Fundamentals of Affinity Chromatography

Penny Hamlyn
Senior Business Development Manager
Bioprocessing Resins
What is affinity chromatography?

Affinity chromatography is a method of separating a biomolecule from a mixture, based on a highly specific interaction between the biomolecule and another substance.

The specific type of binding interaction depends on the biomolecule of interest:

- antigen and antibody
- enzyme and substrate
- receptor and ligand

Interaction: Hydrogen bonding, Ionic, Van der Waals forces, hydrophobic
Advantages of Affinity Chromatography

- Rapid target isolation
- High recovery
- High specificity
- High capacity
- High resolution
- Suitable for large scale purification processes
- Number of purification steps can be reduced
Affinity Chromatography Essentials
What is affinity chromatography?

- Highly specific: Lock and key
- Reversible
- Covalently attach a ligand to a solid support (resin) via a hydrophilic linker
- Resin packed into a glass tube or column
Affinity Chromatography

Steps

1. Equilibration
2. Sample loading
3. Wash
4. Elution
5. Clean and equilibrate
Affinity Chromatography

Steps

1. Equilibration
2. Sample loading
3. Wash
4. Elution
5. Clean and equilibrate
Affinity Chromatography: His Tag

![Graph showing elution buffer and wash](image)

A 280

<table>
<thead>
<tr>
<th>Wash</th>
<th>Elution buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>10.0</td>
</tr>
<tr>
<td>15.0</td>
<td>20.0</td>
</tr>
</tbody>
</table>

A 280 graph with peaks indicating elution and wash.

![Image of gel electrophoresis](image)

- **kDa** markers: 250, 150, 100, 75, 50, 37
- Proteins indicated: L, CL, F, W1, W2, E1, E2, E3
- Highlight on 27 kDa with an arrow labeled 'His'
Base Matrix

Macroporous

Hydrophilic or neutral to prevent non-specific interactions with the base matrix

Chemically stable to withstand harsh conditions during regeneration and maintenance

Physically stable to withstand hydrodynamic stress in packed beds
Derivatisation of the base matrix adding the ligand

**Hydrophilic spacer – green**

It is used to improve binding between ligand and target molecule by overcoming any effects of steric hindrance

Ligand density

Ligand size

Porosity and particle size of the beads

Multi-point vs single point attachment

Ligand Leakage

Ligand lifetime
Ligands for Affinity Chromatography

Mono Specific ligands

• Specific for a single substance (antigen for antibody; hormone for receptor)

• Normally home-made resins, general elution conditions

Group Specific ligands

• Specific for group of molecules: structurally or functionally similar

Lectins for glycoproteins
Protein A for antibodies
Dyes for enzymes

• Normally commercially available, standard elution conditions
### Examples of ligands and their targets

<table>
<thead>
<tr>
<th>Types of ligand</th>
<th>Target molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate analogue</td>
<td>Enzymes</td>
</tr>
<tr>
<td>Heparin</td>
<td>Clotting factors, Cell binding proteins, DNA binding proteins</td>
</tr>
<tr>
<td>Antibody</td>
<td>Antigen</td>
</tr>
<tr>
<td>Lectin</td>
<td>Polysaccharide</td>
</tr>
<tr>
<td>Nucleic acid</td>
<td>Complementary base sequence</td>
</tr>
<tr>
<td>Hormone</td>
<td>Receptor</td>
</tr>
<tr>
<td>Avidin</td>
<td>Biotin/Biotin-conjugated molecule</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>Calmodulin binding partner</td>
</tr>
<tr>
<td>Glutathione</td>
<td>GST fusion protein</td>
</tr>
<tr>
<td>Protein A or Protein G</td>
<td>Immunoglobulins</td>
</tr>
<tr>
<td>Nickel</td>
<td>polyhistidine fusion protein</td>
</tr>
</tbody>
</table>
Capture, purification and polishing

1. **Capture**
   - Speed
   - Recovery
   - Resolution
   - Capacity
   - Cost
   
   "Concentration Isolation"

2. **Purification**
   - Speed
   - Recovery
   - Resolution
   - Capacity
   - Cost
   
   "Removes bulk impurities e.g. HCPs, DNA"

3. **Polishing**
   - Speed
   - Recovery
   - Resolution
   - Capacity
   - Cost
   
   "Remove traces of impurities – modified target molecules (aggregation, incorrect glycosylation etc)"
Affinity: Capture step

