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Efficient Purification of Diverse Single Domain Antibodies Using HyperCel Mixed Mode Resins

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Abstract

This study explores the efficient capture of single-domain antibodies (sdAbs) using MEP & CMM HyperCel, focusing on dimeric sdAbs with neutral pI and a monomeric sdAb with acidic isoelectric point. The dynamic binding capacity at 10% breakthrough (DBC 10%) for dimeric sdAbs significantly surpasses alternative affinity and cation exchange chromatography resins, achieving high product recovery rates. The process maintains low host cell protein and DNA levels, meeting industry specifications. CMM HyperCel emerges as a viable alternative for acidic sdAbs requiring near-neutral pH elution, achieving close to 100% recovery. Beyond the data shown here, optimizing binding conditions at low salt concentrations, contact times, and DBCs can streamline the process, enhancing its economic viability and scalability.

These findings underscore the potential of MEP HyperCel and CMM HyperCel as robust solutions for diverse protein purification needs.

Introduction

Single-Domain Antibodies

VhH fragments, also known as single-domain antibodies (sdAbs) or NANOBODY® molecules, are camelid-derived heavy chain-only antibodies that feature unique characteristics and exhibit several advantages both for research and medicine. sdAbs offer high thermal stability, specificity, and affinity in antigen binding. They also possess a compact design and are smaller than full-length antibodies, which could make them better adapted to hidden targets and tissues,[1] and more suitable for conjugation to other sdAbs or different proteins.[2] Their simplified structure and lack of extensive post-translational modifications make design, engineering, and production easy.[3] As such, they can be produced at high titres in a variety of host cells (both eukaryotic and prokaryotic) as long as secretion is possible.[1]

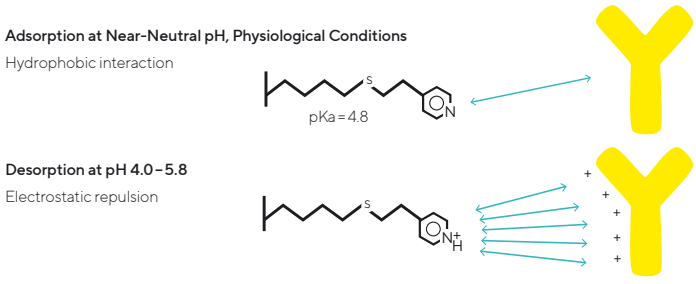
Full-length monoclonal antibodies (mAbs) are easily purified with affinity chromatography utilizing their interactions with Staphylococcal Protein A or Streptococcal Protein G (among other, more specialized molecules).[4] However, VhH purification is more complex, as no universal affinity ligand exists. While some VhHs can be purified with Protein A, many do not bind efficiently, requiring alternative methods such as tag-based chromatography (e.g., IMAC).[3] Given that tags often need to be cleaved after purification, a non-tag-based alternative may be more practical and economical.

HyperCel Mixed Mode Resins

HyperCel mixed mode resins are versatile chromatography consumables designed for the capture and purification of antibodies and recombinant proteins. They feature a proprietary rigid cellulose matrix linked with different mixed mode ligands, providing high porosity, chemical stability, and low non-specific interaction. They ensure excellent flow properties at low backpressures, which is ideal for sdAb capture at high flow rates and low contact times. HyperCel mixed mode resins employ hydrophobic charge induction chromatography (HCIC), a separation mechanism based on the pH-dependent behavior of the mixed mode ligand. On MEP HyperCel, proteins usually bind at pH 6–9 and low or no salt concentration through mild hydrophobic interactions, while the thioether group enhances thiophilic interaction.[5,6] Protein desorption occurs via positive electrostatic repulsion by lowering the pH based on the acid dissociation constant (pKa) of MEP HyperCel and the isoelectric point (pI) of the target molecule.[7] (Figure 1).

Complementary to MEP HyperCel, binding on CMM HyperCel usually occurs at pH 4–6 when the carboxyl group of the ligand is not or only weakly deprotonated. Protein desorption via negative electrostatic repulsion is achieved by increasing the pH to 6–9, leading to deprotonation of both the ligand and the target molecule.

