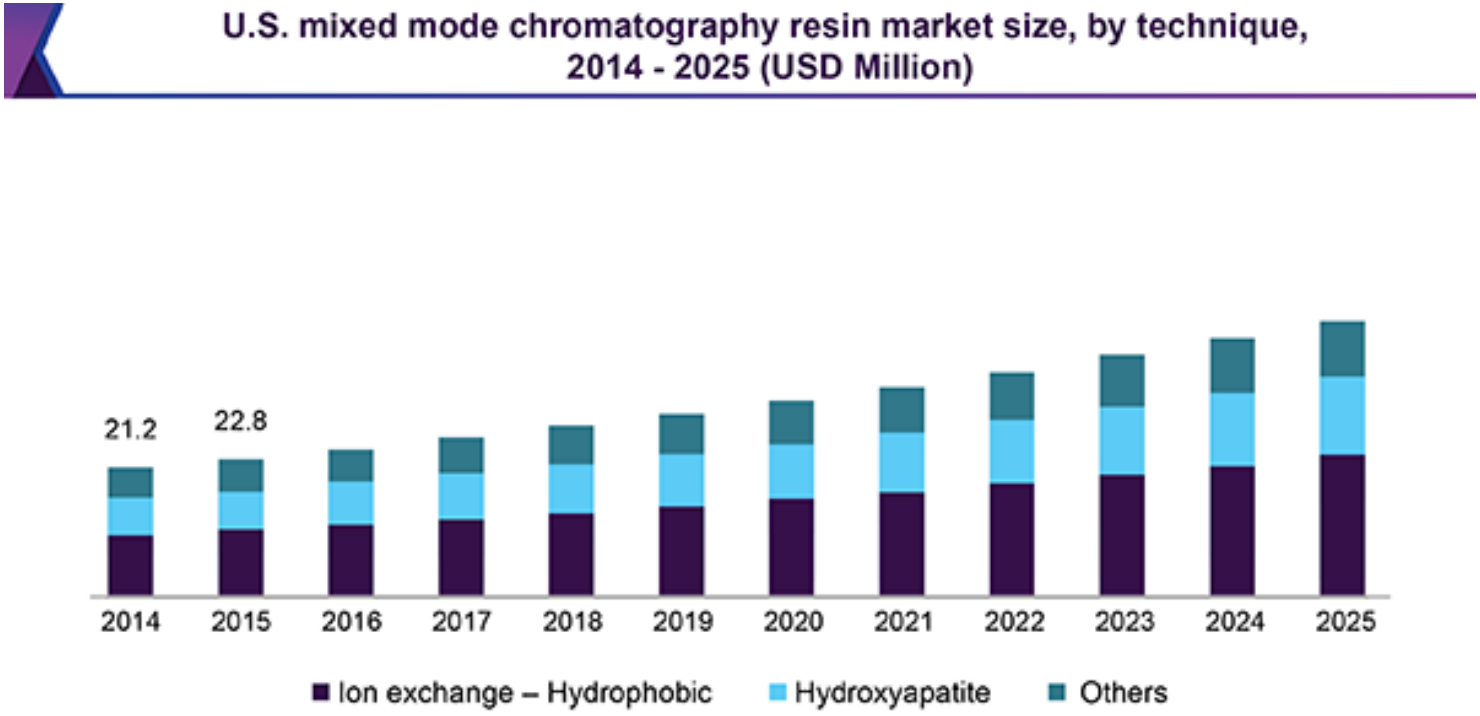
- For not so alkaline stable resin, acidic wash like citric acid (pH 2.2-2.0) followed by 0.05 mol/L NaOH gives more effective cleaning than only 0.1 mol/L NaOH washing
- TOYOPEARL AF-rProtein L-650F is stable over 300 CIP cycles with 0.05 mol/L NaOH

Ref.; N. Eifler et al., Biotechnol. Prog., (2014) Vol. 30 No. 6, p.1311-1318

Salt-Tolerant Ion-Exchange Mixed-Mode (Multi-Mode) Chromatography

- **TOYOPEARL NH_2-750F**
- **TOYOPEARL Sulfate-650F**
- **Hydroxyapatite Ca^{++}Pure-HA**
- **TOYOPEARL MX-Trp-650M**

Global Market in Mixed (Multi)- Mode Chromatography Resin

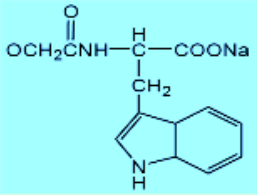


Global market size; USD 75.4 M in 2017

USD 130.3 M by 2025 (CAGR;7%)

Ref.; Grand View Research; Mixed Mode Chromatography Resin Market Analysis Report By Technique (Ion exchange - Hydrophobic, Hydroxyapatite), By End Use (Pharmaceutical & Biotechnology), And Segment Forecasts, 2018 - 2025
https://www.grandviewresearch.com/industry-analysis/mixed-mode-chromatography-resin-market

Salt-Tolerant Ion-Exchangers and Multi (Mixed) Mode Chromatography Resins

Resin	Functional group	Separation mode	Target molecule
TOYOPEARL NH_2-750F	Polyamine -NH_2 (grafted)	・Anion-exchange ・Hydrophobic interaction	Serum albumin
			Antibody (monomer, aggregate, scFv)
			Endotoxin, DNA (removal)
			Virus (removal)
TOYOPEARL Sulfate-650F	Sulfate -O-SO_3 (grafted)	・Cation-exchange ・Hydrophobic interaction	Antibody (monomer, aggregate)
			small antibody (scFv)
			Antithrombin III
TOYOPEARL MX-Trp-650M	Tryptophane 	・Cation-exchange ・Hydrophobic interaction	Antibody (IgG, Bispecific, aggregate)
			Small antibody (scFv, Fab, Fc)
			Serum protein, albumin (recombinant)
			Enzyme
			Insulin-like growth factor (IGF-1)
Ca^{++}Pure-HA®	Hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$	・Cation-exchange ・Metal chelate	Antibody, aggregate
			Antibody fragment
			Enzyme

Salt-Tolerant Anion-Exchanger (Mixed-Mode); TOYOPEARL NH_2-750F

Properties

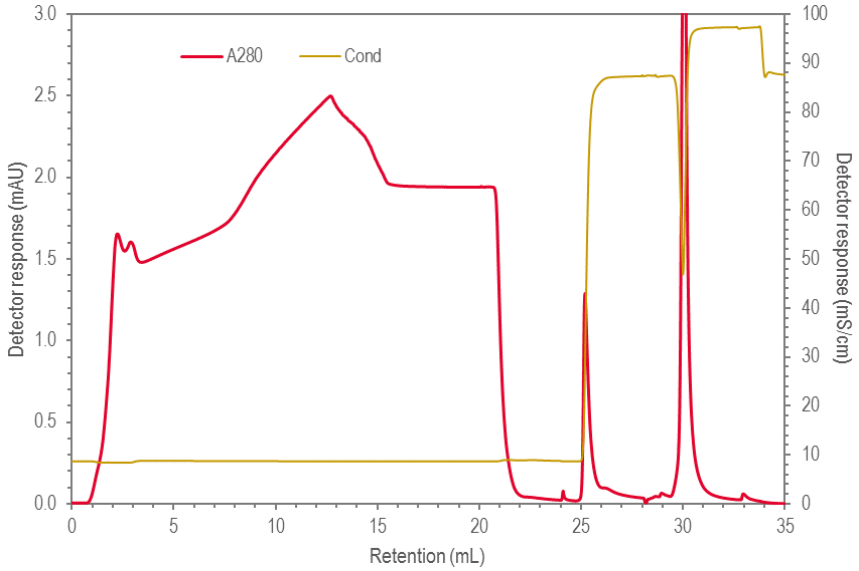
- Salt-tolerant anion-exchanger with huge pore on resin, high capacity
- High selectivity with polyamine functional group (mixed-mode effect)
- Removal HMW impurities like aggregate, virus, endotoxin and DNA
- Flow-through purification after Protein A and/or cation-exchanger
- Application at various pH and salt concentration

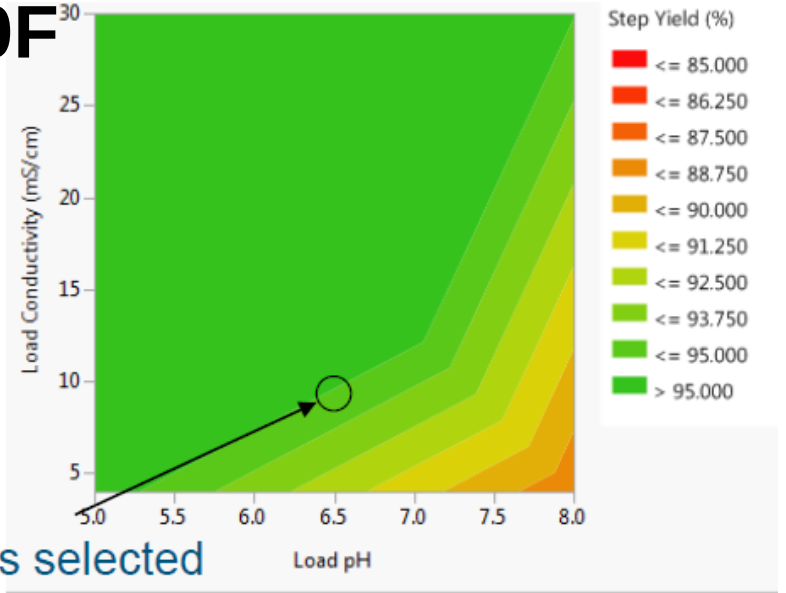
Hints

- Optimization of salt concentration and pH in buffer
  - Applicable to flow-through mode at over 0.15 mol/L NaCl in most cases
- Avoid multivalent anion buffer like phosphate, sulfate, carbonate, citrate, etc. for stronger binding
- Storage of resin with 20 % ethanol solution after washing with acidic solution at pH 4