1. Capture
   - Speed
   - Recovery
   - Resolution
   - Capacity
   - Cost
   Concentration Isolation

2. Purification
   - Speed
   - Recovery
   - Resolution
   - Capacity
   - Cost
   Removes bulk impurities e.g. HCPs, DNA
Antibodies
Antibodies and Protein A

- High Affinity for FC and in some cases the VH3 region of an Antibody
- High capacity
- Proven scalability
- Proven virus removal/inactivation step
- Very robust and generic process
- >90% registered mAbs products for therapeutic use are purified by Protein A
# Purolite Protein A Resin Portfolio

<table>
<thead>
<tr>
<th>Product</th>
<th>Antibody Type</th>
<th>Capacity</th>
<th>Base Matrix</th>
<th>Customer Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Praesto® Jetted A50</strong></td>
<td>NGL-Impact™ ligand with very high capacity and alkaline stability (CIP 0.1 NaOH, 15min R.T.)</td>
<td>Up to 80 g/l</td>
<td>50 μm Jetted base matrix</td>
<td>Improve productivity and costs of goods</td>
</tr>
<tr>
<td></td>
<td>Good binding to FAbs of the VH3 family</td>
<td></td>
<td>Uniform beads with a narrow particle size distribution</td>
<td></td>
</tr>
<tr>
<td><strong>Praesto® Jetted A50 HipH</strong></td>
<td>very high capacity and alkaline stability (CIP 0.1 M NaOH, 15min R.T.)</td>
<td>Up to 60 g/l</td>
<td>50 μm Jetted base matrix</td>
<td>High yield at high elution pH (3.5-5.0) Ideal for labile fc containing mabs, fragment and fusion proteins</td>
</tr>
<tr>
<td></td>
<td>Designed to purify 3rd generation molecules, that would precipitate at normal PrA elution conditions</td>
<td></td>
<td>Uniform beads with a narrow particle size distribution</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fc-binding</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Purification using Protein A
Column Size

- Volume to suit amount (mass) of target protein
- Use 70-100% of available capacity
- Length 10-20 cm
- Scale up on diameter

Residence time (min) = \([\text{bed height (cm)} / \text{linear Flow (cm/h)}] \times 60\)
Most affinity purification protocols follow the same three steps:

1. **Binding**
   A complex solution containing the tagged protein is applied to the column and binds based on the affinity tag - matrix interaction.

2. **Wash**
   Other proteins which bind unspecifically are washed away with suitable buffers.

3. **Elution**
   Specifically bound protein is eluted from the column, typically by competitive binding of a similar molecule (e.g. histidine and imidazole), by cutting off the tag with a protease or by destabilization of the affinity tag - matrix interaction e.g. by a change of pH.
Interaction: Hydrogen bonding, Ionic, Van der Waals forces, hydrophobic

Binding conditions mimic nature: PBS buffer of choice
Wash conditions: aim to get your column as clean as possible whilst still maintaining binding of your target.
Remove as much as possible as early as possible

- Wash to baseline with loading buffer
- Optional intermediate wash step

Ref: *Biotechnol Prog.* 2014 Sep; 30(5): 1114–1124
Research purification: how important is optimizing a wash step

- Can we optimize purification to yield Xtals?
- Wash Step
  Concentration of Imidazole (50-100mM)
- Elution Step
  Salt concentration, flow rate
Apo + Inhibitor
Affinity Chromatography
Elution
Elution from affinity columns: general elution conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH</strong></td>
<td>100 mM glycine-HCl, pH 2.5-3.0</td>
</tr>
<tr>
<td></td>
<td>100 mM citric acid, pH 3.0</td>
</tr>
<tr>
<td></td>
<td>100 mM sodium acetate pH 3.0</td>
</tr>
<tr>
<td><strong>Ionic strength and/or Chaotropic effects</strong></td>
<td>3.5–4.0 M magnesium chloride, pH 7.0 in 10mM Tris</td>
</tr>
<tr>
<td></td>
<td>5 M lithium chloride in 10mM phosphate buffer, pH 7.2</td>
</tr>
<tr>
<td></td>
<td>2.5 M sodium iodide, pH 7.5</td>
</tr>
<tr>
<td></td>
<td>0.2-3.0 M sodium thiocyanate</td>
</tr>
</tbody>
</table>
Elution from affinity columns: specific elution conditions

competitive ligand – competing free ligand or competing binding substance

https://www.researchgate.net/figure/Competitive-elution-of-the-target-by-adding-a-competitive-free-ligand-triangle_fIg5_221929569
Elution is crucial to success
Elution

$K_D$ is the equilibrium dissociation constant, a ratio of $k_{off}/k_{on}$, between the antibody and its antigen. $K_D$ and affinity are inversely related.

