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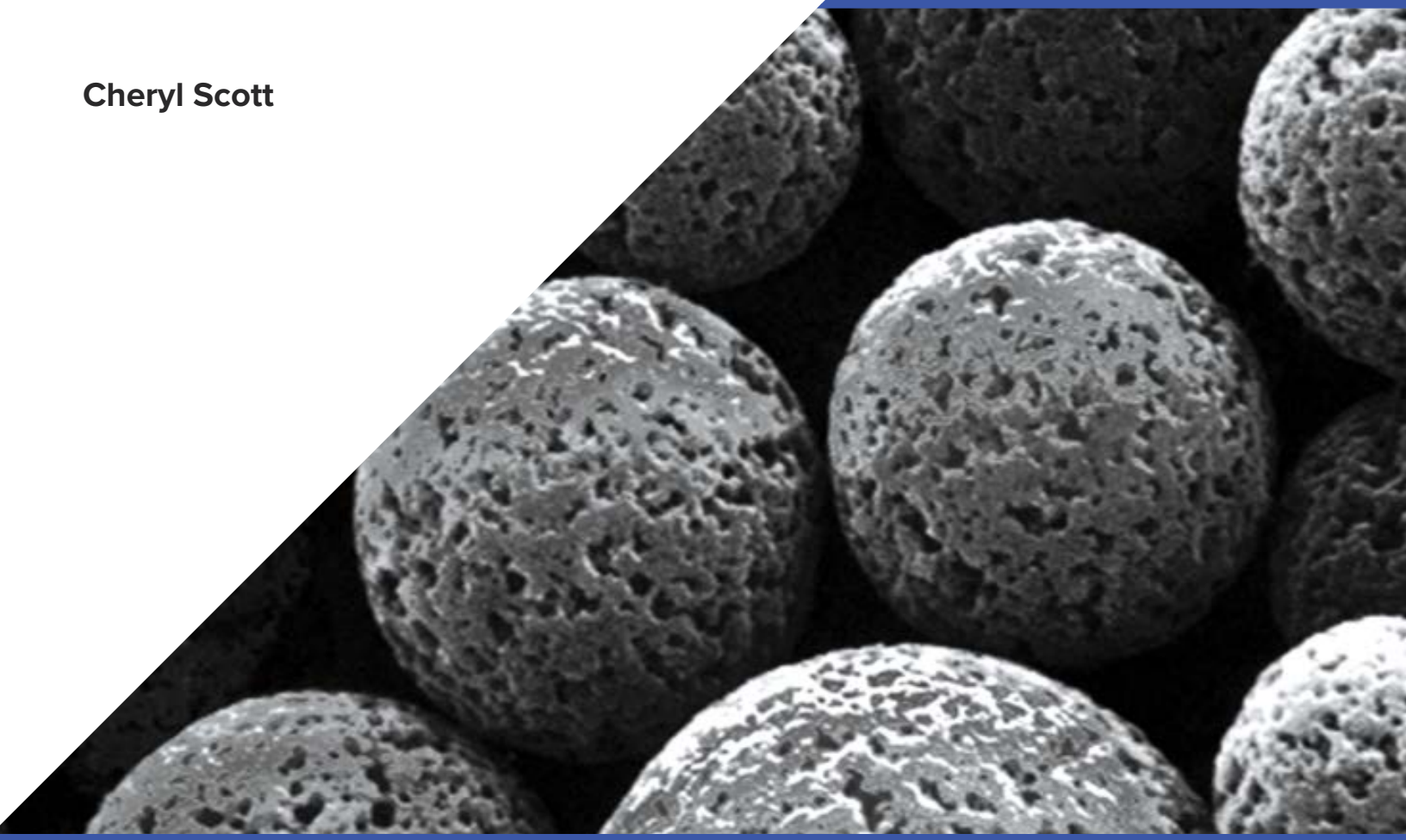
MIXED-MODE CHROMATOGRAPHY

FOR PURIFICATION OF BIOPHARMACEUTICALS

Cheryl Scott



October 2020

Mixed-Mode Chromatography

For Purification of Biopharmaceuticals

by Cheryl Scott

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Successful development and manufacture of biotherapeutics requires maximum product recovery from downstream processes along with effective clearance of product-variants, host-cell proteins and nucleic acids, and process-related impurities. To accomplish this, a number of chromatography steps typically are used for capture, intermediate, and polishing steps based on different modes of separation such as affinity, ion exchange, size exclusion, and hydrophobic interaction.

As upstream workflows intensify, downstream purification processes must keep pace to handle increased protein titers and impurity concentrations that can compromise therapeutic efficacy and/or patient safety, potentially increasing the risk of immunogenicity. Given the need to process more highly concentrated feed streams and remove a range of impurities, a single step chromatographic process for biotherapeutic manufacturing has been infeasible. In recent years, however, mixed-mode (or multimodal) chromatography has emerged as an important purification method for biomolecules that are otherwise difficult to purify with single-mode resins. The technology leverages multiple interactions between analytes and ligands and, as a result, it can selectively clear impurities in a single step for increased product recovery, accelerated throughput, and improved process economics.

Mixed-mode resins use ligands that are capable of at least two modes of interaction with solutes such as hydrophobic, ion exchange, and metal affinity. The interactions between stationary and mobile phases that result from those combinations enhance chromatographic selectivity, facilitating separation efficiencies that are not possible using other chromatography media.

Although intentional use of multimodal technology is relatively new, mixed-mode effects can be observed with single-mode resins such as ion exchangers. In ion exchange resins, a functional group is coupled to base beads with a spacer, and the spacer can contribute a hydrophobic interaction to the overall separation. This phenomenon also was observed in early reports on hydrophobic-interaction chromatography (HIC), through which HIC ligands typically are coupled to chromatographic material by amino groups that are capable of carrying a charge.

Mixed-mode chromatography offers several advantages in downstream processing of biotherapeutics. The multimodal approach can save developers time and money by enabling robust purification of biopharmaceuticals in a single chromatographic step. As a result, this technology offers the potential to purify proteins for which single-mode approaches are insufficient even when used sequentially.

Types of Mixed-Mode Functionalities

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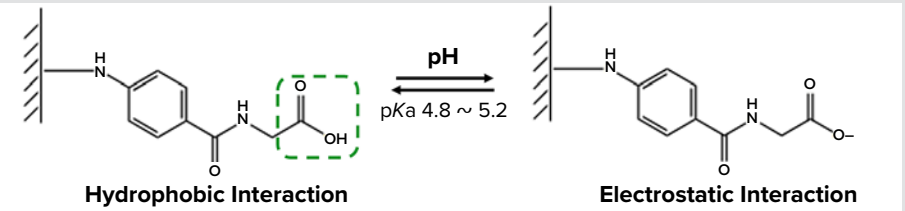
A number of mixed-mode media combining different interaction principles are commercially available (Table 1). Commonly combined modes of interaction are electrostatic interaction (ion exchange) with either metal affinity or hydrophobic-complex formation.