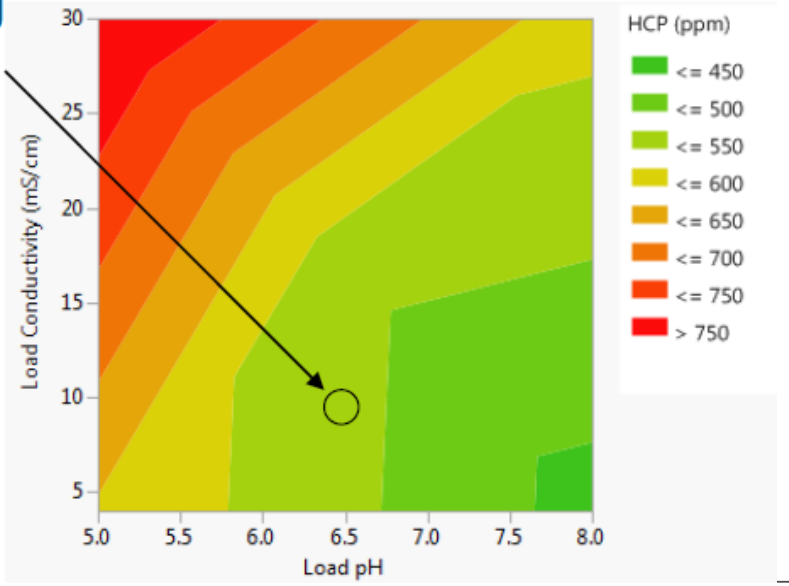
Optimizing Flow-Through (FT) Conditions on TOYOPEARL NH$_2$-750F

- Load sample
  - Aggregate; 3.1%
  - CHO-HCP; 3,422 ppm
  - DNA; 0.70 %





Conditions selected for testing



Ref.; W. Evans, PITTCON 2019, Philadelphia PA

Analysis of Flow-Through Fraction by SEC

	Recovery (monomer)	Ratio				
		HMW1 (Aggregate)	HMW2 (Dimer)	Monomer	LMW1 (Fragment)	LMW2 (Fragment)
Load		10.2 %	6.5 %	76.6 %	4.7 %	2.0 %
30 g/L	76.2 %	0.1 %	3.5 %	90.7 %	3.6 %	2.1 %
60 g/L	86.5 %	1.2 %	4.7 %	87.6 %	4.4 %	2.0 %
90 g/L	95.3 %	6.6 %	6.3 %	80.7 %	4.5 %	1.8 %
150 g/L	98.7 %	8.1 %	6.7 %	78.7 %	4.7 %	1.7 %

- Excellent removal of HMW1 impurity at ≤ 45 mg/mL load ratio, breakthrough at > 60 mg/mL
- Significant HMW 1 breakthrough noted starting at 60 mg/mL load ratio
- Poor fragment removal

Conditions
Coumn; TOYOPEARL NH_2-750F, 5 mm I.D. x 5 cm (1 mL)
Flow through; 50 mmol/L MES-0.1 mol/L acetate (pH 6.5), at 9 mS/cm
Elution; 50 mmol/L MES + 1.0 mol/L NaCl (pH 6.5)
Sanitization: 0.5 mol/L NaOH
Flow rate; Flow rate: 0.25 mL/min (4 min residence time)
Temperature: ambient
Detection: UV (280 nm), conductivity (mS/cm)
Sample; mAb-01A, 7.5 mg/L, 9 mS/cm, pH 6.5, 20 mL (150 mg/mL load ratio)
Instrument: ÄKTA avant 25 (Unicorn 7.3)

Ref.; W. Evans, PITTCON 2019, Philadelphia PA (data modified)

Salt-Tolerant Cation-Exchanger (Mixed-Mode); TOYOPEARL Sulfate-650F

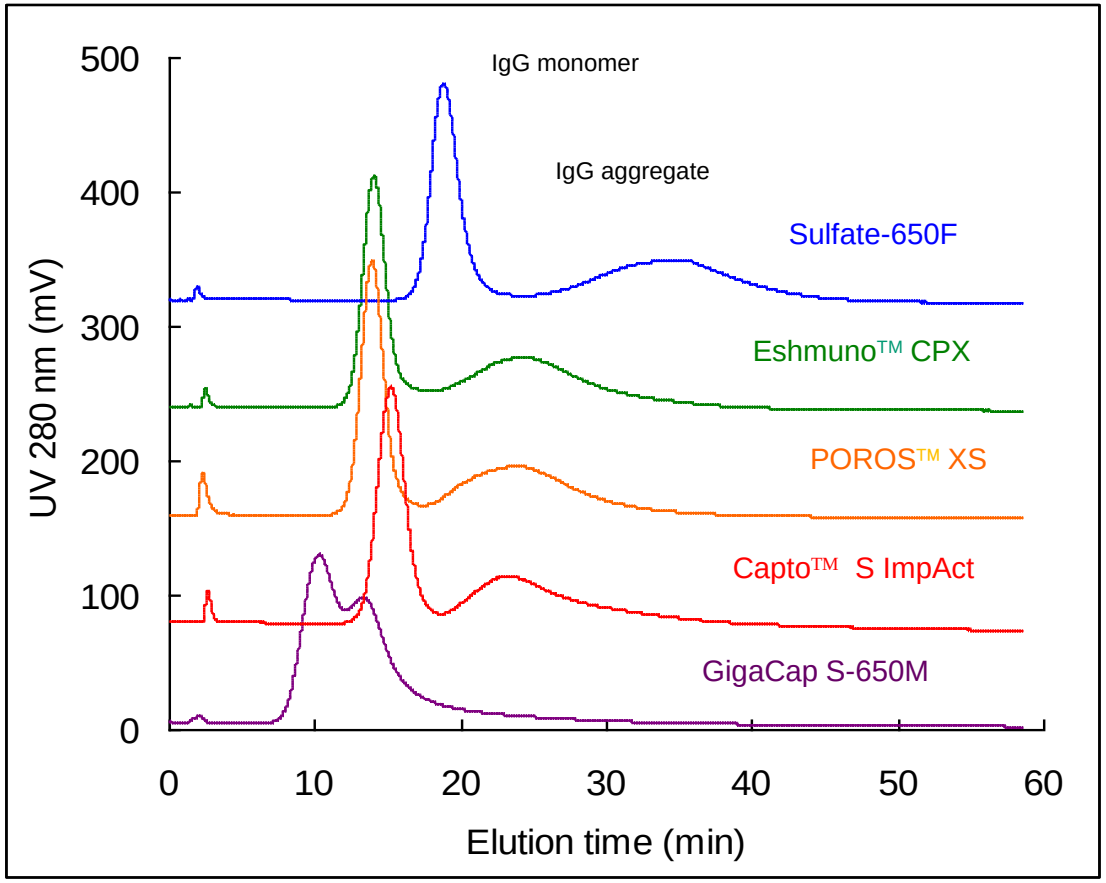
Properties

- Salt-tolerant cation-exchanger with larger pore size, high capacity
- High selectivity by Sulfate functional group (mixed-mode effect)
- Best removal of aggregate antibody（HMW）in cation-exchangers
- Removal of impurities like virus, endotoxin and DNA
- Bind/Elute separation after Protein A and anion-exchanger
- Heparin-like affinity interaction with sulfate functional groups

Hints

- Optimization of buffer pH and salt concentration
  - In most cases, buffer at pH 5.0 or lower, salt-tolerant separation can be achieved at 0.15 mol/L NaCl or higher.
- Operation at pH 4 - 12, CIP/short term cleaning at pH 3 - 13
- Storage of resin with 20 % ethanol including 0.2 mol/L sodium acetate at neutral or weak acidic pH

Salt-Tolerant Cation-Exchanger (Mixed-Mode) TOYOPEARL Sulfate-650F



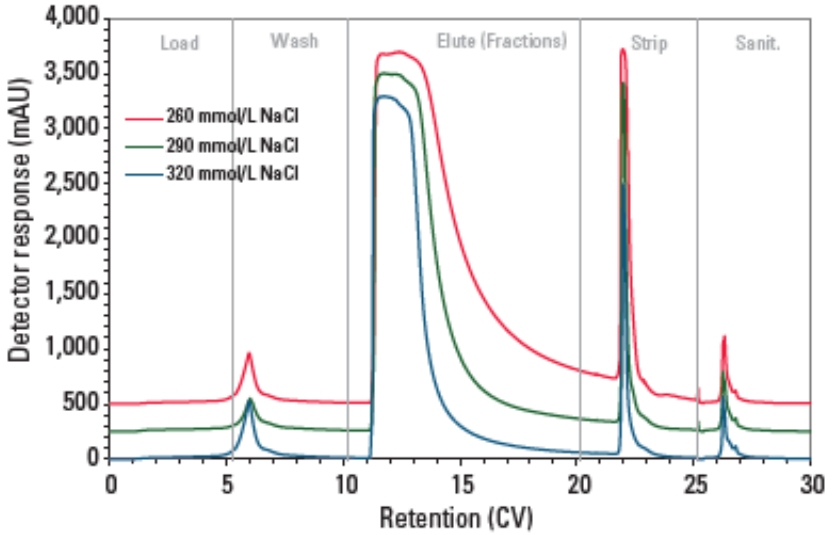
Column; TOYOPEARLSulfate-650F
（7.5 mm I.D. X 7.5 cm）
Elution;
Buffer A: 0.054 mol/L acetate buffer (pH 5.5)
Buffer B: 1.0 mol/L NaCl + Buffer A
Linear gradient:
Buffer A (95%)- Buffer B (100%), 58.5 min
Flow rate; 1.0 mL/min
Temperature; ambient
Sample; 3 g/L monoclonal humanized IgG acid/high temperature treatment (Injection volume 90 uL)

- TOYOPEARL Sulfate-650F showed best resolution for antibody between monomer and aggregate.

