SARTORIUS

Instructions for Use

HEA and PPA HyperCel

Mixed-mode Chromatography Resins

1 Product Description

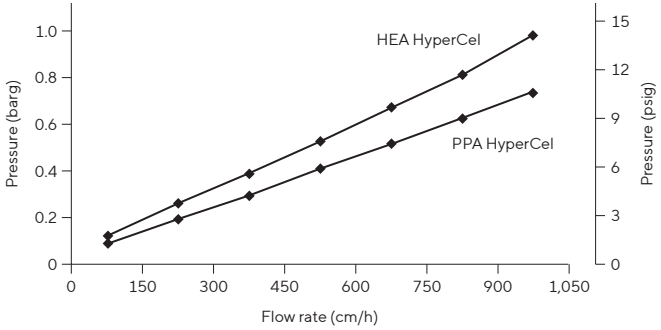
HEA and PPA HyperCel resins are novel industry-scalable chromatography resins designed for protein capture and impurity removal in a biopharmaceutical environment. They represent new "mixed-mode" chromatography selectivities, sharing some features of Hydrophobic Interaction Chromatography (HIC), but can be used in a "physiological-like" environment (no need for pH adjustment of the feedstream, no — or limited — addition of salt). HEA and PPA HyperCel resins provide users with additional selectivities for their process, as well as a mixed-mode, bidimensional separation mechanism (first dimension based on isoelectric point of the protein, second dimension on relative hydrophobicity) which can be exploited, for example, to achieve discrimination of proteins having similar or very close isoelectric points, a separation which cannot be done by methods like ion exchange chromatography. Two different robust synthetic ligands, aliphatic (HEA — hexylamine), and aromatic (PPA — phenylpropyl amine) exist to offer different selectivity options, according to the composition of the feedstock. HEA and PPA HyperCel resins are orthogonal to other conventional chromatography steps.

2 Properties

Particle size range	80 – 100 µm
Bead composition	High porosity cross-linked cellulose
Dynamic binding capacity for BSA[(1)]	40 – 60 mg/mL
Ligand	— Aliphatic (HEA): Hexylamine — Aromatic (PPA): Phenylpropylamine
BSA recovery	≤ 90%
Adsorption pH	7.0 – 9.0
Elution pH	By gradient or step elution, e.g., 7.0 – 2.6
Cleaning pH	3 – 14
Pressure resistance	< 3 barg (44 psig)
Typical working pressure	< 1 barg (14 psig)

(1) Determined using 5 mg/mL BSA in PBS, 10% breakthrough, flow rate: 100 cm/h.

Figure 1: Pressure vs. Flow Rate



3 Technical Overview

Protein binding on both ligands can be achieved in physiological-like conditions (PBS) without feedstream conductivity adjustment, especially for proteins that are more acidic and hyrdophobic in character. This is in contrast to conventional HIC or ion exchange resins, where lyotropic salt addition and | or conductivity adjustments must be made.

Protein binding to HEA and PPA HyperCel resins is achieved by a mixed-mode interaction. In certain cases, especially for proteins that are more basic and | or hydrophilic in nature, salts like sodium chloride or ammonium sulphate may be added to act on either the ionic or the hydrophobic effects (salt decreases the ionic component and enhances the hydrophobic component). Even when salt does need to be used to promote binding, lower concentrations of less lyotrophic salts can be used as compared to conventional HIC.

Elution is prompted by decreasing the pH, conductivity, or both in a gradient or step elution mode. Typically, there is no need for extensive desalting | diafiltration following the HEA or PPA HyperCel resin step, in contrast to conventional HIC.

4 Column Packing

4.1 General Considerations

HEA and PPA HyperCel resins are supplied as a slurry | suspension in 1 M NaCl containing 20% (v/v) ethanol or as a moist cake for process-scale applications. The moist cake resin facilitates the resin transfer, avoiding the agitation and suspension of large material volumes. The resins are also available in other configurations : in PRC prepacked columns for rapid selectivity screening under reliable and reproducible conditions and in miniaturized RoboColumn®* for fully automated and parallel chromatographic separations. For process-scale column packing support, contact Technical Service.

4.2 Packing a Small Column (≤ 5 cm I.D.)

HEA and PPA HyperCel resins are compatible with traditional low or medium pressure chromatography columns and equipment. For preliminary laboratory studies, a column equipped with an adjustable piston of dimensions such as 1 cm I.D. x 20 cm length facilitates optimal packing. Direct scale-up is accomplished by maintaining bed height constant while diameter is increased. We recommend a chromatography system able to deliver a linear flow rate of 1,000 cm/h (600 cm/h minimum for packing operations).