Figure 1: Example of a hydrophobic cation exchanger ligand.



Hydrophobic Ion-exchange Chromatography: Hydrophobic ion-exchange resins combine hydrophobic and electrostatic interactions to deliver simultaneous purity and yield of therapeutic proteins that are difficult to purify by conventional chromatographic approaches.

Hydrophobic cation exchangers typically consist of a ligand with a hydrophobic moiety (e.g., a benzene ring in Figure 1) and a carboxylate functional group. The pH of the buffer used during purification can change the charge state of the ligand. For example, if the pH is lower than the pK_a of the ligand, then the ligand will be neutral and behave like a traditional HIC resin. By contrast, if the buffer is more basic, the carboxyl group of the ligand will be fully ionized and carry a negative charge, causing the resin to function more like a classical cation exchanger.

Figure 2 compares the unique selectivity of hydrophobic cation exchange with a classical cation exchanger using a pair of basic proteins: ribonuclease A (RNase A, pI 8.7) and cytochrome C (pI 10.7). For this comparison, a mixture of the two proteins was loaded on a cation exchanger with a gradient between two buffers: 20 mM sodium acetate with 150 mM sodium chloride at pH 5.5 (buffer A), and 20 mM sodium phosphate with 1 M sodium chloride at pH 7 (buffer B).

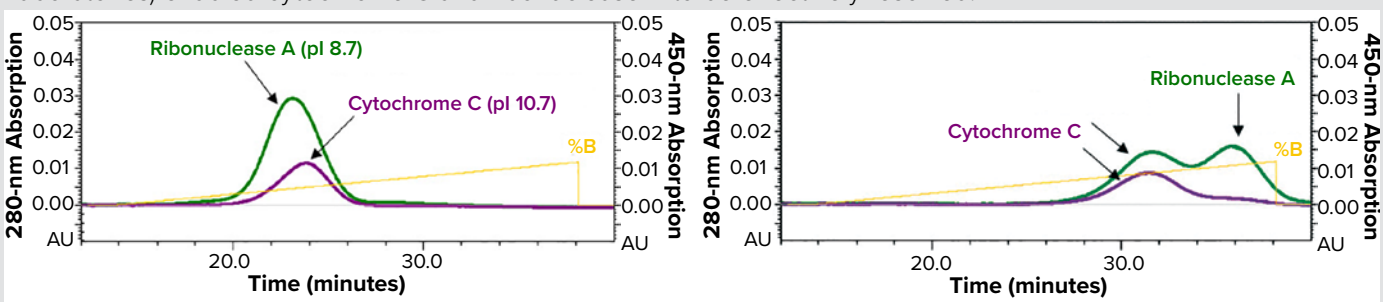
Cytochrome c has a higher pI than RNase A and, as expected, required more salt for elution. However, these two proteins were not separated by classical cation-exchange purification. RNase A contains more nonpolar and neutral amino acids than cytochrome c. When the two proteins were eluted from a hydrophobic cation-exchange resin, the ability of the ligand to interact with both positively charged amino acids and hydrophobic residues provided significant separation.

Hydrophobic anion exchangers have a hydrophobic moiety such as a phenyl group and a positive charge on the ligand (Figure 3A). At neutral pH, basic proteins generally bind less well (because of electrostatic repulsion) than do acidic proteins or species such as

Table 1: Mixed-mode chromatography media combine different interaction principles.

Mode	Principle
Anion exchange	Electrostatic interaction
Cation exchange	Electrostatic interaction
Hydrophobic interaction	Hydrophobic complex formation
Hydrogen bonding	Hydrogen bonding
Metal affinity	Metal chelation
Phosphate-specific interactions	Electrostatic interaction

Figure 2: Comparison of a classical cation exchanger (LEFT) and a hydrophobic cation exchanger (RIGHT) for the separation of two basic proteins. Use of the hydrophobic cation exchanger (Nuvia cPrime resin from Bio-Rad Laboratories) enabled cytochrome C and ribonuclease A to be effectively resolved.



nucleic acids, endotoxins, and some viruses. Therefore, the acidic molecules are likely to bind stronger than the basic ones. Figure 3B provides an example. Both buffer pH and conductivity can be manipulated to achieve desired selectivity and recovery of target molecules.

Figure 3A: Structure of a hydrophobic anion exchanger.

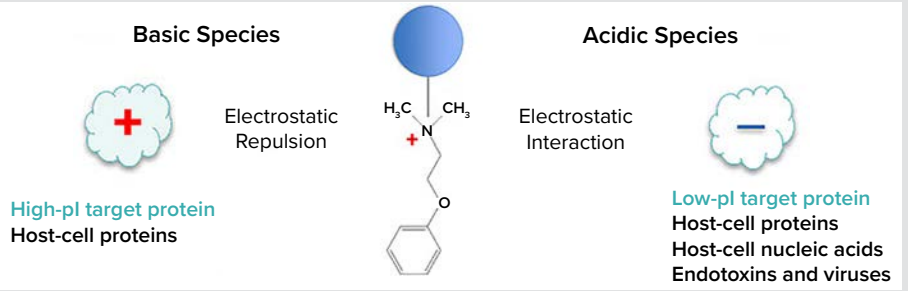
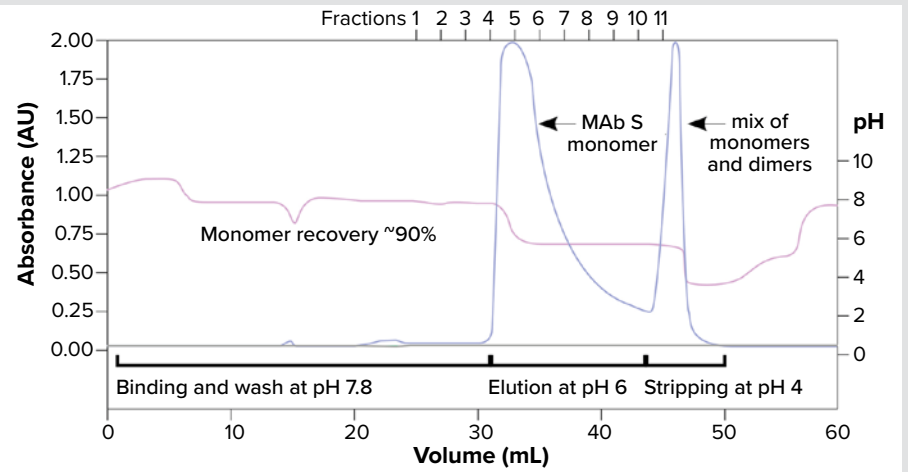