New Salt-Tolerant Cation-Exchanger;

mAb Aggregate Removal by Bind/Elute Method

- Salt tolerant
- High capacity
- Aggregate removal
- Viral clearance
- Heparin-like affinity



Purification results	Protein A	Sulfate-650F
Sample loading (mg/mL resin)	48	101
Recovery, monomer (%)	n/a	83
Dimer (%)	3.90	2.40
Aggregate (%)	0.54	0.07
HCP (ppm)	1,260	134
Protein A (ppm)	1.20	0.04

*Elution at 260 mmol/L NaCl

Resin:	TOYOPEARL Sulfate-650F
Column:	6.6 mm ID × 3.0 cm (1.0 mL)
Mobile phase:	A: 50 mmol/L acetate-Tris, 100 mmol/L NaCl, pH 5.2
	B: 50 mmol/L acetate-Tris, pH 5.2, NaCl as indicated
	C: 50 mmol/L acetate-Tris, 1.0 mol/L NaCl, pH 5.2
	D: 0.5 mol/L NaOH
Flow rate:	45 cm/hr (4 min residence time)
Detection:	UV @ 280 nm (mAU)
Temperature:	ambient
Injection vol.:	5.3 mL (97 mg/mL-resin load ratio)
Sample:	TBL-mAb-01, 19.1 mg/mL
Instrument:	ÄKTA avant 25

Adsorption Capacity for scFv on Various Cation-Exchangers after Desalting

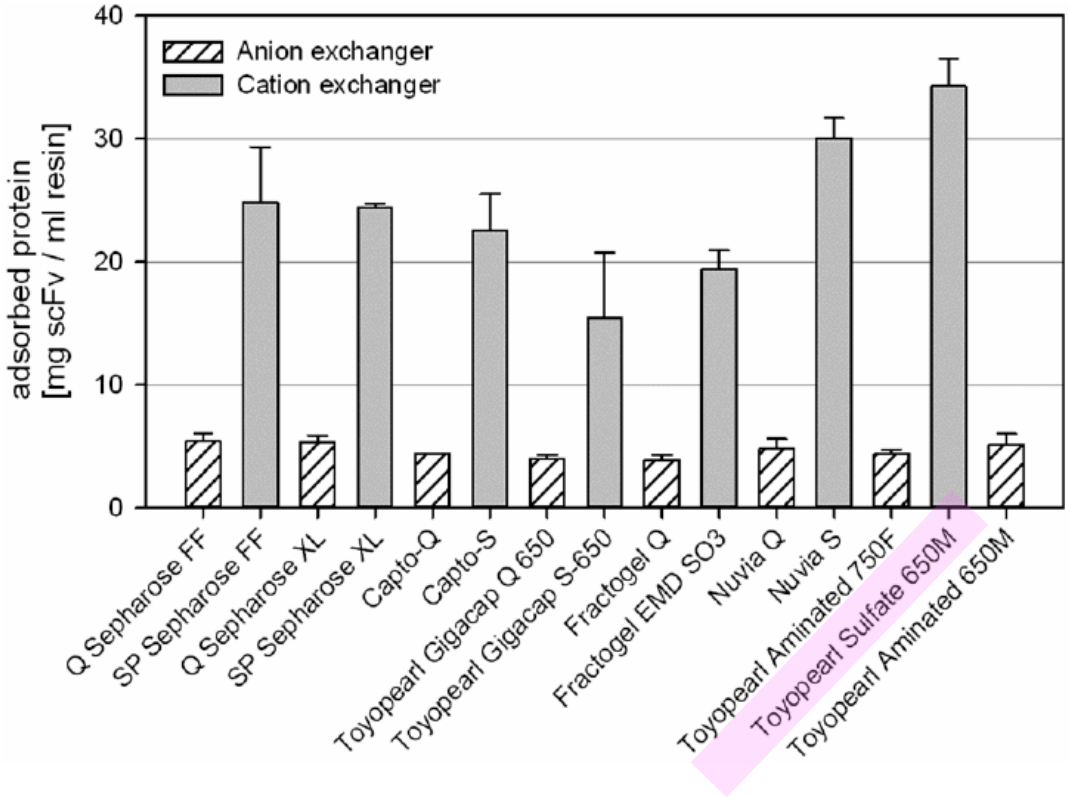


Figure 4. Screening of macropore ion exchange resins for equilibrium binding capacity of scFv. Hatched bars: direct capturing of scFv from refolding solution with anion exchangers; gray bars: capturing of scFv from desalted refolding solution with cation exchangers.

- TOYOPEARL Sulfate-650F showed the best binding capacity for scFv.

Ref. ; N. Walch et al., Biotechnol. J., 2017, 12, 1700082

Multi (Mixed) -Mode (Cation-Exchange Type) Resin; TOYOPEARL MX-Trp-650M

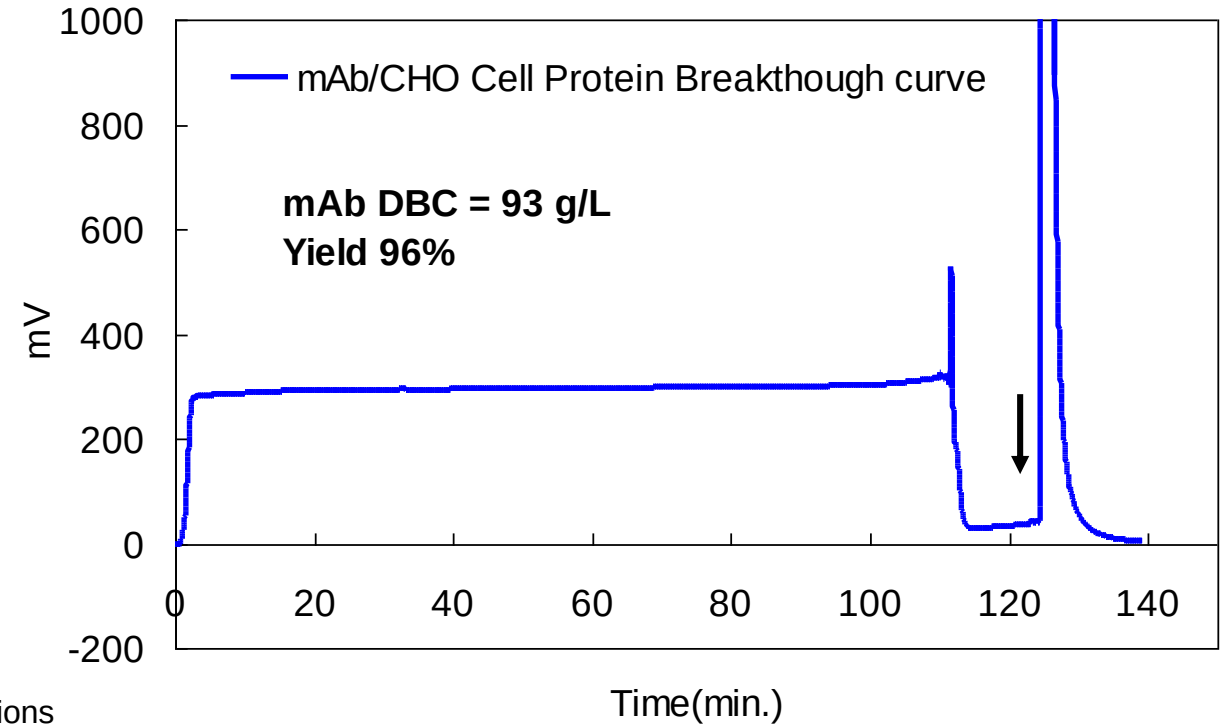
Properties

- Tryptophan (amino acid) immobilized
- Multi-mode (Cation-exchange/hydrophobic interaction) separation
- High capacity; IgG ≧ 75 g/L
- Sharper elution peak and higher recovery
- Salt-tolerant separation for antibody and other proteins

Hint for separation

- Optimization of salt concentration and pH in eluent
- Adsorption at pH 4.0 - pH 5.5, elution at pH 7.0 - 8.0 with salt
- CIP, short term cleaning at pH 3 - pH 13
- Storage by shading container, ethanol solution without oxidative reagent