Figure 1: *HCIC Binding and Elution on MEP HyperCel Resin*



Note. Binding is facilitated by a mix of hydrophobic interactions and molecular recognition by thiophilic interaction, while elution is triggered by electrostatic charge repulsion when the pH of the mobile phase is lowered

The advantages of MEP HyperCel and CMM HyperCel over traditional Ion Exchange (IEX) and Hydrophobic Interaction (HIC) resins in sdAb capture include:

- High dynamic binding capacities (DBCs) of sdAbs, with weak or no Protein A affinity
- Protein binding in low-salt conditions, reducing process costs and waste
- Differentiated selectivity, effectively separating host cell proteins (HCPs), DNA, aggregates, and misfolds
- Mild elution conditions (usually between pH 5.5–4.0 for MEP HyperCel and 7.0–8.0 for CMM HyperCel), minimizing aggregate formation and preserving biological activity
- Long resin lifespan and low bioburden risk, capable of enduring harsh cleaning methods (up to 1 M NaOH at 60 minutes contact time)
- Low contact times and high flow rates enabling rapid sdAb capture

In this study, we evaluated the capture capabilities of MEP HyperCel and CMM HyperCel for sdAbs. The work was performed in collaboration with VALIDOGEN, a leading contract research and development organization (CRO) specializing in recombinant protein expression using its exclusive UNLOCK PICHIA® protein expression technology. With its UNLOCK PICHIA® platform, VALIDOGEN applies a broad and unique portfolio of technologies for the successful and economically viable production of recombinant proteins in the yeast *Pichia* pastoris (also known as *Komagataella phaffii*), serving many industries including biopharmaceutical, food and feed, industrial biotechnology, and diagnostics.

VALIDOGEN provides advanced *Pichia* strain generation, fermentation, and protein purification process development for the manufacture of biopharmaceuticals, enzymes, and other recombinant proteins.

Based on their strong *Pichia* expertise, customers from different industries have success-fully scaled up commercial production processes with highly effective *Pichia* strains to produce recombinant proteins for pharmaceutical and non-pharmaceutical applications.

Materials

Sample Preparation

Table 1 lists the sdAbs used in this study.

Table 1: *List of Different sdAbs Purified on MEP HyperCel and CMM HyperCel*

Name	Molecular Weight [kDa]	Isoelectric Point [pI]
dV_hH1	28	6.8
dV_hH2	27	7.1
mV_hH	13	5.6

Chromatography Columns

Table 2 lists the chromatography columns used in this study.

Table 2: *List of Used Chromatography Columns*

Resin	Description	Volume	Article Number
MEP HyperCel	prepacked column	5 mL	PRC08X100MEPHCEL
CMM HyperCel	prepacked column	5 mL	PRCCMMHCEL5ML

After clarification by centrifugation at 12,000 × g for 20 minutes and filtration by Sartopure® PP3 0.45 µm, dV_hH1 & dV_hH2 were adjusted to 50 mS/cm using NaCl and pH 7.0 using NaOH. mV_hH was adjusted to 35 mS/cm, pH 7.0.

Chromatography Buffers

All buffers used for chromatography are listed in Table 3.

Table 3: *Buffers Used to Purify Different sdAbs on MEP HyperCel and CMM HyperCel*

Target Molecule	Resin	Phase	Ingredients	pH
dV_hH1, dV_hH2	MEP HyperCel	Equilibration	50 mM Bis-Tris, 500 mM NaCl	6.0
		Elution	50 mM Na-acetate	4.5
		Regeneration	50 mM Na-acetate	3.0
mV_hH	MEP HyperCel	Equilibration	50 mM Bis-Tris, 300 mM NaCl	6.0
		Elution	50 mM Na-citrate	2.5
	CMM HyperCel	Equilibration	50 mM Na-acetate, 250 mM NaCl	4.5
		Elution 1	50 mM tris	7.0
		Elution 2	50 mM tris	8.0
		Elution 3	50 mM tris	9.0
All	All	Clean-in-place	500 mM NaOH	14
All	All	Storage	20% Ethanol	n/a

Chromatography Protocols

Chromatography protocols are displayed in Tables 4 and 5.