Figure 3B: Purification of MAb S (pH ~6.9) on a hydrophobic anion-exchange resin



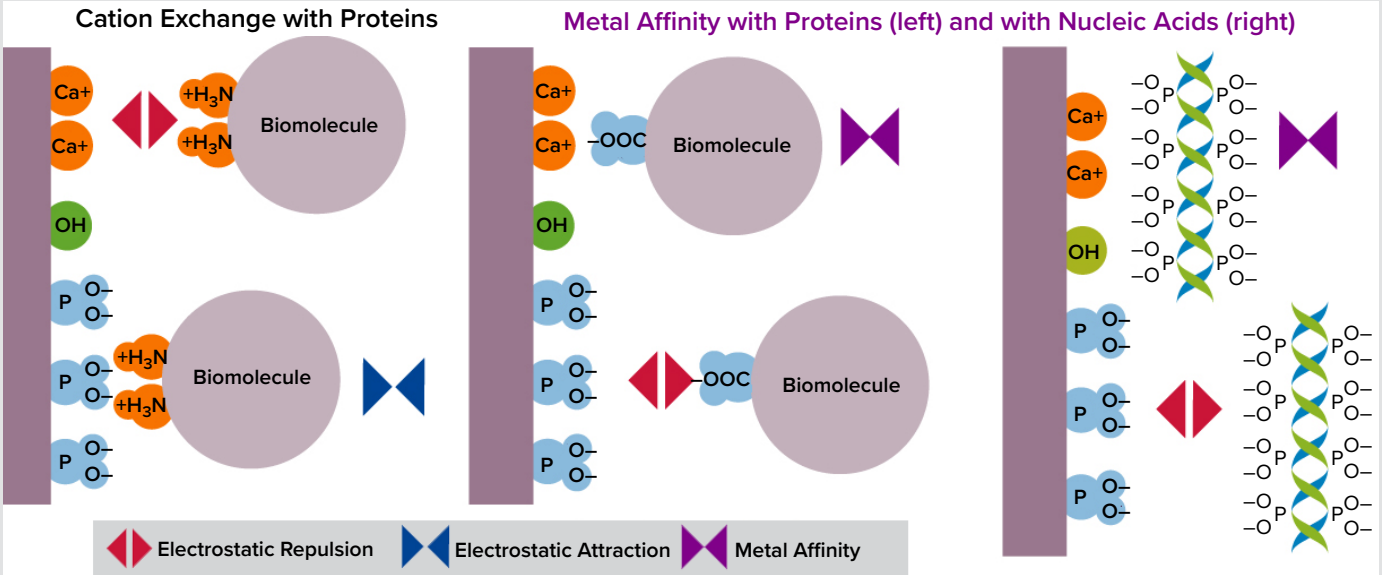
Hydroxyapatite Chromatography: The base matrix used in hydroxyapatite chromatography (HAC) is mixed mode by nature, combining ion exchange and metal affinity. As a result, it offers unique separation properties with unparalleled selectivity and resolution and has the potential to separate proteins otherwise shown to be homogeneous by electrophoretic and other chromatographic techniques.

Hydroxyapatite — $Ca_{10}(PO_4)_6(OH)_2$ — is a form of calcium phosphate with sets of five positively charged calcium pairs (C-sites) and pairs of phosphate triplets (P-sites) arranged in a repeating geometric pattern. Carboxyl groups on biomolecules are attracted to the C-sites and repelled by the P-sites; amino groups are attracted to the P-sites and repelled by the C-sites (Figure 4A). Cation-exchange interactions can be disrupted by adding neutral salts such as sodium chloride (NaCl) or buffering species such as sodium phosphate to the mobile phase. These interactions also weaken with increasing pH.

Meanwhile, calcium affinity occurs through interactions with carboxyl clusters and/or phosphoryl groups on proteins or other molecules such as nucleic acids. Those groups are repelled simultaneously by the negative charge of the phosphate groups. Dominantly acidic proteins (e.g., albumin) bind chiefly by metal-affinity interaction, whereas dominantly basic proteins (e.g., immunoglobulins) bind chiefly through cation-exchange interactions. Elution is induced with elevated concentration of sodium phosphate, which in this case serves as both a buffer and an eluting agent. Figure 4B illustrates an example HAC method, and the "General Guidelines" box (next page) suggests an approach to method development.

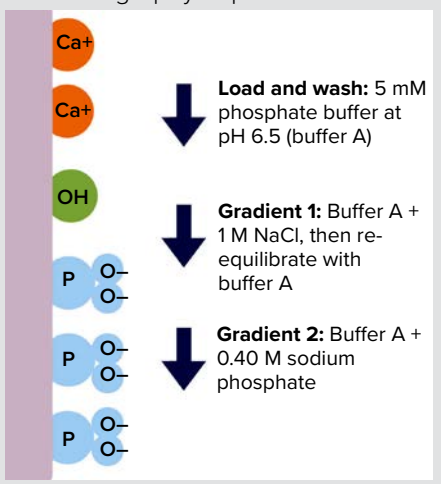
Packing Mixed-Mode Chromatography Columns: Generally, hydrophobic ion-exchange chromatography resins can be packed into columns using standard methods because they are compressible. By contrast, HAC resins are incompressible and have high specific

Figure 4A: Hydroxyapatite is unique in that the base matrix itself provides multiple modes of interaction



gravity, a rapid settling rate, and sensitivity to mechanical shear. Those variations from traditional compressible resins must be considered when designing HAC column-packing protocols (see box, right).

Figure 4B: Example hydroxyapatite chromatography separation method



Advantages Over Classical Ion Exchange

Ion-exchange chromatography has been a mainstay in biotherapeutics purification; mixed-mode resins offer several advantages over it, however. Mixed-mode technology enables selective clearance of impurities in a single unit operation, which can accelerate downstream processes and increase yield and purity. Protein activity and yield are improved through advantageous use of charge–charge repulsion between ligand and protein (**1**).

Multimodal resins that include electrostatic interaction also help users overcome a key limitation of single-mode ion exchange, which is the inability to load high-conductivity feed materials. Cell culture harvests often contain high levels of salt, which compromise binding of target proteins to chromatography media (**2**). With classical ion-exchange chromatography, the feed typically must be prepared in a low-conductivity buffer to facilitate binding. Elution from mixed-mode resins can be achieved by changing buffer conductivity and/or pH (Figure 5):

- If the subsequent step is ion exchange, then a low-conductivity buffer at a different pH will be used

CHT Column Packing Dos and Don'ts

Do ensure column leveling.

Do use plastic paddles for manual mixing and low-shear hydrofoil impeller for automated mixing.

Do use only a diaphragm pump for media transfer.

Do leave a headspace of 1–5 mm.

Do restrict settling time to <10 minutes for optimal packing.

Don't compress.

Don't include defining/decanting steps.

Don't allow mechanical shear or compression.