Purification of Monoclonal Antibody from Cell Culture Media by TOYOPEARL MX-Trp-650M

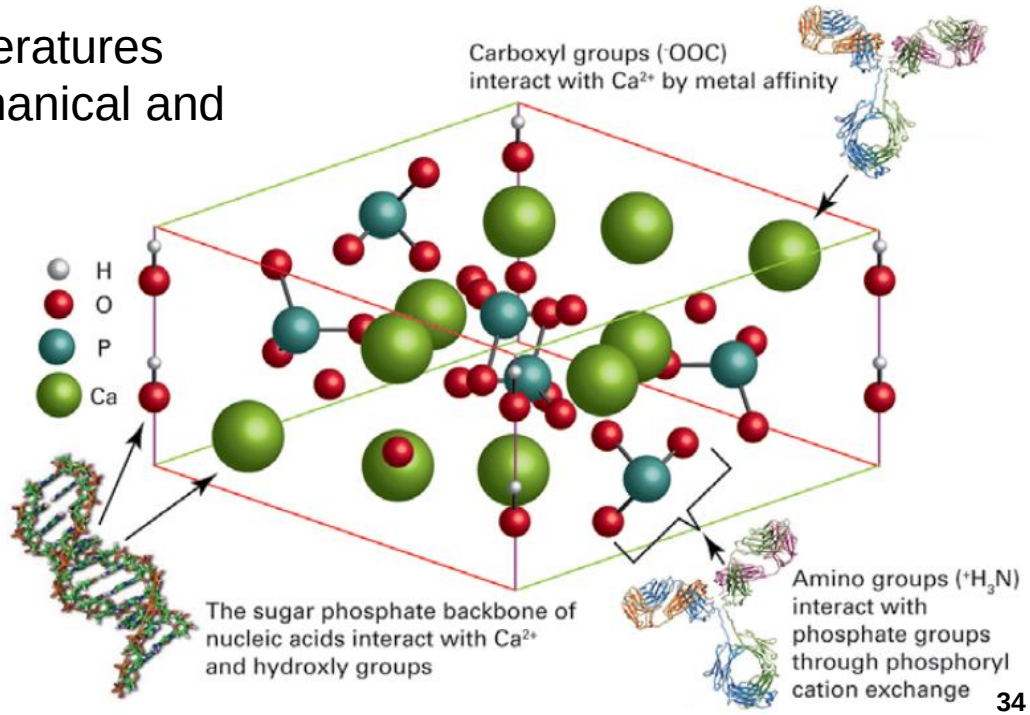


Conditions

Column; TOYOPEARL MX-Trp-650M (6 mm I.D. x 4 cm)
Binding buffer；0.05 mol/L acetate buffer (pH 4.7) + 0.1 mol/L NaCl
Elution buffer；0.1 mol/L Tris-HCl buffer (pH 8.5) + 0.3 mol/L NaCl
Flow rate (binding)；1.0 mL/min (212 cm/h)
(elution) ；2.0 mL/min （at 124 min）
Detection； UV(280 nm)
Sample； CHO cell culture media, monoclonal antibody (1 mg/mL), diluted with binding buffer
Sample conductivity; 12 mS/cm

Hydroxyapatite Ca^{++}Pure-HA

- Media is composed of a unit cell comprised of $Ca_{10}(PO_4)_6(OH)_2$
- 10 nm x 100 nm hexagonal cross section crystals are agglomerated into particles and then heated to create stable welds at the crystal contact points
- Spherical, macroporous form of a hexagonal crystalline structure
- Sintered at high temperatures
- for increased mechanical and
- chemical stability



Properties on Hydroxyapatite Ca^{++}Pure-HA

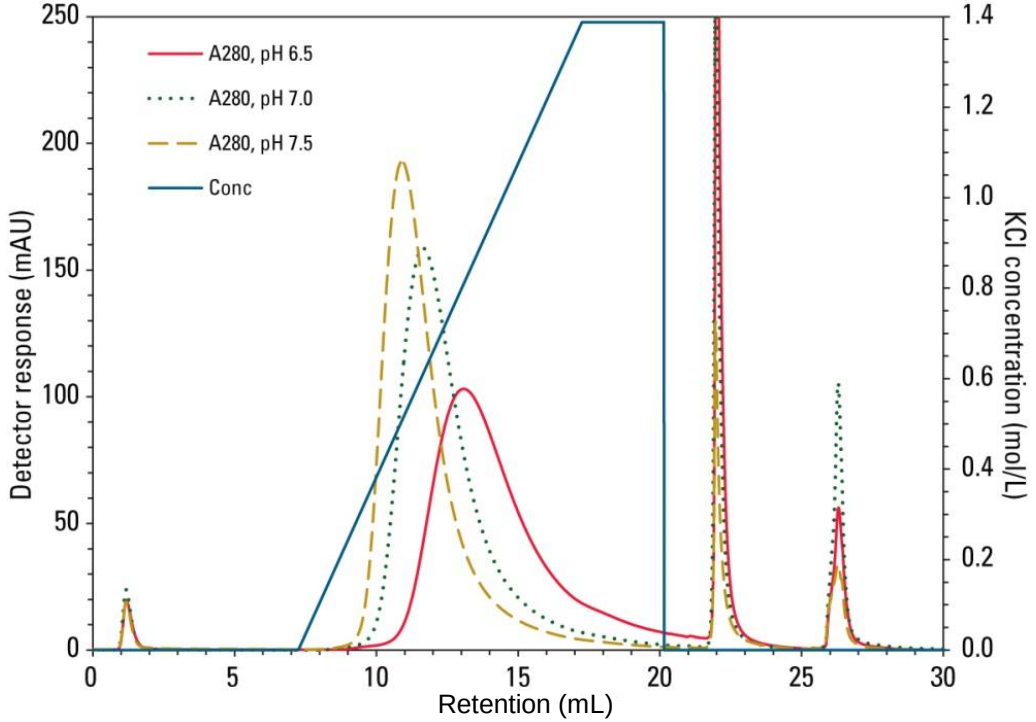
- Effective to higher MW biomolecule like antibody by large 50 nm pore size of matrix
- DBC on antibody; 55 g/L (IgG; 2 g/L, residence time; 5 min)
- Similar or better capacity and mechanical stability than commercial hydroxyapatite
- CIP over 65 cycles with 1 mol/L NaOH

  (operational buffer pH above 6.5, unstable in acidic condition)
- Mixed-mode matrix (cation-exchange type) effective to separate antibody and removal of impurities
- 30 % lower price than commercial hydroxyapatite to replace existing process matrix
- Target molecules

  Antibody and its fragment, recombinant protein, enzyme, virus, vaccine, nucleic acid (plasmid, double stranded DNA)

Excellent Selectivity on Hydroxyapatite

Aggregate removal from purified IgG using KCl for elution



- Ca^{++}Pure-HA is compatible with potassium phosphate buffer as a loading buffer and potassium chloride as an elution buffer at different pH.
- Similar mAb aggregate removal performance across all three pH values.
- The separation profiles show high resolution between the monomer peak and the aggregate peaks, and increased pH makes earlier elution

High Purity & Yield for mAb Purification

Salt	pH	Peak molarity (mmol/L)	Pool Volume (CV)	Recovery monomer (%)	Aggregate (%)	Fragment (%)	CHO-HCP (ppm)
				Load	6.6	0.6	2,200
KCl	6.5	814	5.3	73	1.3	0.5	500
	7.0	615	3.9	80	1.8	0.3	590
	7.5	509	3.9	81	2.2	0.3	910

- > 80% recovery of mAb monomer at optimal pH (7.0)
- KCl elution buffer shows good aggregate removal across pH conditions.
- This elution buffer shows that the lower eluting buffer pH gives better aggregate removal.
- Ca^{++}Pure-HA significantly reduces host cell proteins (HCP) at pH of 6.5 and 7.0.

Attention fo Usage of Ca^{++}Pure-HA

- Some limitation of mobile phase usage due to patent infringement

- Gradient elution by salt
  - Use 25 mM or higher concentration of phosphate buffer
  - Use KCl as salt (avoid use of NaCl)

    （KCl has better solubility than NaCl for salt gradient elution.)
- Gradient elution by phosphate buffer
  - No limitation for usage
- Neutralization of hydroxyapatite in initial cleaning
  - At first cleaning should be achieved with phosphate buffer, not to use NaOH solution for neutralization of hydronium ion in hydroxyapatite.