4.2.1 Preparing the Resin Slurry

1. Gently agitate the container to fully suspend the resin. Do not use magnetic stirrers at any point in the procedure.
2. Depending on the desired bed volume, transfer a suitable volume of slurry to a graduated beaker, including a "practical" excess of resin (20 – 25% of the desired bed volume is recommended).
3. Allow the resin to settle, and remove the supernatant. Add 3 to 5 volumes of packing buffer (equilibration buffer).
4. Gently agitate the slurry and allow resin to settle. Remove the supernatant and add 3 to 5 volumes of fresh packing buffer.
5. Proceed to buffer-exchange steps 3 times minimum in total.
6. Gently agitate the slurry, pour it into a measuring cylinder and allow the resin to settle.
7. Remove the supernatant and add a volume of packing buffer equal to one-half the volume of settled resin. A slurry of 67% (v/v) — the concentration recommended for packing — is obtained.

Optional: the final slurry concentration can be estimated by centrifuging between 20-g and 200-g about 10 mL of homogenized slurry in a graduated vial tube for 5 minutes.
The concentration of slurry expressed as [resin volume | total volume (resin + supernatant) x 100] can be directly estimated in the tube. Pour the 10 mL slurry back into the total suspension before packing.

4.2.2 Preparing Solumn and System for Packing

1. Determine pressure | flow characteristics for the empty system, taking into account recommendations on flow rate during packing and chromatographic operation.
2. Prime the solvent delivery system, lines and valves to assure that all air is displaced. Assure that top and bottom frits or nets are fully wetted and free of air. Fill the column with packing buffer and operate the system over a range of flow rates representative of values that will be used during packing and anticipated chromatographic procedures. Record pressure associated with the empty column and system (including detectors, etc.).
3. Stop the pump, close the column outlet, and remove the upper flow adaptor or piston.
4. Open the outlet and drain buffer from the column, leaving 1 – 4 cm of buffer above the bottom frit.

Following completion of blank pressure | flow measurements, proceed with column packing as described in Section 4.2.3.

4.2.3 Packing the Column

1. Homogenize gently the slurry and pour it into the column in one continuous motion against the wall of the glass tube to minimize introduction of air bubbles (if the pouring process is done in several motions, gently homogenize the slurry in the column using a plastic rod).
2. Complete with packing buffer (i.e., equilibration buffer such as PBS, pH 7.4) up to the top of the glass tube. Allow the suspension to settle so that a layer of clear supernatant ≤ 1 cm is visible at the top of the column.
3. Connect the upper piston to the system and prime with packing buffer to ensure that no air is trapped under the net or frit. Stop the pump and insert the adjustable piston into the column. Tighten it.
4. Open the column outlet and operate the pump at a selected linear velocity of 1,000 cm/h (~13.1 mL/min for a 1 cm I.D. column) for efficient packing; in any case, flow rate must not be lower than 600 cm/h for guaranteeing high packing performance.
5. When the top of the bed stabilizes, stop the pump and untighten the piston. Position the adjustable piston at the top of the packed resin, leaving no visible space between the frit and the packed resin at any point around the circumference.
6. Operate the pump again and repeat the adjustment of the piston until no visible space appears under flow (1,000 cm/h) between the frit and the top bed.

4.2.4 Evaluating Column Performance

Column performance is evaluated by determining column efficiency, expressed as either plates/meter (N/m), or HETP (height equivalent to one theoretical plate). Additionally, the asymmetry factor is calculated.

Required formulas are shown below. Measurements are made as follows:

1. Equilibrate a column of I.D. 1 cm x 12 cm length (10 mL) with packing buffer (equilibration buffer like PBS, pH 7.4).
2. Inject a sample of 5% (v/v) acetone in packing buffer corresponding to 1% (v/v) of total column volume (i.e., 100 µL for a 10 mL column). Apply a flow rate of 100 cm/h. Record UV absorbance at 280 nm.
3. Alternatively to acetone, a sample of 1 – 2 M NaCl in packing buffer can be injected (corresponding to 1% (v/v) of the column volume). A flow rate of 100 cm/h is applied and conductivity (mS/cm) is recorded instead of absorbance.

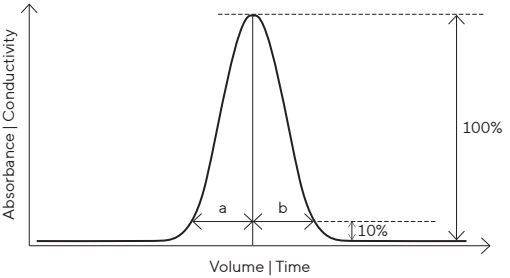
The HETP and the number of plates/m (N/m) are calculated as follows:

$$HETP = \frac{d^2 \times L}{5.54 \times t^2} \qquad N/m = \frac{5.54 \times t^2}{d^2 \times L}$$

where: t = Retention value of acetone or sodium chloride peak expressed in units of time, volume or measured distance on the chromatogram.
d = Peak width at half height expressed in same units as "t".
L = Column length (m).

Figure 2: Peak Trace in a Typical Test Evaluation of Column Performance

$$AF = \frac{b}{a}$$



"a" and **"b"** are respectively first and second half peak width at 10% of peak height. Typical values at 100 cm/h for N/m are 1,500 – 3,000 plates/m. Typical values at 100 cm/h for asymmetry factor (AF) range from 0.8 and 1.4 at 10% of peak height. These values are given as the average of experimental values. More important than the values by itself, the reproducibility of the values over the successive packing operations is critical.