Don't perform buffer upflow with packed CHT (except during unpacking).

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- If the next step is hydrophobic interaction, high-salt elution is used.

Such flexibility facilitates the transition to the next purification step. Moreover, the salt tolerance of multimodal resins often allows for direct loading of feed streams at high conductivity. Hydrophobic ion-exchange resins also work within a large design space for binding and elution, allowing for development of highly robust methods in a commercial manufacturing setting (**3**). With hydrophobic ion exchange, a protein may interact with the chromatography media by alternative modes in response to buffer conductivity changes. That provides a larger window for buffer pH manipulation and conductivity variation to achieve desired binding and elution selectivities.

Method Development

Multimodal matrices are designed to resolve target proteins and impurities, which are complex molecules themselves. As such, one or more interaction modes can be involved in the binding or elution of target proteins. The complexity of these interactions complicates predicting the behavior of a protein based on its pI or amino acid sequence. A relatively simple design of experiment (DoE) approach can be used to determine the effects of buffer pH and conductivity on selectivity, recovery, and robustness of protein purification. DoE also can help end users define a design space for their target protein purification processes.

Binding and elution mechanisms of hydrophobic ion-exchange resins are affected chiefly by buffer pH and salt. Changes in ionic strength also induce and/or optimize elution, and the final defined method often will be a combination of a change in pH and/or a change in salt concentration. In some cases, though, a mobile-phase modifier or a different salt in the elution buffer might be necessary for optimal results.

Guidelines for HAC Method Development

Proteins with Unknown pI

Binding occurs at pH 6.5–7.5; an increase in pH stabilizes CHT; add phosphate ≥5 mM.

Elution: With phosphate and/or NaCl, gradient or step elution, add phosphate ≥5 mM.

Basic Proteins

Binding: Bind primarily to phosphate sites by cation exchange. Calcium affinity may also play a role. Consider flow-through for nonbinding targets.

Elution: NaCl elution might provide more selectivity than phosphate. Try 0–1 M NaCl gradient and convert to step. Low levels of phosphate eliminate weak Ca^{2+} interactions. Try 5 mM, 10 mM, and then 15 mM.

Acidic Proteins

Binding: Bind primarily to calcium sites by metal affinity. Binding may be possible in high-salt conditions. Consider flow-through for nonbinding targets.

Elution: Phosphate alone will elute target from calcium as well as phosphate sites. Try 0–400 mM phosphate gradient and convert to step. Elution could be achieved with or without NaCl.

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Figure 5: General method development rationale for hydrophobic ion exchangers.

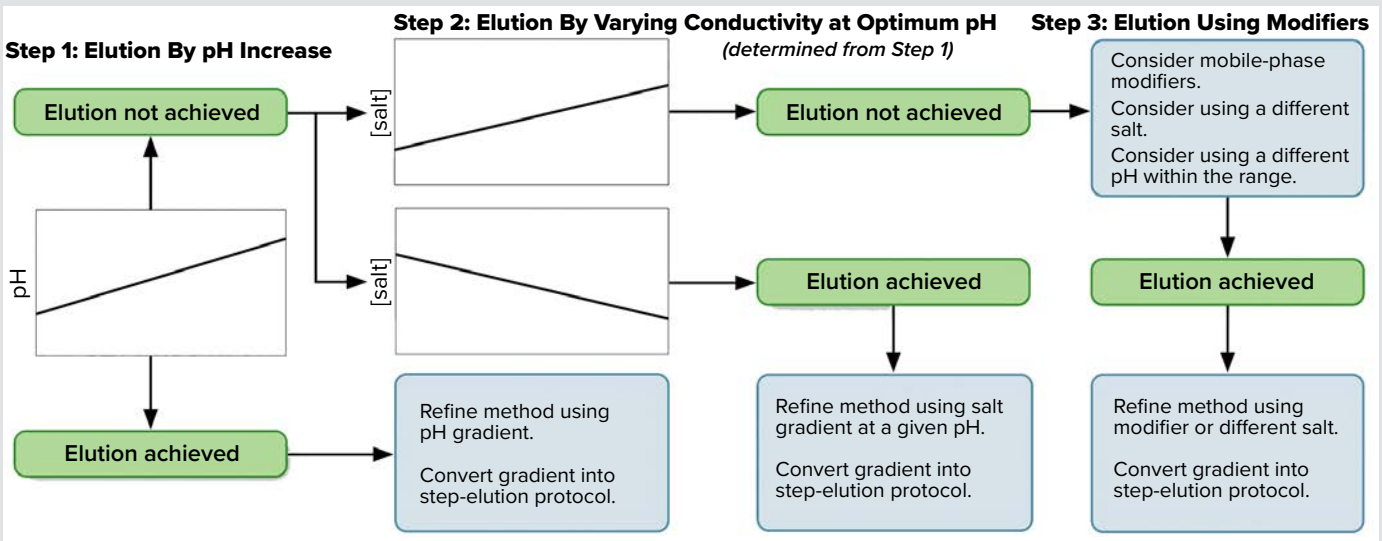
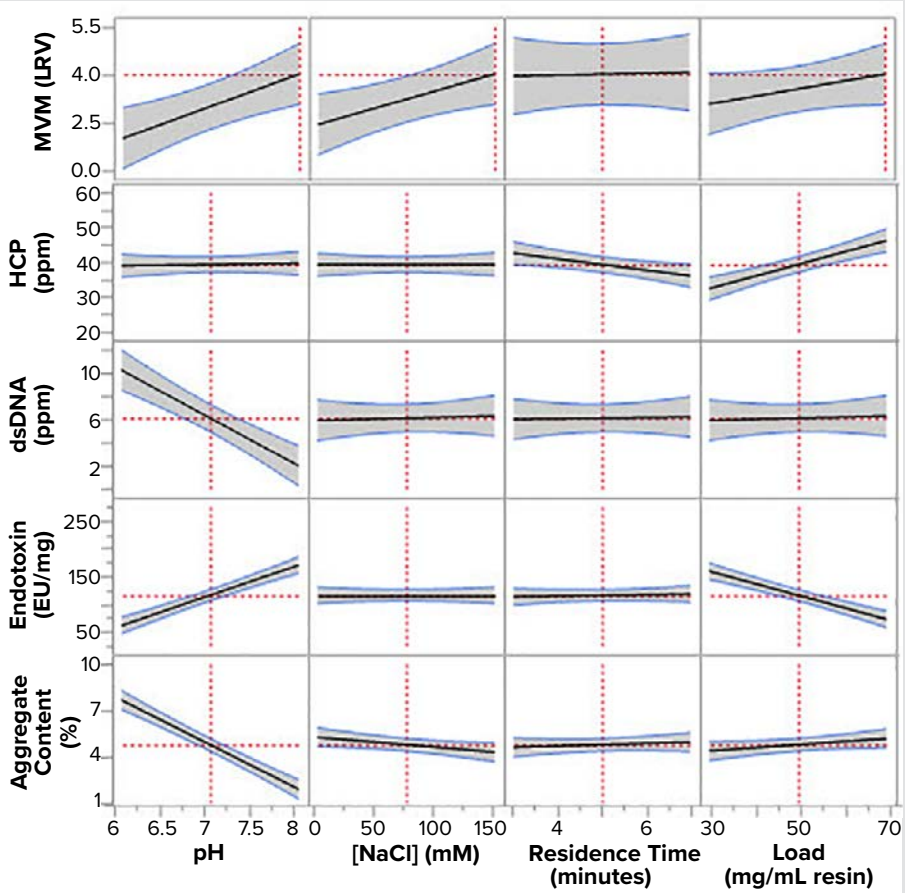