Selection of TOYOPEARL Ion-Exchangers and/or Hydroxyapatite (HA)

Customer already using hydroxyapatite

- Recommend Ca^{++}Pure-HA
- Cost advantage (30 % less price)
- No significant difference in separation
  （replacement of existing matrix)
- Slightly better binding capacity
- Slightly better mechanical stability
- FOC sample demonstration
  (Anyway, ask trial resin with bulk matrix or MiniChrom)

Customer not yet used hydroxyapatite

- Recommend TOYOPEARL Sulfate-650F, or MX-Trp-650M, (or NH_2-750F, in case)
- TOYOPEARL is chemically stable in alkaline and acid.
  (HA is unstable in acidic solution.)
  (HA only is used at pH above 6.5)
- FOC sample demonstration
  (Anyway, ask trial resin with bulk matrix or MiniChrom)

Summary of Viral Clearance of Antibody by Chromatographic Resins

Chromatography Mode	Resin	Separation	Log Reduction Value (LRV)*			
			MuLV	PRV	Reo	MVM
Anion-exchange (AIEC)	TOYOPEARL GigaCap Q-650M	Flow through	5.8	5.7	5.7	6.0
Cation-exchange (CIEC)	TOYOPEARL GigaCap S-650M	Bind-elute	4.3	5.5	1.4	-0.2
Mixed-mode (MXC)	TOYOPEARL NH_2-750F**	Flow through	4.9	5.9	5.7	6.4
Mixed-mode (MXC)	TOYOPEARL Sulfate-650F**	Bind-elute	4.7	5.4	6.0	1.2
Mixed-mode (MX)	TOYOPEARL MX-Trp-650M	Bind-elute	2.8	3.5	1.8	0.6
Mixed-mode (MXC)	Hydroxyapatite Ca^{++}Pure-HA	Flow through	3.3	2.3	2.7	-0.3
Hydrophobic interaction (HIC)	TOYOPEARL PPG-600M	Bind-elute	2.4	0.9	4.8	0.8
Hydrophobic interaction (HIC)	TOYOPEARL Hexyl-650C	Flow through	3.4	1.8	6.2	0.0
Affinity (AFC)	TOYOPEARL AF-rProtein A HC-650F	Bind-elute	1.7	1.2	0.0	2.2
Affinity (AFC)	TOYOPEARL AF-rProtein L-650F	Bind-elute	2.5	1.7	3.0	2.5

* LRV was measured independently for each product by optimized conditions for model sample as monoclonal antibody or albumin (Hexyl-650C).

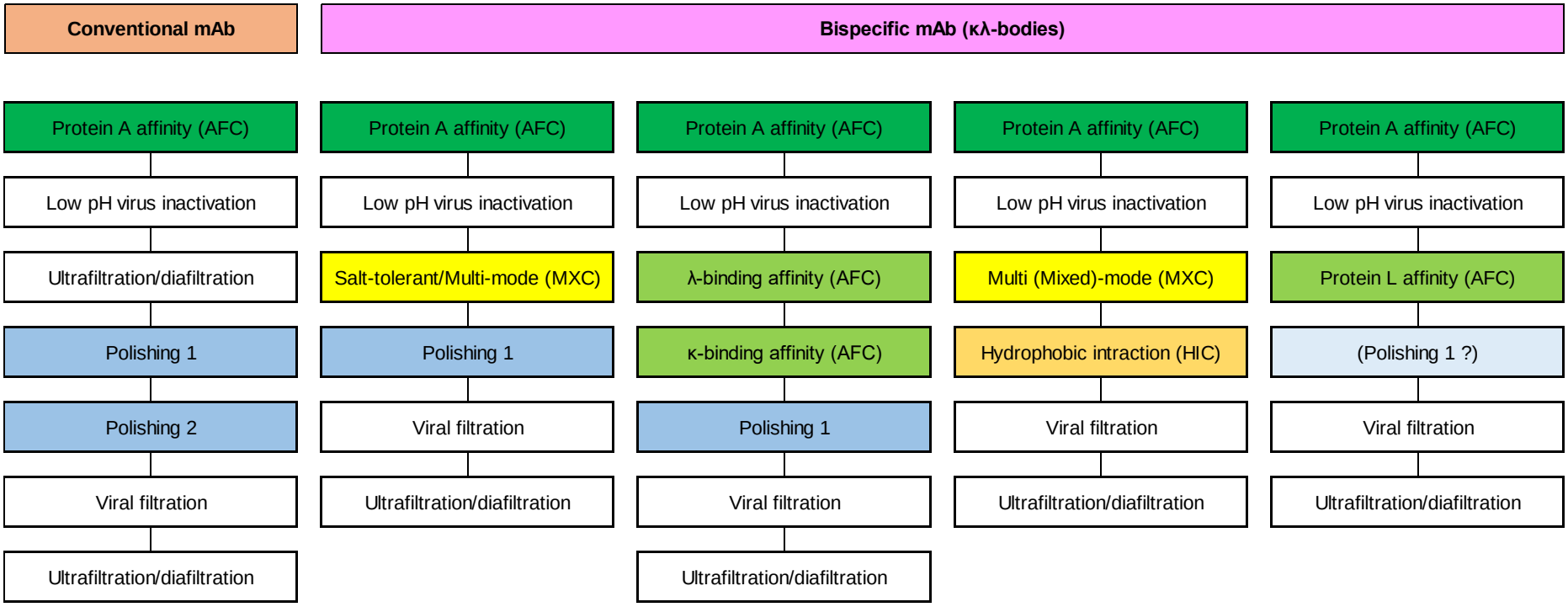
**Salt-tolerant ion-exchanger

- Anion-exchanger and salt-tolerant (mixed-mode) resins show better viral clearance.

Other New Application to mAb Separation

- Bispecific mAb separation
  - One-step purification by TOYOPEARL AF-rProtein L-650F by linear gradient of both pH and salt,（Chugai, 2018, PCT WO 2018/159615 A1）
  - Two-step purification by TOYOPEARL MX-Trp-650M and Butyl-600M
    （J. Vadja et al., Bioprocess Int. 16 (10) (2018) 59-62), Novimmune SA, US patent 2016/0264685 A1）
- Antibody-Drug Conjugate (ADC) separation by HIC
  - Separation by less hydrophobic resin, TOYOPEARL PPG-600M or Ether-650M
- Continuous production of mAb
  - PEG treatment followed by TOYOPEARL GigaCap S-650M
  - TOYOPEARL AF-rProtein A HC-650F followed by AEC and MXC
- Flow-through HIC application
  - New flow-through HIC resin for mAb (trial resin)
  - Removal of detergent in cell culture feedstock prior to Protein A affinity purification on TOYOPEARL Butyl-600M

New Process Development for Bispecific mAb



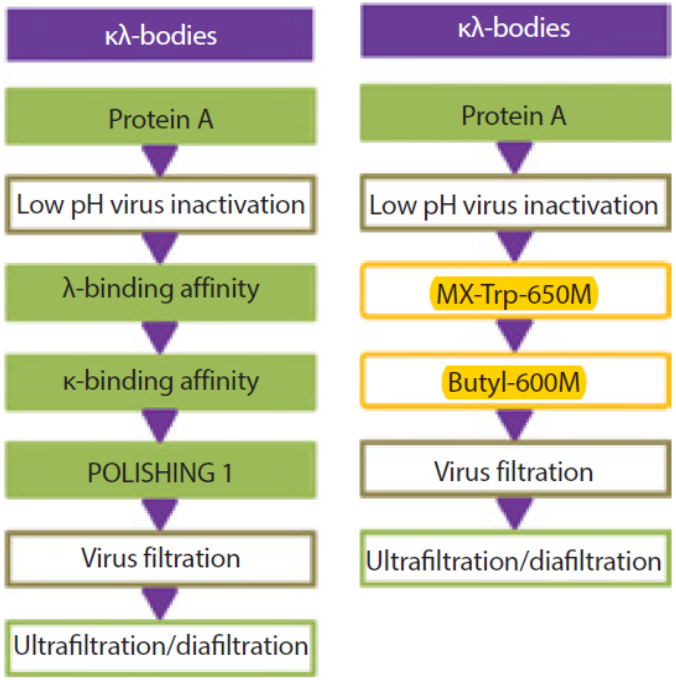
POROS MabCapture A; 25 g/L
MabSelect SuRe
TOYOPEARL AF-rProtein A-650F
CaptoMMC; 23 g/L
A. D. Tustian et al., Mabs (2016)
Vo. 8 (4) 828-838

KappaSelect; 15 g/L (Fab)
LambdaSelect; 20 g/L (Fab)
Sample load; 20 g/L

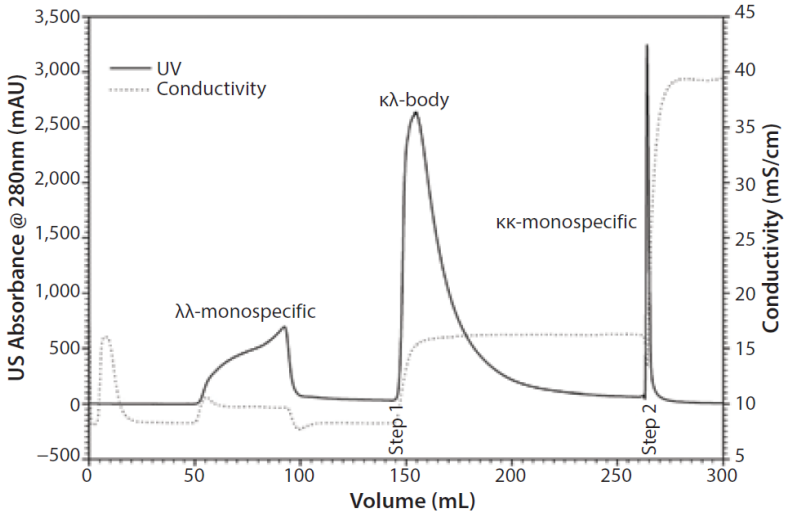
MX-Trp-650M; DBC; 87 g/L
Sample load; 70 g/L (80 % load)

