TN0006 Development of Preparative and Analytical Fractionation Methods with CIMmultus™ and CIMac™ H-Bond ADC

H-Bond ADC is the first of a new class of chromatography ligands from BIA separations that exploits hydrogen bonding as the dominant mode of biomolecule retention. The ligand consists of a terminal series of hydrogen donors grafted to a root series of hydrogen acceptors.

H-Bond ADC brings a unique new selectivity to all fractionation tasks but is especially distinctive in its ability to retain large biomolecules more strongly than small ones. This can be of great value for removing fragments, aggregates, and viruses from protein products, or for removing proteins and other small contaminants from large biologics like viruses and extra-cellular vesicles.

Although H-Bond ADC carries a weak positive charge, it supports stronger retention at acidic pH. The lower the pH, the greater the differentiation between large and small biomolecules. Method development should generally begin at the lowest pH where the product of interest is known to be stable. If unknown, a pH value of 4.5 is a reasonable place to start. Selectivity at pH 6.0 and 7.5 (or higher) may also be worth checking as H-Bond ADC is known to give very different selectivities compared to anion exchangers even at alkaline pH values.

Biomolecules retained by hydrogen bonding can be eluted with simple sodium chloride gradients except that elution will require much higher salt concentrations than are customary with ion exchangers. Reliance on hydrogen bonding as the dominant retention mechanism also enables elution methods that cannot be used with any other mode of adsorption chromatography. Products can be eluted with soluble nonionic hydrogen donor-acceptors, including sorbitol and urea among others.

Remarkably, H-Bond ADC can also be eluted with *ascending* pH gradients. This highlights the role of hydrogen bonding since positively charged media are normally eluted with *descending* pH gradients. If maximum

separation of large and small solutes is a particular objective, polyethylene glycol can be added to the buffers.

Sample preparation:

Titrate the sample to the operating pH. A residual salt concentration of 50 mM is typically well tolerated at all pH values. Tolerated salt concentration may increase to 100 mM at pH 6.0, and 200 mM at pH 4.5. Samples should generally be filtered to 0.45 µm before application to the monolith.

Basic selectivity screening.

The following protocols are starting points. Acetic acid provides adequate buffer capacity from about pH 3.5 to 5.5. For experiments at other pH values, try MES from pH 5.0 to 7.0; HEPES from pH 6.0 to 8.0, or Tris from pH 7.0 to 9.0.

Equilibrate column: 50 mM acetic acid, 50 mM NaCl, pH 4.5. Note that equilibration buffers containing salt will reduce the column equilibration volume. If the product binds very strongly consider increasing the equilibration salt concentration.

Load sample. Wash the column with equilibration buffer. Elute with a linear NaCl gradient. For biologics up to about 100 kDa, 1 M NaCl may be adequate. For large biologics, a gradient ending at 2–3 M NaCl may be required.

Elution with hydrogen donor-acceptors.

Use the initial screening experiments to determine a concentration of NaCl where the product of interest will be still retained. Keeping that level constant, apply a gradient to the same concentration of NaCl plus the hydrogen donor-acceptor of choice. Sorbitol may be used at concentrations up to 20%. Urea may be used up to concentrations of 8.0 M. Urea imposes a risk of denaturation but sorbitol is a well-known stabilizer.

Elution with pH gradients.

Use the initial screening experiments to determine a concentration of NaCl where the product of interest will be still retained. Keeping that level constant, apply an ascending pH gradient. Gradients from pH 6–8 can be generated with a mixture of 20 mM MES and 20 mM HEPES. Formulate this buffer at the indicated salt concentration, divide it into two containers, titrate one to pH 6.0 for use as the gradient start buffer, and the other to pH 8.0 as the gradient endpoint buffer. To extend the gradient down, include acetate at the same

concentration and adjust pH accordingly. To extend it up, include Tris at the same concentration and adjust pH accordingly.

To increase resolution among species of different size classes within any gradient format, include 5–10% PEG with a molecular weight of 3500 to 8000. Higher concentrations of larger PEG polymers have a stronger effect but also increase viscosity and may require reducing the flow rate. PEG should not be added directly to samples since it may cause precipitation.

Cleaning and sanitization.

H-Bond ADC tolerates repeated exposure to organic solvents, acids, and bases. A solution of 1 M NaOH, 2 M NaCl is recommended for routine cleaning and sanitization. CIMmultus and CIMac monoliths may be autoclaved to support aseptic processing or for sterilization prior to disposal.

Ordering information:

Catalogue #	Description
110.5130-2	CIMac™ H-Bond ADC -0.1 Analytical Column (Pores 2 um)
311.5130-2	CIMmultus™ H-Bond ADC -1 Advanced Composite Column (Pores 2 um)

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