Figure 5 provides a general method development rationale (**4**). In most cases, conducting a DoE study to identify optimal binding and elution conditions will yield an effective, robust, and scalable method. A packed column often is used to verify and optimize the resulting chromatography conditions.

Figure 6 summarizes DoE results from a method-development exercise for flow-through polishing of a monoclonal antibody (MAb, pI 9.0) using a hydrophobic anion exchanger. DoE was used to identify the chromatography conditions that would provide efficient clearance of impurities, providing >90% recovery of this pI 9.0 MAb. A spin column format was used to perform the DoE, and 5-mL Foresight prepacked chromatography columns (Bio-Rad) were used to verify the DoE results.

Figure 6: Use of DoE in development of a process for impurity clearance with a hydrophobic anion exchanger.



Buffer pH had the greatest effect on clearance efficiency for host-cell proteins (HCPs), double-stranded DNA, endotoxins, and aggregates of the target protein, as well as log reduction of minute virus of mice (MVM). In terms of salt tolerance, NaCl concentration did affect the log reduction value (LRV) for MVM. Additionally, the load level affected HCP and MVM clearance. According to the DoE results, the greatest log reduction of MVM occurred at pH 8 with 150 mM NaCl in the buffer, whereas recovery of the target antibody monomer was highest at pH 6 (Figure 7). Because the goal of the process was to ensure virus reduction, the purification method ran at pH 8 with 10 mM Na_2SO_4 and 150 mM NaCl, which provided an MVM log reduction of 4.2 and a target monomer recovery at >90%.

Applications

Given its ability to enhance separation of target molecules from impurities, multimodal chromatography has become an essential tool for downstream purification of biotherapeutics. Both hydrophobic ion exchange and HAC have been used for a wide range of applications in development and manufacturing of biopharmaceutical products (Table 2).

Recombinant Protein Capture: Purification of recombinant proteins, especially those lacking affinity "handles," can require

Table 2: Mixed-mode chromatography can be used to deliver robust purification of biotherapeutics and a wide range of contaminants present in the manufacturing process.

Application	References
Monoclonal antibodies	5, 7, 20–22
Charged antibody isomers	6
Antibody fragments	7
Recombinant proteins	7, 8
Viral particles	9
Recombinant adenoassociated (AAV) virus	10
Recombinant adenovirus vectors	24, 25
Vaccines	11
Isozymes	12
Supercoiled DNA from linear duplexes	13
Single-stranded from double-stranded DNA	13, 25

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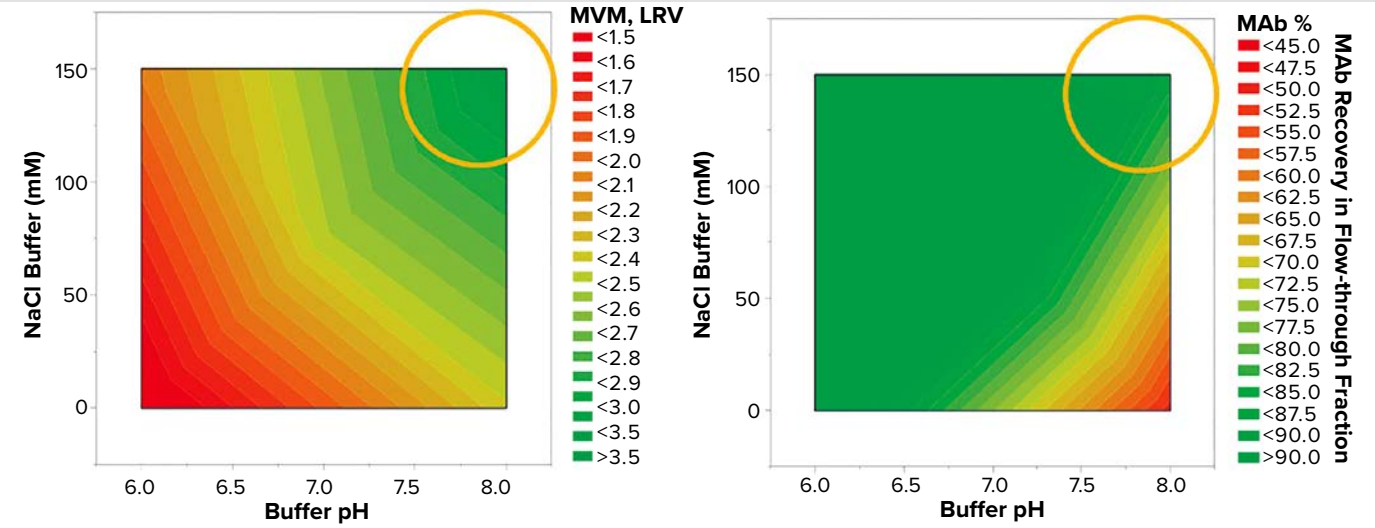
Figure 7: Balancing virus reduction with target monomer recovery.



multiple chromatography steps and complicated feed-stream conditioning. These factors lead to high production costs and relatively low recovery rates and are exacerbated by low protein expression, pH sensitivity, and limited protein stability. Mixed-mode is well-suited for purifying such proteins, and its gentle chromatography conditions allow for maximum protection of target protein integrity (**14**).

Antibody Purification, Aggregate, Endotoxin, and DNA Removal: Impurities that must be removed by downstream purification processes include aggregates of the target protein, host-cell DNA, and bacterial endotoxins. The presence of aggregates can increase the risk of immunogenicity for patients and/or give a product different bioactivity, potency, and pharmacodynamic/pharmacokinetic properties compared with the correct monomer presentation. The presence of host DNA in a feedstream can interfere with purification, whereas endotoxins pose a serious safety risk.

Techniques that use ion-exchange or HIC resins can induce formation of aggregates or multimers through increased protein concentrations or the salt/pH requirements for elution. Multimodal resins offer a robust approach to removing these impurities while minimizing the risk of inducing aggregates.