C. Chen et al., Mabs, 2019,
May/June, 11(4) 632-638

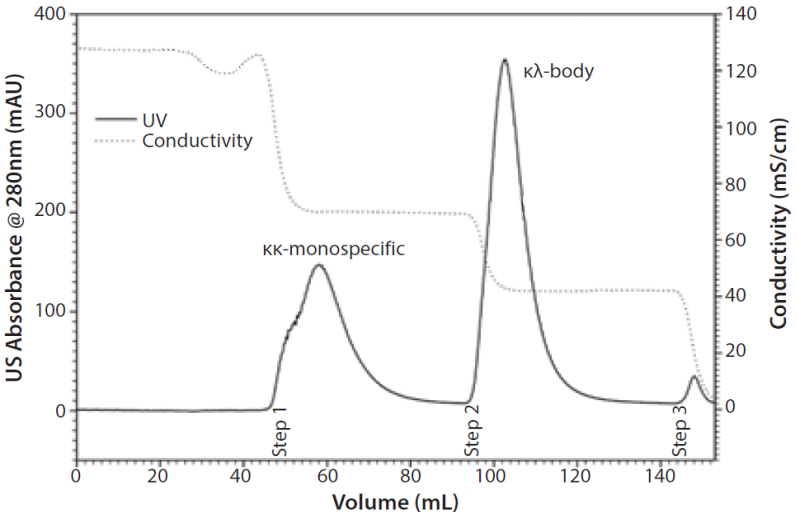
Purification of Bispecific mAb by Multi (Mixed)-Mode and HIC



TOYOPEARL MX-Trp-650M

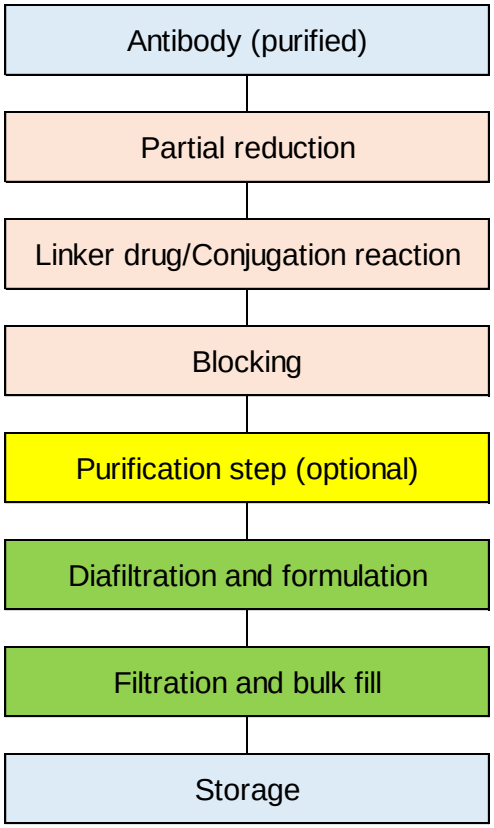


TOYOPEARL Butyl-600M

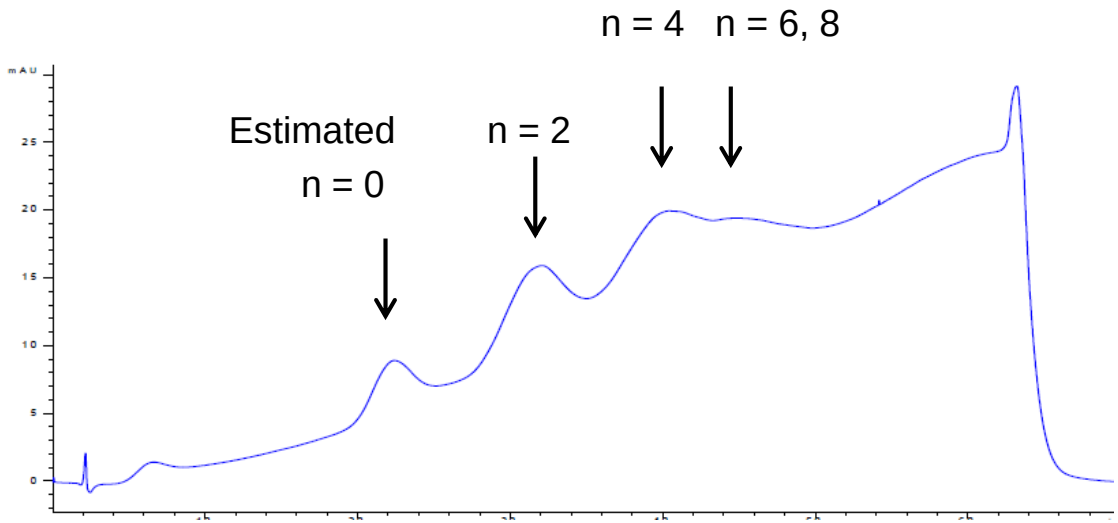


Ref.; J. Vadja et al., BioProcess International, October (2018) 16 (10) 59-62

Purification of ADC by HIC on TOYOPEARL PPG-600M

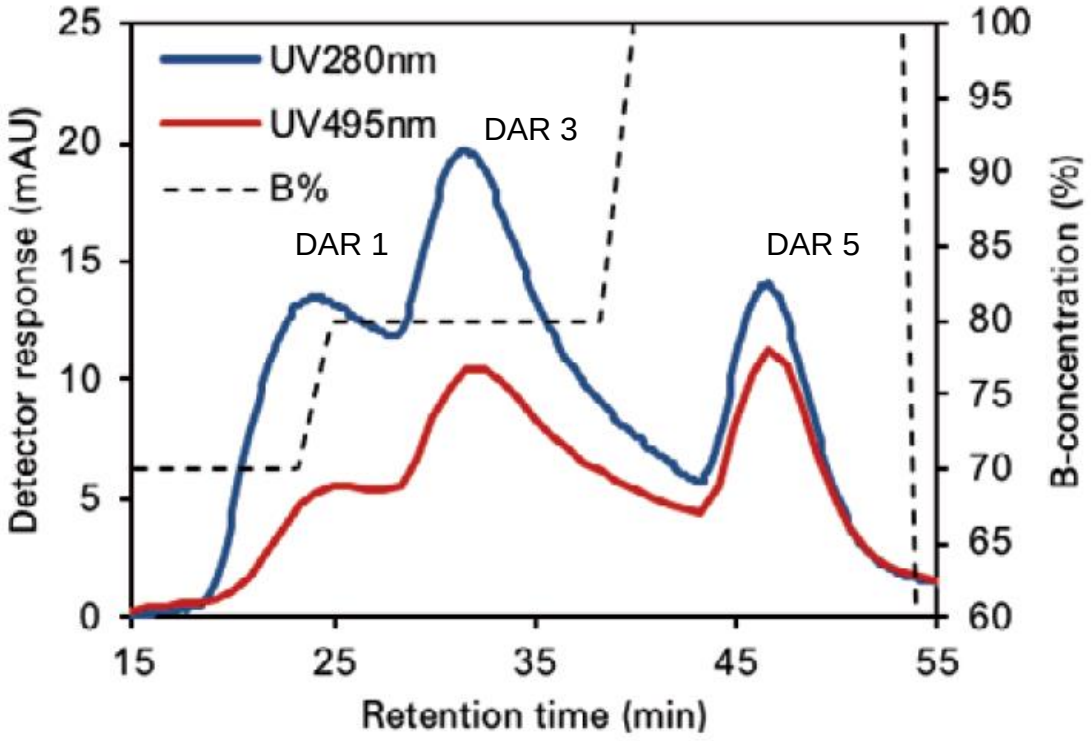


Ref. V. Leur et al., Bioprocess International, 16(12)E2, Dec. 2018, modified



Column; ToyoScreen® PPG-600M 6.4 mm I.D. x 3 cm (1 mL)
Elution; A: 25 mmol/L sodium phosphate (pH 7.0)
+ 1.5 mol/L ammonium sulfate
B; 25 mmol/L sodium phosphate (pH 7.0)/iso-propanol = 75/25
60 min linear gradient from Buffer A to B
Flow rate; 1 mL/min, Detection; UV (280 nm)
Sample; ADC (1 g/L, 50 uL)

Separation of Antibody Drug Conjugate Mimic by HIC on TOYOPEARL PPG-600M



Conditions

Column; TOYOPEARL PPG-600M (6.6 mm I.D. x 10 cm)

Elution; Buffer A: 0.1 mol/L sodium phosphate (pH 6.5) containing 1.5 mol/L ammonium sulfate

Buffer B: 0.1 mol/L sodium phosphate (pH 6.5)

Step gradient, 100 % Buffer A (5CV), 70 % Buffer B (5CV), 80 % Buffer B (5 CV), 100 % Buffer B (5 CV)

Flow rate; 250 cm/hr for equilibration and washing, 175 cm/hr for step elution

Detection; UV (280 nm), VIS (495 nm)

Sample; adalimumab, conjugated with FITC (5 mg/mL resin)

Ref.; Tosoh Bioscience Application Note (2019)

Summary

- Best-in-Class process chromatography resins as of high capacity, high selectivity and alkaline stability for purification of antibody.
- TOYOPEARL Protein A and Protein L affinity chromatography, and salt-tolerant ion-exchangers showed good purification of various antibodies like intact IgG and single chain fragments (scFv).
- Combination of capture step, Protein A/Protein L affinity chromatography and salt-tolerant ion-exchangers would be powerful tool to purify monoclonal antibody and to remove impurities like aggregate, HCP, host DNA, virus and Protein A/Protein L leachable.