Table 4: *MEP HyperCel Recipe for the Capture of dV_hH1 & dV_hH2*

Phase	Buffer	Volume [CV]	Flowrate [mL/min]	Contact Time [s]
Equilibration	Equilibration	5	3.4	90
Loading	Feed	Variable	3.4	90
Wash	Equilibration	8	3.4	90
Elution	Elution	5	3.4	90
Regeneration	Regeneration	5	3.4	90
Clean-in-place	Clean-in-place	5	1.7	180

Table 5: *CMM HyperCel Recipe for the Capture of mV_hH*

Phase	Buffer	Volume [CV]	Flowrate [mL/min]	Contact time [s]
Equilibration	Equilibration	5	3.4	90
Loading	Feed	Variable	3.4	90
Wash	Equilibration	5	3.4	90
Elution 1	Elution 1	5	3.4	90
Elution 2	Elution 2	5	3.4	90
Elution 3	Elution 3	5	3.4	90
Clean-in-place	Clean-in-place	5	1.7	180

Methods

Analytical Methods

Microfluidic Capillary Electrophoresis

Approximate protein concentration was determined by microfluidic capillary electrophoretic (mCE) separation (GXII, Revvity). A correction factor, derived from human serum albumin (HSA) mixed in similar buffer conditions, has been applied to account for matrix effects on the measurement of different fractions from chromatography.

PicoGreen™ DNA Assay

Host cell DNA (hcDNA) was measured with Quant-iT™ PicoGreen™ (Thermo Fisher Scientific) dsDNA Reagent and Kit according to the manufacturer's protocol using a fluorescence photometer (SpectraMax Plus 384, Molecular Devices).

ELISA Assay

Pichia HCPs were measured using an HCP ELISA kit (*Pichia pastoris* 2nd Generation HCP ELISA Kit, F640, Cygnus Technologies) following the manufacturer's protocol.

Results

Efficient Capture of Dimeric sdAbs With Neutral pI on MEP HyperCel

In the first set of experiments, the capture of two sdAbs with near-neutral pI and similar size was assessed (Table 6).

First, the dynamic binding capacity at 10% breakthrough (DBC10%) at 90 seconds contact time was determined (Table 6). The DBC10% values of 23.4 (dV_hH1) and 44.4 g/L (dV_hH2) significantly exceed the binding capacities of alternative affinity and cation exchange (CEX) chromatography resins. Differences in the DBC10% across different molecules fall within anticipated ranges, attributed to diverse protein structures and their interactions with the ligand.

After washing with about 10 column volumes (CVs) of equilibration buffer (Figure 2), a small wash peak was observed with both tested sdAbs. However, this contributes only to 0–6.7% of the total yield. Overall, the estimated product recovery is 75% (dV_hH1) & 110% (dV_hH2).

The recovery was determined with mCE, including a correction factor for matrix effects. As this assay is not as product-specific as an ELISA, some degree of error is expected. Nonetheless, the data reveal remarkably high recoveries for both molecules (Figure 3).

Furthermore, the resulting HCP levels in the eluates, at 0.35 µg/mg and 1.2 µg/mg product, along with final hcDNA levels of 1.3 and 1.4 ng/mg product, comfortably meet specifications. These results correspond to an HCP log reduction of 1.20–1.65 and an hcDNA log reduction of 1.9–2.8. These data demonstrate that MEP HyperCel can be used as an efficient capture medium with a DBC 10% significantly surpassing alternative chromatography resins, achieving high product recovery and meeting specifications for HCP and hcDNA.

Table 6: *Dynamic Binding Capacity of Different sdAbs Purified on MEP HyperCel*

	Molecular Weight [kDa]	Isoelectric Point	Contact Time [s]	DBC 10% [g/L Resin]	Capacity of Alternative Resin [g/L]
dV_hH1	28	6.8	90	23.4	2 – 3 (Protein A Resin, JSR Life Sciences)
dV_hH2	27	7.1	90	44.4	35 (CEX Resin, Cytiva)

Note. Dynamic binding capacity at 10% target protein breakthrough at 90 seconds contact time. CEX = cation exchange resin