HAC is an excellent choice for intermediate or final polishing in biotherapeutic purification workflows. For example, it can improve immunoglobulin G (IgG) purification quality, efficiency, yield, and process economics because of its large capacity for high-titer feed streams. Diverse selectivity enables robust removal of aggregates and fragments and one-step clearance of multiple process-related impurities including DNA, HCPs, endotoxins, viruses, and protein A from a common affinity capture step (**15**). Table 3 lists impurities typically found in a bioprocess feed stream with observed clearances by HAC. It has been used for aggregate removal, which could not be achieved by either ion exchange or HIC alone, and it also is well-

Table 3: Observed clearances of process feed stream impurities using HAC (CHT Ceramic Hydroxyapatite media, Bio-Rad Laboratories).

Impurity	Method	Observed Clearance*
Aggregates	HP-SEC	1–2 logs (<0.03%)
Protein A	ELISA	1–2 logs
CHO protein	ELISA	2 logs
DNA	PicoGreen assay	>3 logs
Endotoxin	LAL assay	>4 logs
XMuLV	Infectivity or PCR	>4 logs
MVM	Infectivity	2 logs

* NaCl gradients at constant phosphate concentration

CHO = Chinese hamster ovary (cells)

ELISA = enzyme-lined immunosorbent assay

LAL = *Limulus* amebocyte lysate

PCR = Polymerase chain reaction

MVM = minute virus of mice

XMuLV = xenotropic murine leukemia virus

PicoGreen is a trademark of Thermo Fisher Scientific

suited to endotoxin depletion (**16–18**). Endotoxins are highly acidic, containing many phosphoryl and carboxyl residues that have a strong affinity to the calcium ions in HAC resins.

Hydrophobic cation-exchange media provide robust recovery of MAbs at high flow rates in commercial manufacturing settings (**19**). With a higher affinity for full-length antibodies relative to process impurities and by-products, these resins are ideal for the polishing step in MAb workflows. This approach is demonstrated to be highly effective in clearing DNA contamination from a CHO cell culture harvest in a final polishing step for a MAb product (**20**).

Hydrophobic cation-exchange resins can also be used for purification of recombinant protein therapeutics that lack affinity handles. In a workflow without affinity capture, the multimodal resin provides maximal clearance of HCPs and double-stranded DNA from host cells with minimal aggregate content (**21**).

Similarly, hydrophobic anion-exchange media can be used to purify both acidic and basic antibodies in either bind-elute or flow-through mode, simultaneously delivering high purity and good yield. As with other multimodal resins, this salt-tolerant resin minimizes the need for feed-stream dilution before column loading or transfer to a subsequent column in a workflow. The broad mixed-mode design space in terms of pH and salt enables use of conditions required for difficult-to-purify proteins, including constructs that lack affinity handles (**22**).

Virus Purification: Conventional techniques for mammalian virus purification — either for vaccine production or biological studies — can produce material of varying quality and quantity, often with a significant loss of particle infectivity. By contrast, HAC is a simple and scalable approach for separation of virus particles with diverse sizes and from different families. The method delivers a concentrated preparation of highly active and pure viruses (**23**).

Purification of adenoviruses, the vehicle of choice for gene therapies, warrants use of hydrophobic ion-exchange resins. The large virus particles (165 MDa, 0.1-µm diameter) combined with thousands of protein subunits and a lack of stability at low pH create a challenge for conventional approaches to purification such as filtration, density gradients, and ultracentrifugation. Mixed-mode chromatography enables high-quality purification of adenoviruses in two steps to yield active, concentrated vectors with purity, HCP levels, and DNA contamination levels comparable to those of existing clinical-grade products (**24**).

Viral Clearance: The possible presence of adventitious agents and virus contaminants from source materials necessitates removal or inactivation during biotherapeutics manufacturing. As described above, mixed-mode chromatography can provide robust viral clearance even for MVM, which is otherwise resistant to inactivation and often the most difficult adventitious virus to remove during bioprocessing.

Advantages Over Classic Ion Exchangers

Mixed-mode resins offer

- higher resolution compared to single mode interactions
- improved yield
- consolidated purification steps due to orthogonal interaction modes
- a large design space for protein interaction
- minimal feed manipulation prior to binding
- mild operating conditions which preserve activity.

Discover our online purification library

Our digital hub has a variety of resources to support your purification at each phase of biotherapeutic development, complete with resin information, technical videos and tutorials, application notes, and strategy guides for method development and purification success. Register to attend or view webinars focused on bioprocessing challenges and solutions and meet our purification experts at the next industry event.

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A Powerful Alternative

Downstream processing is an essential part of all biomanufacturing workflows. Robust recovery of target biological molecules is essential; impurity clearance is necessary to help ensure therapeutic efficacy and patient safety. Although classical or "single-mode" chromatography resins are used throughout the biopharmaceutical industry to achieve these objectives, shortcomings to that approach can be overcome using multimodal chromatography. The combined action of multiple functionalities on the ligands of multimodal resins enhances separation of target molecules from impurities and offers significant advantages in purification of biotherapeutics:

- Resolution of biomolecules that appear homogeneous using other chromatographic methods
- Selective clearance of impurities in a single step, which can accelerate downstream processes and improve overall yield and purity
- Intuitive development of robust methods for manufacturing, with a large design space for determining acceptable binding and elution conditions
- Loading of feed streams at high conductivity due to robust salt tolerance
- Preservation of target-molecule activity through mild operating conditions
- Simplifying bioprocesses by reducing purification steps and minimizing feed manipulation.

Given the compelling benefits of mixed-mode chromatography, this approach should be considered by anyone developing methods for impurity clearance and purification of biotherapeutics.

References

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1 Simmonds RJ, Yon RJ. Protein Chromatography on Adsorbents with Hydrophobic and Ionic Groups: Purification of Human Erythrocyte Glycophorin. *Biochem. J.* 163(2) 1977: 397–400; https://dx.doi.org/10.1042%2Fbj1630397.

2 Halan V, et al. Multimodal Chromatography for Purification of Biotherapeutics: A Review. *Curr. Peptide Prot. Sci.* 20(1) 2019: 4–13; https://doi.org/10.2174/1389203718666171020103559.

3 Burton SC, Harding DR. Hydrophobic Charge Induction Chromatography: Salt Independent Protein Adsorption and Facile Elution with Aqueous Buffers. *J. Chromatogr.* 814(1–2) 1998: 71–81; https://doi.org/10.1016/s0021-9673(98)00436-1.

4 *Using Design of Experiments (DOE) with Nuvia cPrime to Elucidate Capture Conditions of a Recombinant Protein.* Bio-Rad Protein Purification Bulletin #7128. https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_7128.pdf.

5 Cummings LJ, Frost RG, Snyder MA. Monoclonal Antibody Purification By Ceramic Hydroxyapatite Chromatography. *Meth. Mol Biol.* 1131, 2014: 241–251; https://doi.org/10.1007/978-1-62703-992-5_15.