Commercial Ion-Exchangers and Mixed-Mode Resins

Resin particle size	TOSOH	GE Life Science	Merck	Bio-Rad	Others
AEC 50–200 um	TOYOPEARL GigaCap Q-650M TOYOPEARL QAE-550C TOYOPEARL Q-600C AR TOYOPEARL NH_2-750F	Capto Q Q Sepharose XL Q Sepharose Fast Flow Q Sepharose Big Beads MacroCap Q CaptoCore Q	Eshumuno Q Fractogel EMDTMAE Hicap (Fractogel EMD TMAE (M)	Nuvia Q MacroPrep High Q UNOsphere Q	POROS 50 micron HQ Pall Q Hypercel YMC BioPro QA
AEC 20–35 um	TOYOPEARL GigaCap Q-650S TSKgel SuperQ-5PW (20), (30)	Capto Q ImPress Q Sepharose HP Source Q15, Q30	Fractogel EMD TMAE (S)	MacroPrep 25 Q	
AEC 50–100 um	TOYOPEARL GigaCap DEAE-650M TOYOPEARL DEAE-650M/C	Capto DEAE DEAE Sepharose Fast Flow	Fractogel EMD DEAE	MacroPrep DEAE	
AEC 20–35 um	TOYOPEARL DEAE-650S TOYOPEARL GigaCap DEAE-650S TSKgel DEAE-5PW (20), (30)	DEAE Sepharose HP			
CEC 50–200 um	TOYOPEARL GigaCap S-650M TOYOPEARL SP-550C TOYOPEARL SP-650M/C TOYOPEARL MegaCap II SP-550EC TOYOPEARL Sulfate-650F	Capto S SP Sepharose XL SP Sepharose Fast Flow SP Sepharose Big Beads MacroCap SP	Eshumuno S Fractogel EMD SE Hicap (M) Fractogel EMD SO3-	Nuvia S MacroPrep High S UNOsphere S	POROS XS POROS 50 micron HS Pall S Hypercel YMC BioPro SP
CEC 20–35 um	TOYOPEARL GigaCap S-650S TOYOPEARL SP-650S TSKgel SP-5PW (20), (30)	Capto S ImPress S Sepharose HP Source S15, S30	Fractogel EMD SO3-(S)	MacrpPrep 25 S	
CEC 50–100 um	TOYOPEARL GigaCap CM-650M TOYOPEARL CM-650M/C	CM Sepharose Fast Flow	Fractoge EMD COO-	MacrpPrep CM	
CEC 20–35 um	TOYOPEARL CM-650S	CM Sepharose HP			
MMC 50–100 um	(TOYOPEARL NH_2-750F)	Capto Adhere			Pall MEP HyperCel HEA HyperCel, PPA HyperCel
MMC 50–100 um	TOYOPEARL MX-Trp-650M (TOYOPEARL Sulfate-650F)	CaptoMMC	Eshmuno HCX	Nuvia cPrime	
Hydroxyapatite 20–55 um	Ca^{++}Pure-HA			CHT Ceramic hydroxyapatite Type-I, II	

BioSeparation on TSKgel® Columns and TOYOPEARL® Resins

Mode	Target	TSKgel Columns	TOYOPEARL, Preparative HPLC Resins	Remarks
Size-Exclusion Chromatography (SEC)	Peptide	TSKgel UP-SW2000 (2019) TSKgel SuperSW2000 TSKgel G2000SWXL	TOYOPEARL HW-40S/F	•High resolution on TSKgel SW series •Separation range on TOYOPEARL HW-40 similar to TSKgel G2500PWXL
	Protein	TSKgel UP-SW3000 TSKgel SuperSW mAb HR/HTP TSKgel G3000SWXL	TOYOPEARL HW-50S/F TOYOPEARL HW-55S/F	•High resolution on TSKgel SW series. •Separation range on TOYOPEARL HW-55 simiar to TSKgel G3000PW
	Aggregate High Molecular Bio-complex	TSKgel UltraSW Aggregate, G4000SWXL TSKgel G4000PWXL, G5000PWXL TSKgel G6000PWXL/G-DNA-PW	TOYOPEARL HW-65S/F TOYOPEARL HW-75S/F	•TSKgel UltraSW Aggregate separates aggregates up to 5 mer •TOYOPEARL HW-75 has higher pore size.
	Oligosaccharide Polysaccharide	TSKgell SuperOligo-PW®, G-Oligo-PW TSKge; G2500PWXL, G3000PWXL TSKgel G5000PWXL, G6000PWXL TSKgel GMPWXL, SuperMultiporePW-H, -M	TOYOPEARL HW-40S/F TOYOPEARL HW-50S/F TOYOPEARL HW-55S/F TOYOPEARL HW-65S/F, HW-75S/F	•TSKgel Oligo-PW series for non-ionic saccharide •Separation range on TOYOPEARL HW-40 up to 8mer
Ion-Exchange Chromatography (IEC)	Acidic Molecule (Strong Anion-Exchange)	TSKgel Q-STAT, DNA-STAT, DNA-NPR® TSKgel SuperQ-5PW TSKgel BioAssist® Q	TOYOPEARL GigaCap® Q-650M/S TOYOPEARL SuperQ-650S/M TOYOPEARL Q-600C AR, QAE-550C TSKgel SuperQ-5PW(20), (30)	•TOYOPEARL GigaCap series with higher adsorption capacity •TOYOPEARL Q-600C AR with higher alkaline stability
	Acidic Molecule (Weak Anion-Exchange)	TSKgel DEAE-NPR TSKgel DEAE-5PW	TOYOPEARL GigaCap DEAE-650M TOYOPEARL DEAE-650S/M TOYOPEARL NH_2-750F TSKgel DEAE-5PW (20), (30)	•TOYOPEARL GigaCap series with higher adsorption capacity •TOYOPEARL NH2-750F is salt-tolerant ion-exchanger (and mixed-mode) with higher resolution of huge bio-molecule
	Basic Molecule (Strong Cation-Exchange)	TSKgel SP-STAT TSKgel SP-NPR TSKgel SP-5PW	TOYOPEARL GigaCap S-650M TOYOPEARL SP-650S/M, SP-550C TOYOPEARL MegaCap II SP-550EC TOYOPEARL Sulfate-650F TSKgel SP-5PW(20), (30), SP-3PW(30)	•TOYOPEARL GigaCap series with higher adsorption capacity •TOYOPEARL Sulfate-650F is salt-tolerant ion-exchanger (mixed-mode). •TOYOPEARL SP-550 series and TSKgel SP-3PW(30) for insulin separation
	Basic Molecule (Weak Cation-Exchange)	TSKgel CM-STAT TSKgel CM-5PW	TOYOPEARL GigaCap CM-650M TOYOPEARL CM-650S/M	•TSKgel CM-STAT with higher resolution
Mixed-Mode Chromatography (MXC)	Acidic Molecule (Weak Anion-Exchange)	N/A	TOYOPEARL NH_2-750F	•Salt-tolerant ion-exchanger
	Basic Molecule (Cation-Exchange) (Hydroxyapatite)	N/A	TOYOPEARL Sulfate-650F TOYOPEARL MX-Trp-650M Ca^{++}Pure-HA	•TOYOPEARL Sulfate-650F is salt-tolerant ion-exchanger •Hydroxyapatite
Hydrophobic-Interaction Chromatography (HIC)	High Hydrophobic Protein	TSKgel Ether-5PW	TOYOPEARL Ether-650M, PPG-600M	•TOYOPEARL 600 series have higher binding capacity for antibody.
	Medium Hydrophobic Protein	TSKgel Butyl-NPR TSKgel Phenyl-5PW	TOYOPEARL Phenyl-600M, Phenyl-650S/M TOYOPEARL Butyl-600M, Butyl-650S/M TOYOPEARL SuperButyl-550C	•TOYOPEARL 600 series have higher binding capacity for antibody. •TOYOPEARL 550 series for low molecule protein separation
	Low Hydrophobic Protein Nucleic Acid	N/A	TOYOPEARL Hexyl-650C	•Plasmid DNA and oligosaccharide separation
Affinity Chromatography (AFC)	Antibody (Fc, glycan) Fc Fusion Protein	TSKgel FcR-IIIA-NPR TSKgel Protein A-5PW	TOYOPEARL AF-rProtein A HC-650F	•TSKgel FcR-IIIA-NPR can separate antibody according to activity.
	Antibody (Kappa Light)	N/A	TOYOPEARL AF-rProtein L-650F	•For artificial small antibody
	Heparin-Affinity Molecule	TSKgel Heparin-5PW	TOYOPEARL AF-Heparin HC-650M	•For plasma protein with heparin affinity
	Glycoprotein (Diol compound)	TSKgel Boronate-5PW	N/A	•For glycated protein separation
	Protein, Peptide	TSKgel Chelate-5PW	TOYOPEARL AF-Chelate-650M	•Metal ion chelated affnity chromatography (IMAC)
	Nucleotide-Dependent Affinity	N/A	TOYOPEARL AF-Red-650M	•For NADP-dependent enzyme and protein
	Activated Affinity (Ligand Immobilization)	TSKgel Tresyl-5PW	TOYOPEARL AF-Tresyl-650M, AF-Formyl-650M TOYOPEARL AF-Epoxy-650M, AF-Amino-650M TOYOPEARL AF-Carboxy-650M	•Tresy resin requires no additive for reaction
Reversed-Phase Chromatography (RPC)	Protein, Peptide	TSKgel Protein C_4-300 TSKgel ODS-120H, ODS-100V TSKgel Octadecyl-NPR, Octadecyl-2PW, -4PW TSKgel Phenyl-5PW RP	N/A	•Separation of protein and peptide with molecular weight below 50,000. •TSKgel Phenyl-5PW RP shows better resolution and recovery for protein with molecular weight over 30,000.
Normal-Phase Chromatography (NPC, HILIC)	Peptide Oligosaccharide, Polysaccharide	TSKgel Amide-80 TSKgel NH_2-100	N/A	•Gycan analysis depleted from antibody and glycoprotein

Attention

- Most of Tosoh products are not inspected for safety and toxicity. Tosoh products should not be assured as harmless nor non-toxic without any specific warning and/or caution.
- When products or those solution purified by Tosoh products are applied to finished or intermediate products, please fully confirm its safety by yourselves.
- Data described on this presentation are Tosoh data or reference data, but not assured ones.
- Reconfirmation or taking data by yourselves should be highly appreciated according to your environment, conditions and judgement criteria.