Figure 2: *Chromatograms of dV_hH1* ***(A)*** *dV_hH2 on MEP HyperCel* ***(B)***

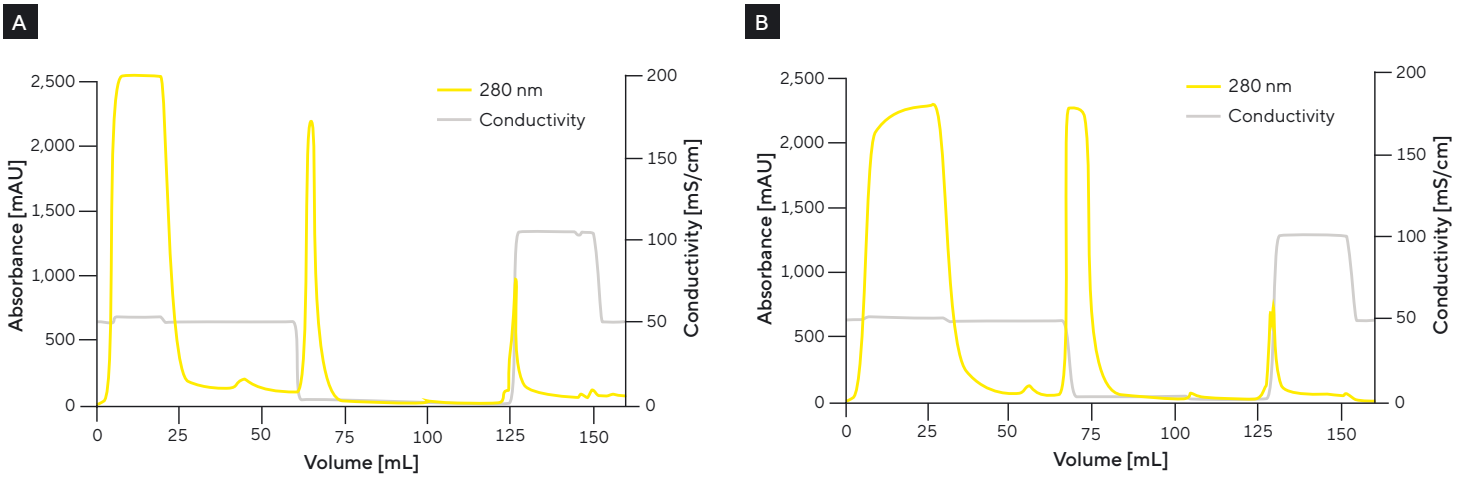
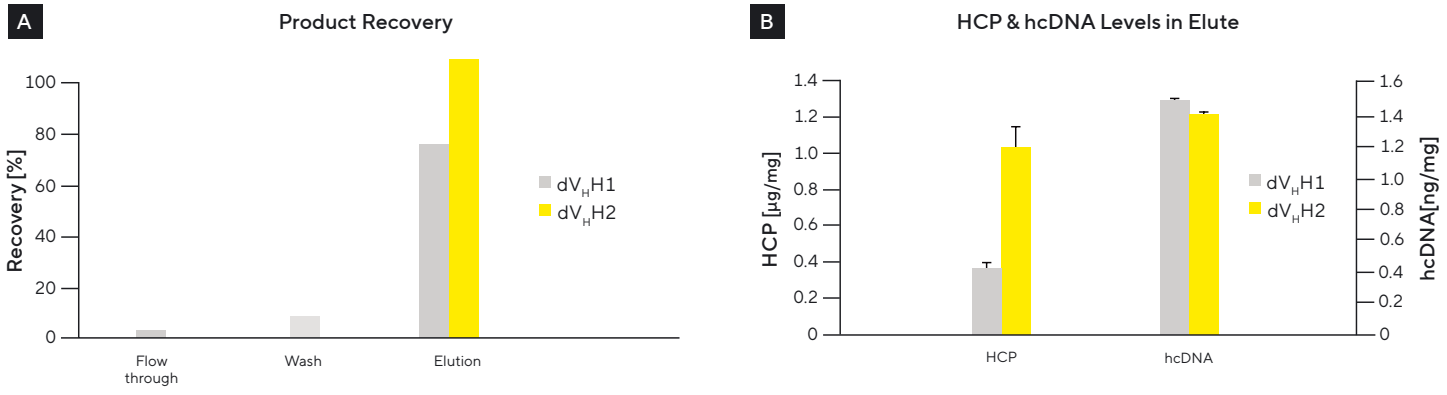