4.3 Packing a Large Column (> 5 cm I.D.)
Please contact technical service.

5 Working Conditions and Basic Protocol
Use only samples and buffers that are previously filtered on a 0.2 µm membrane. Make sure that changes in pH and ionic strength to be used during the chromatography do not cause precipitation of sample components.

5.1 Equilibration
Equilibrate the column in equilibration buffer (5 – 8 CV). PBS, pH 7.4 is typically recommended.
Alternatively, a phosphate buffer at neutral pH containing 0.5 M NaCl can increase hydrophobic interactions. If binding needs optimization, it is advised to use a more basic pH (50 mM Tris-HCl, pH 8 – 9) and | or to add 0.5 – 1.0 M sodium chloride or ammonium sulphate in equilibration buffer.

5.2 Sample Application
Load prefiltered sample at typical residence times of 5 – 8 minutes to reach optimal capacity. The feedstock can be loaded directly onto HEA or PPA HyperCel columns without preliminary dia filtration or concentration.
Due to the mechanical stability of the resin, large volumes of sample can be processed rapidly and efficiently, while keeping low backpressures (< 3 bar).

⚠ **Temperature:** The hydrophobic component of the mixed-mode mechanism is sensitive to temperature (capacity increases when temperature rises), and significant variations in capacity may be observed. Make sure to allow buffer equilibration at desired temperature to avoid variations.

⚠ **Chaotropic Agents:** The presence of chaotrophic agents such as 4 – 8 M urea or detergents such as CHAPS may alter performance on HEA and PPA HyperCel resins. This may occur through changes in protein conformation and | or competitive interactions with the ligands

5.3 Working Flow Rate
Typical residence times of 5 – 8 minutes correspond to linear flow rates between 75 – 120 cm/h for a column of 10 cm height (0.98 – 1.57 mL/min volumetric flow rates for 1 cm I.D. column).
Optimization of the residence time may lead to an increase of the linear velocity (shorter loading time) while loading capacity is maintained.

5.4 Elution
Elution is performed either by gradient or step elution. For gradient elution, decrease the pH — typically between pH 6.5 to 3.0. For example, use 5 CV of 50 mM sodium acetate, pH 5.5 to pH 3.0 in 60 minutes. At process scale, step elution using acetate buffer is the preferred choice. Avoid citrate since it may delay protein desorption. Check that changes in pH and ionic strength during elution do not cause precipitation. Salt concentration: In some cases, a decrease of the salt concentration can also contribute to elution. Optimal conditions may be a combination in the decrease of pH and ionic strength. Screening conditions using 96-well plate would help to optimize elution performances.

6 Regeneration and Cleaning
We recommend to regenerate the column with a wash at low pH (e.g., 50 mM sodium acetate, pH 3.0 or 4.0) followed by a general cleaning-in-place (CIP) with 5 CV of 1 M sodium hydroxide, 60 min contact time.
After CIP, re-equilibration of the column in 2 CV of an acidic solution (sodium acetate pH 3.0 or 4.0) hastens the neutralization of the column before equilibration in the desired buffer for the next cycle (e.g., PBS, pH 7.4).
For specific CIP challenges (strongly adsorbed contaminants), 3 – 5 CV of denaturing agents (6 M guanidine-HCl or 8 M urea) or 3 – 5 CV of 40% isopropanol are recommended prior to the step in 1 M NaOH.
If CIP sequence requires optimization, please contact technical service.

7 Thermal Stability and Storage

Temperature of use	2 – 30 °C
Storage temperature	2 – 8 °C
Storage solution	Neutral buffer containing 1 M NaCl and 20% (v/v) ethanol
⚠	Product must never be frozen. Avoid long exposure to light.
i	Product is shipped at ambient temperature

8 Ordering Information

Bottled Resin

Pack Size	Part Number	
	HEA HyperCel	PPA HyperCel
25 mL	20250-026	20260-025
100 mL	20250-033	20260-030
1 L	20250-041	20260-040
5 L	20250-042	20260-045
10 L	20250-056	20260-052

Prepacked Columns

Description	Part Number
PRC Columns	
PRC Prepacked Column 5 x 50 HEA HyperCel, 1 mL, 1/pkg	PRC05X050HEAHCEL
PRC Prepacked Column 8 x 100 HEA HyperCel, 5 mL, 1/pkg	PRC08X100HEAHCEL
PRC Prepacked Column 5 x 50 PPA HyperCel, 1 mL, 1/pkg	PRC05X050PPAHCEL
PRC Prepacked Column 8 x 100 PPA HyperCel, 5 mL, 1/pkg	PRC08X100PPAHCEL
RoboColumn®*	
RoboColumn®* HEA HyperCel 200 µL, row of 8	SR2HEA
RoboColumn®* HEA HyperCel 600 µL, row of 8	SR6HEA
RoboColumn®* PPA HyperCel 200 µL, row of 8	SR2PPA
RoboColumn®* PPA HyperCel 600 µL, row of 8	SR6PPA

* RoboColumn is a trademark of Repligen GmbH

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