6 Saito M, Yoshitake T, Okuyama T. Separation and Analysis of Charged Isomers of Monoclonal Immunoglobulin G By Ceramic Hydroxyapatite

Chromatography. *Prep. Biochem. Biotechnol.* 46(3) 2016: 215–221; https://doi.org/10.1080/10826068.2014.995811.

7 Moro A, et al. Single-Step Purification of Pepsin-Derived Monoclonal Antibody Fragments from Crude Murine Ascitic Fluids By Ceramic Hydroxyapatite High-Performance Liquid Chromatography. *J. Biochem.* 144(6) 2008: 733–739; https://doi.org/10.1093/jb/mvn128.

8 Islam T, et al. A Novel Strategy for the Purification of a Recombinant Protein Using Ceramic Fluorapatite-Binding Peptides As Affinity Tags. *J. Chromatogr. A.* 1339, 2014: 26–33; http://dx.doi.org/10.1016/j.chroma.2014.02.079.

9 Masuda T, et al. Purification of Virus Particles By Ceramic Hydroxyapatite Chromatography on Microfluidic Chip. *14th IEEE International Conference on Nanotechnology, Toronto, ON, 2014*: 131–134; https://doi.org/10.1109/NANO.2014.6968054.

10 Qu W, et al. Calcium-Ion-Modulated Ceramic Hydroxyapatite Resin for the Scalable Purification of Recombinant Adeno-Associated Virus Serotype 9. *J. Chromatogr. B* 990, May 2015:15–22; https://doi.org/10.1016/j.jchromb.2015.03.003.

11 Saito M, Kurosawa Y, Okuyama T. Scanning Electron Microscopy-Based Approach to Understand the Mechanism Underlying the Adhesion of Dengue Viruses on Ceramic Hydroxyapatite Columns. *PLoS One* 8(1) 2013: e53893; https://doi.org/10.1371/journal.pone.0053893.

12 Pentia DC, et al. Purification of PDE6 Isozymes From Mammalian Retina. *Methods in Molecular Biology, Vol. 307: Phosphodiesterase Methods and Protocols*. Lugnier C, Ed. Humana Press: Totowa, NJ, 2005; https://doi.org/10.1385/1592598390.

13 Alberin CS, Franklin SG. *Plasmid Purification Using CHT Ceramic Hydroxyapatite Support*. Bio-Rad Tech Note 2731; https://www.bio-rad.com/webroot/web/pdf/psd/literature/BULLETIN_2731.pdf.

14 Frisch D, et al. *Efficient Purification of a Recombinant Protein from* Esherichia coli *Fermentate with Nuvia aPrime 4A Resin*. Bio-Rad Bulletin 7302; https://www.bio-rad.com/webroot/web/pdf/ps/literature/Bulletin_7302.pdf.

15 Gagnon P, et al. A Ceramic Hydroxyapatite–Based Purification Platform: Simultaneous Removal of Leached Protein A, Aggregates, DNA, and Endotoxins from MAbs. *BioProcess Int.* 4(2) 2006: 20; http://www.bio-rad.com/webroot/web/pdf/ps/literature/Bulletin_RP0033.pdf.

16 Gagnon P, Beam K. Antibody Aggregate Removal By Hydroxyapatite Chromatography. *Curr. Pharm. Biotechnol.* 10, 2009: 440–446; https://doi.org/10.2174/138920109788488833.

17 Morrison CJ, Gagnon P, Cramer SM. Purification of Monomeric MAb from Associated Aggregates Using Selective Desorption Chromatography in Hydroxyapatite Systems. *Biotechnol. Bioeng.* 108, 2011: 813–821; https://doi.org/10.1002/bit.22971.

18 Kandelwal P. *Practical Guide: Selecting the Optimal Resins for Endotoxin Depletion in Process Purification*. Bio-Rad Bulletin #6813; http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6813.pdf.

19 Kandelwal P. *Practical Guide: Selecting the Optimal Resins for Monoclonal Antibody Process Purification*. Bio-Rad Bulletin #6875; http://www.bio-rad.com/webroot/web/pdf/psd/literature/Bulletin_6875.pdf.

20 Kandelwal P. *Practical Guide: Selecting the Optimal Resins for Removal of DNA Contamination During Process Purification*. Bio-Rad Bulletin #6681; http://www.bio-rad.com/webroot/web/pdf/psd/literature/Bulletin_6881.pdf.

21 He X, Snyder M. *A Purification Strategy for Clinical-Grade Monoclonal Antibody Using Hydrophobic Cation Exchange Chromatography*. Bio-Rad Tech Note #6241; http://www.bio-rad.com/webroot/web/pdf/ps/literature/Bulletin_6241A.pdf.

22 He X, et al. *Purification of Antibodies with Nuvia aPrime 4A Hydrophobic Anion Exchange Resin*. Bio-Rad Bulletin #7193; https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_7193.pdf.

23 Kurosawa Y, et al. *Mammalian Virus Purification Using Ceramic Hydroxyapatite*. Bio-Rad Tech Bulletin #6549; http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6549.pdf.

24 Khandelwal P. *Practical Guide: Selecting the Optimal Resins for Adenovirus Process Purification*. Bio-Rad Bulletin #6807; http://www.bio-rad.com/webroot/web/pdf/psd/literature/Bulletin_6807.pdf.

25 Aberin C, Franklin S. *Plasmid DNA Adsorption Behavior on CHT Ceramic Hydroxyapatite Type II Support*. Bio-Rad Bulletin #2946; https://www.bio-rad.com/webroot/web/pdf/psd/literature/Bulletin_2946.pdf.

About the Author

Cheryl Scott *is cofounder and senior technical editor of* BioProcess International, *part of Informa Connect, PO Box 70, Dexter, OR 97431; 1-646-957-8879; cheryl.scott@informa.com.*

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Bio-Rad Experts Discuss Multimodal Chromatography

Xuemei He

William Rushton

Mark Snyder

Daniel Yoshikawa

Xuemei He (R&D manager, chromatography media chemistry): With a PhD in biological chemistry, He has over 20 years of experience in biomolecule separation and characterization. Her laboratory concentrates on development of chromatography media for process-scale production of biopharmaceuticals, with an emphasis on viral safety and removal of residual process- and product-related impurities during polishing stages. She also provides application support on chromatography media screening, method development, and process optimization based on molecular interactions and mechanistic understanding.

William Rushton (process chromatography support scientist): Rushton has over 23 years of industry experience in downstream process development. Before Bio-Rad, he spent 10 years in the process development group at Centocor (a wholly owned subsidiary of Johnson & Johnson). In 2007 he joined Charles River Laboratories as manager of its process evaluation/validation group, performing viral clearance studies for pharmaceutical and biotechnology clients. In 2009, he moved to Auxilium Pharmaceuticals as a senior scientist in process development. He holds a master of science degree in biomedical sciences from Philadelphia College of Osteopathic Medicine and a bachelor of science in biology from St. Joseph's University.