Figure 3: *Recovery* ***(A)*** *HCP, and hcDNA Levels in Eluates* ***(B)*** *After the Capture of dV_hH1 (grey) & dV_hH2 (yellow) on MEP HyperCel*



Capturing an Acidic Monomeric sdAb on MEP HyperCel and CMM HyperCel

To showcase the versatility of MEP HyperCel, we performed a proof-of-concept study to test its capability to capture an acidic monomeric sdAb (Figure 4). Successful binding was achieved at a pH of 5.6 and 300 mM NaCl. The DBC 10% was not determined due to the limited feed stream, but it is above 12 mg/mL of resin. A significant wash peak was observed during washing with equilibration buffer after loading. However, only 0.2% of the product was found, highlighting that contaminants are the primary cause of the wash peak. Due to the acidic nature of this molecule, a low pH of 2.5 was necessary for elution, which led to an impressive 98% product recovery.

If the target molecule does not withstand the low pH needed to elute from MEP HyperCel, CMM HyperCel can be used as an alternative. Here, successful binding was achieved at pH 4.5 and 250 mM NaCl. Full elution with recovery close to 100% was achieved at pH 8 (Figure 5).

This method could be further optimized to streamline the process. Nevertheless, the data highlights that CMM HyperCel can be used as an alternative to MEP HyperCel for acidic sdAbs.

Figure 4: *Capture of mV_hH on MEP HyperCel*

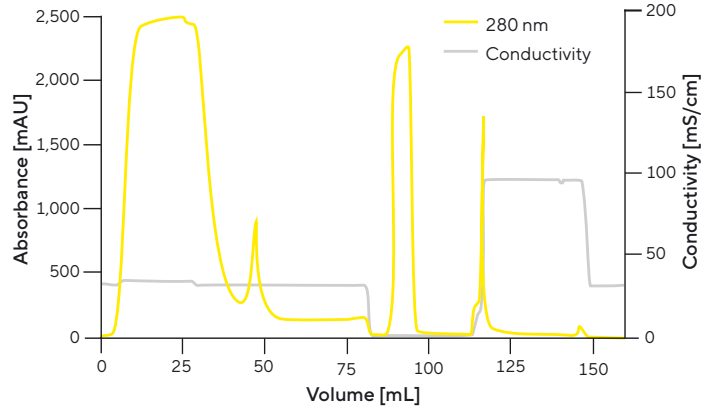
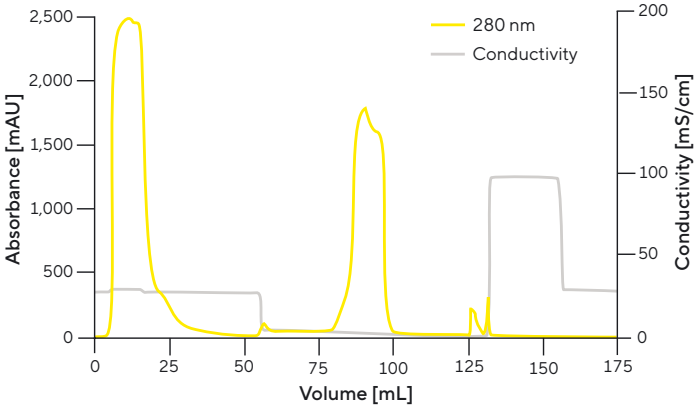


Figure 5: *Capture of mV_hH on CMM HyperCel*



Conclusion

This study demonstrates the efficacy and versatility of MEP HyperCel as a capture platform for diverse sdAbs with neutral to acidic pI, achieving superior DBCs and high product recovery rates compared to alternative chromatography resins. The successful capture and elution of both dimeric and monomeric sdAbs, with minimal HCP and hcDNA contamination, underscore MEP HyperCel's potential as a robust solution for diverse protein purification needs. Additionally, CMM HyperCel presents a viable alternative for acidic sdAbs requiring near-neutral pH elution, offering flexibility in optimizing purification processes.

By further refining binding conditions at low salt concentrations, along with contact times and dynamic binding capacities, the MEP HyperCel and CMM HyperCel capture platforms could be further streamlined.

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