Thank you for your attention.

Web Site;

https://www.separations.us.tosohbioscience.com/
https://www.separations.eu.tosohbioscience.com/

Flow-Through Purification of mAb Fraction on TOYOPEARL NH_2-750F

Purification Process	Conditions	Monomer Yield (%)	Aggregate (%)*	HCP Log Reduction	DNA (pg/mg)	Protein A (ppm)
TOYOPEARL NH_2-750F (Flow through)	Start **	N/A	3.1	1.7	0.3	<0.05
	A	95	2.6	2.3	not detected***	<0.05
	B	88	1.0	2.5	not detected***	<0.05

* Aggregate determined according to Bond et al.
** Starting material; TOYOPEARL AF-rProtein A HC-650F capture eluate with 0.1 mol/L glycine
Sample materials (Protein A eluate) were adjusted with Tris to keep pH 6.8 – 7.0, after 30 min incubation for viral inactivation.
*** Less than the lower limit of quantification using resDNASEQ Quantitative CHO DNA Kit (Life Tech)

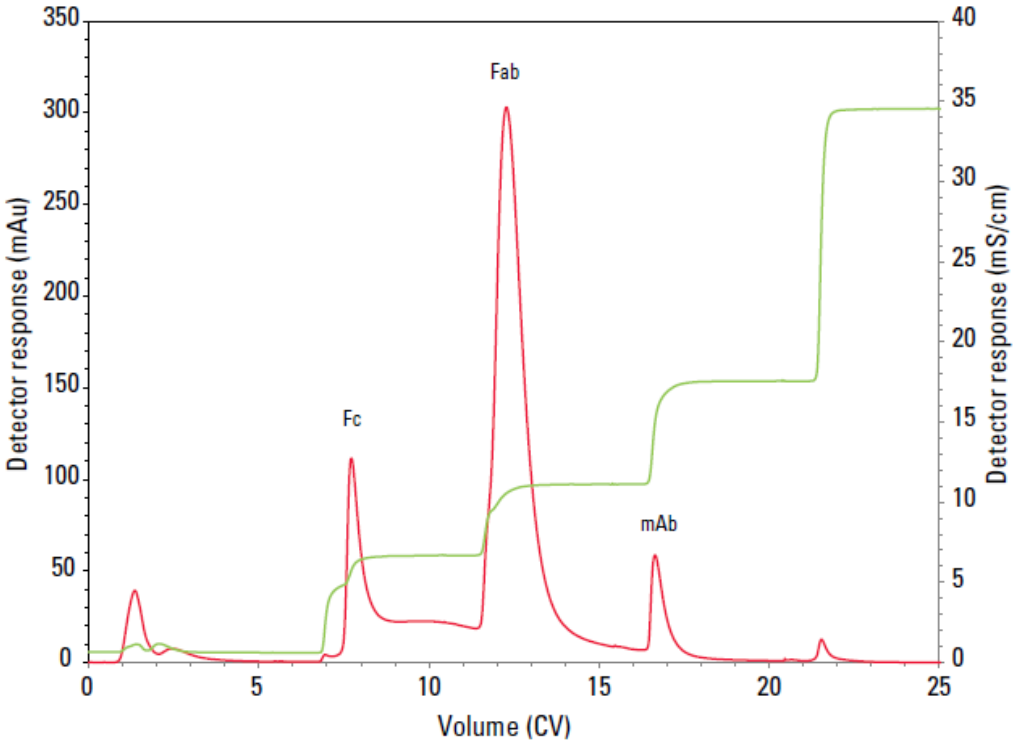
- Improvement of removal of impurities from Protein A eluent by flow-through
- 2.5 Log reduction of HCP
- Host DNA not detected, Protein A leacheable less than 0.05 ppm

Reference; A. Grabski et al., PREP 2016, Poster, Presentation data was slightly modified.

- Flow-through conditions would be more effective at NaCl over 0.15 mol/L in buffer at neutral pH on TOYOPEARL NH_2-750F

Reference; Tosoh Bioscience, Chromatography Application Note

Purification of mAb Digests on Ca^{++}Pure-HA



Resin:	CaPure-HA
Column size:	6.6 mm ID × 15 cm (5.1 mL)
Mobile phase:	A: 5 mmol/L sodium phosphate, 0.5 mmol/L $CaCl_2$, pH 6.5
	B: mobile phase A + 500 mmol/L sodium phosphate
Gradient:	12% B (5CV), 22.5% B (5CV), 40% B (5CV)
Flow rate:	2.57 mL/min (450 cm/hr)
Detection:	UV @ 280 nm, conductivity
Sample:	papain digested mAb, 5.0 g/L
Sample load:	2.0 g/L-resin

Flow-Through (FT) HIC Purification of mAb （resin under development）

・*Condition-1 : 50 mM Phosphate buffer (5 mS/cm)*

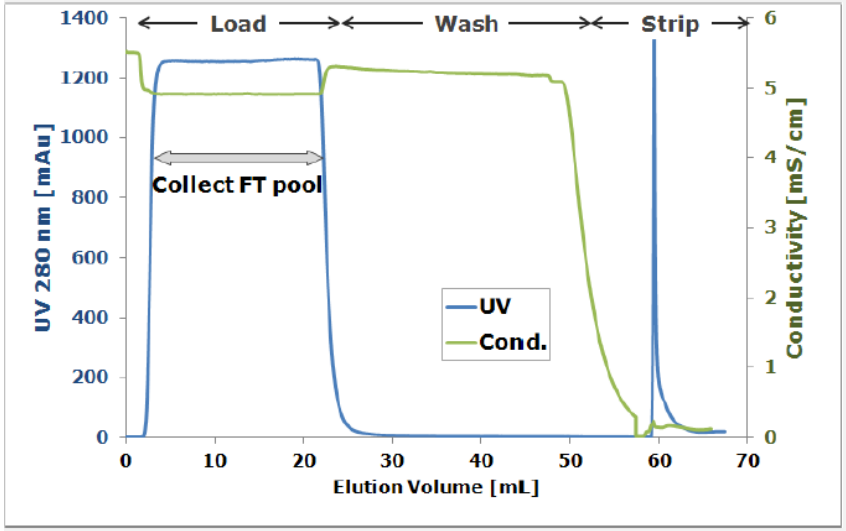


Fig. 2. Chromatogram of Flow through purification

Resin	: Prototype HIC resin
FT buffer	: 50 mM Phosphate buffer, 5 mS/cm
Strip buffer	: Acetate buffer, 0.2 mS/cm
Flow rate	: 0.8 mL/min (Residence time = 2.0 min)
Sample	: 5 g/L Humanized mAb @ FT buffer
Load Vol.	: 20 mL

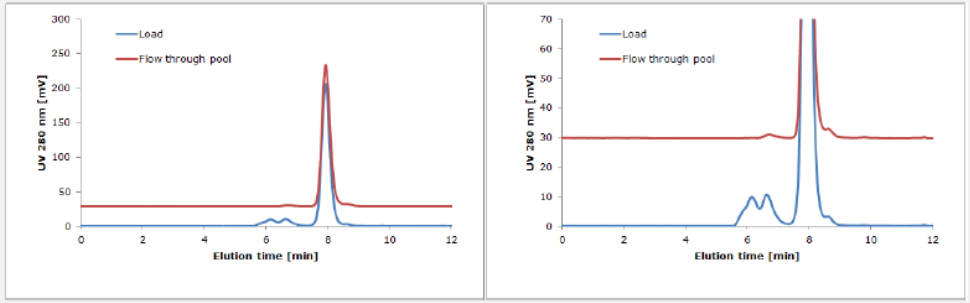


Fig. 3. SEC chromatograms of Load and Flow through pool

Column	: TSKgel® G3000SWXL (7.8 mm I.D. x 30 cm)
Mobile phase	: 100 mM Phosphate buffer + 200 mM Sodium sulfate pH 6.8
Flow rate	: 1.0 mL/min

Table 1. Purity and recovery rate by SEC analysis

	Monomer [%]	Dimer [%]	HMW [%]	Recovery [%]
Load	87.7	6.1	6.2	-
Flow through pool	99.4	0.6	0.0	96