Mark Snyder (R&D applications manager, process chromatography): Snyder earned his bachelor of science degree at the Massachusetts Institute of Technology and his doctorate in biochemistry at the University of California, Berkeley. He has been responsible for many developed processes, among them Bayer's licensed recombinant factor VIII purification process. He is experienced and widely published in process troubleshooting, optimization, and validation.

Daniel Yoshikawa (global product manager, process chromatography): Yoshikawa received his doctorate in pharmacology from the University of Rochester, New York. He is responsible for bringing Bio-Rad's mixed-mode process resins to market while connecting with life-science researchers to stay abreast of industry needs.

The Conversation

BPI: To what extent have you seen uptake of multimodal chromatography by the biopharmaceutical industry?

Rushton: Many of my customer interactions involve some discussion of mixed-mode resins. This has increased significantly over the past two to three years.

Snyder: I think that interest is continuing to grow. Uptake was slow for a while because of inexperience with multimodal ligands and also because of the (at least perceived) need for design of experiments (DoE).

He: Customers' initial hesitation most likely comes from the term mixed-mode itself. Most scientists working on protein purification are not trained as organic chemists, and sometimes they are frustrated by the chemical structures of the ligands. From presenting the unique selectivity of mixed-mode resins and the use of DoE for method development, I have seen increasing acceptance.

Yoshikawa: I agree that uptake was slow when the technology was first introduced, but acceptance has increased swiftly in recent years. In fact, according to Strategic Directions International, mixed-mode is among the fastest growing technologies (https://strategic-directions.com/product/laboratory-process-scale-purification-pharmabiotech-revolution).

BPI: Which applications are generating the greatest interest — and why?

Rushton: In gene therapy and purification of larger molecules, process engineers need to be able to separate empty and full capsids.

Snyder: Mixed-mode is attractive for those molecules that don't have a readily available purification platform.

He: Some people are looking for simple step transitions without extensive adjustment of feed-stream conductivity, with selectivity that cannot be achieved by single-mode chromatography media (e.g., species of very similar pI), or simply to reduce purification steps. Multimodal resins generally show better salt tolerance than ion exchangers and better

Bio-Rad Experts Discuss Multimodal Chromatography

target recovery than hydrophobic-interaction resins.

Yoshikawa: Purifications of antibody-related molecules are among the greatest interests. These include classic monoclonal antibodies (MAbs) and derivatives such as antibody–drug conjugates, bispecifics, fragments, and others. The industry is turning toward mixed-mode resins because those molecules are becoming more and more difficult to purify.

Links for Further Reading

• On CHT media and virus purification (https://www.bio-rad.com/webroot/web/pdf/psd/literature/Bulletin_6790.pdf)

• On adenovirus purification (https://www.bio-rad.com/webroot/web/pdf/psd/literature/Bulletin_6807.pdf).

BPI: What are some considerations that a first-time user of multimodal chromtaography should keep in mind?

Rushton: Users need to understand that you cannot think of a traditional ion-exchange (IEX) step when working with mixed-mode resins. With mixed-mode resins, you might be able to load at a much higher conductivity than with a traditional IEX and still be able to separate your product from its impurities.

Snyder: First, mixed-mode is not a black box. It's just a combination of what you already know: IEX and hydrophobic-interaction chromatography (HIC). Second, because each ligand is different, it's a virtual certainty that your target protein and/or impurities will behave differently on each one.

He: I always suggest that users start with DoE instead of trying to use the predicted pI or hydrophobicity of a protein to guide their method development.

Yoshikawa: Do not be intimidated by mixed-mode. The technology simply uses what you already know from traditional single-mode chromatography modes. You're simply combining multiple modes into one.

Links for Further Reading:

• On Nuvia cPrime method development (https://www.bio-rad.com/webroot/web/pdf/ps/literature/Bulletin_6242.pdf)

• On CHT method development (https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6086.pdf)

• On effects of buffers on Nuvia aPrime 4A method development (https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_7207.pdf).

BPI: Tell us about Bio-Rad's background and current multimodal offerings.

Snyder: For decades, Bio-Rad has been selling the original mixed-mode ligand, CHT ceramic hydroxyapatite media. We also have launched hydrophobic cation-exhange (HIC-CEX) and hydrophobic anion-exchange (HIC-AEX) mixed-mode resins, with more to come.

Yoshikawa: Bio-Rad has been in the chromatography business since the 1950s and has supported hydroxyapatite calcium affinity/cation-exchange mixed-mode chromatography for the past 27 years.

Links for Further Reading:

• On the history of Bio-Rad Laboratories (https://www.bio-rad.com/en-us/corporate/our-history?ID=MR8ISY4VY).

BPI: What is unique about Bio-Rad's hydrophobic ion-exchange resins and HAC resin?

Rushton: For the Nuvia cPrime and Nuvia aPrime 4A resins, the hydrophilic nature of their base beads is unique in the resin space. CHT ceramic hydroxyapatite media's uniqueness stems from the fact that the bead is the ligand (rather than a traditional resin with ligands attached to base beads).

Snyder: Every ligand performs differently for every protein, so there's no way to predict how one resin will necessarily behave in a given feed stream.

Yoshikawa: Essentially, each ligand performs differently with individual molecules. Therefore, what end users are being offered is a range of selectivities for purifying their molecules of interest.

Links for Further Reading:

• On Nuvia aPrime 4A resin (https://www.bio-rad.com/webroot/web/pdf/ps/literature/Bulletin_7142.pdf)

• On Nuvia cPrime resins (https://www.bio-rad.com/webroot/web/pdf/ps/literature/Bulletin_6242.pdf)

• On CHT XT media (https://www.bio-rad.com/webroot/web/pdf/psd/literature/Bulletin_7080.pdf).

BPI: Do you have data to show improvements in process economics or timelines that can be achieved using multimodal chromatography in downstream processing?

Rushton: Bio-Rad has studied many applications to help introduce mixed-mode resins into different types of processes.

Links for Further Reading:

• On a nonaffinity-based purification platform (https://www.bio-rad.com/webroot/web/pdf/psd/literature/Bulletin_7134.pdf) and https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6887.pdf)

• On MAb purification cost efficiencies (https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_7099.pdf).

BPI: What new applications for multimodal chromatography do you see on the horizon?

Rushton: I think that the next set of applications will be in more nontraditional expression systems with which other purification platforms will have difficulty in meeting the required quality outputs.

He: It will be in purification of biomolecules for which no affinity purification tools are available.

Yoshikawa: Biotherapeutic molecules will continue to evolve. Mixed-mode technology increasingly will be required for their purification.