The lower the $K_D$ value the higher the affinity of the antibody.
**K_D in summary**

\[
\frac{[\text{Bound target}]}{[\text{Total Target}]} \approx \frac{\text{Lo}}{K_D + \text{Lo}}
\]

Lo is the concentration of ligand (Protein A)

For good binding, \(K_D\) needs to be at least 2 orders of magnitude less than Lo: \(10^{-6} \quad \text{to} \quad 10^{-4} \text{ M}\)

Ref: Graves, DJ; Wu, YT; Methods in enzymology 34 (1974) 140-164
Elution by changing the $K_D$

- **Ideal situation**
  - Target elutes as a sharp peak
  - $K_D$ Low $10^{-6}$ M

- **Target elutes under binding Conditions**
  - Change binding buffer
  - $K_D$ too high $10^{-3}$ M

- **Target elutes long low peak**
  - Change elution buffer
  - $K_D$ Low $10^{-6}$ M

- **Difficult to increase the $K_D$ enough to elute without destroying the activity**
  - Change ligand
  - $K_D$ very Low $10^{-15}$ M

- **$K_D$ still too low $10^{-5}$ M**
Kinetics of binding

Ideal situation
Target elutes as a sharp peak

Target elutes under binding Conditions
Change load flow rate or stopped flow binding

Target elutes long low peak
Change elution flow rate or stopped flow elution
Step Elution first choice for affinity
Gradient can be useful for affinity
Praesto® Jetted A50 HipH

- New resin for elution of pH sensitive mAbs or other Fc containing proteins
- Elution up to pH 5
- Alkaline stable ligand
- High capacity - 60 g/L for IgG₁
Elution chromatogram for an IgG1

<table>
<thead>
<tr>
<th>Step</th>
<th>Buffer</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibration</td>
<td>20 mM Sodium Phosphate, 0.15 M NaCl, pH 7.0-7.4</td>
<td>5</td>
</tr>
<tr>
<td>Sample Load</td>
<td>70-90 % of the dynamic binding capacity (DBC)</td>
<td>N/A</td>
</tr>
<tr>
<td>Chase</td>
<td>Equilibration buffer</td>
<td>5</td>
</tr>
<tr>
<td>Column Wash</td>
<td>100 mM Sodium citrate, pH 7.0</td>
<td>5</td>
</tr>
<tr>
<td>Elution</td>
<td>100 Mm Sodium citrate, pH 3.0</td>
<td>0 - 100 % 10 CV 100 % B 5 CV</td>
</tr>
<tr>
<td>Regeneration</td>
<td>100 Mm Acetic Acid</td>
<td>3</td>
</tr>
<tr>
<td>CIP</td>
<td>0.1 M NaOH</td>
<td>3</td>
</tr>
<tr>
<td>Equilibration</td>
<td>20 mM Sodium Phosphate, 0.15 M NaCl, pH 7.0-7.4</td>
<td>3</td>
</tr>
</tbody>
</table>
Fc fusion screening with Praesto® Jetted A50 HipH

Early Regeneration

- Fc fusion protein
- Basic ProA method, no HCP wash
- 6 min RT
- DBC of 33.8 g/L
- 7 different 50 mAU – 50 mAU

<table>
<thead>
<tr>
<th>Elution pH</th>
<th>Elution CV</th>
<th>Eluate pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 3.5</td>
<td>1.68</td>
<td>4.46</td>
</tr>
<tr>
<td>pH 3.8</td>
<td>1.76</td>
<td>4.57</td>
</tr>
<tr>
<td>pH 4.0</td>
<td>1.80</td>
<td>4.71</td>
</tr>
<tr>
<td>pH 4.2</td>
<td>1.80</td>
<td>4.86</td>
</tr>
<tr>
<td>pH 4.5</td>
<td>1.76</td>
<td>5.10</td>
</tr>
<tr>
<td>pH 4.8</td>
<td>4.04</td>
<td>5.13</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>5.32</td>
<td>5.26</td>
</tr>
</tbody>
</table>

- Platform Protein A yields are typically >90%
- HipH Similar trend but seen to drop at elution pH > 5.0
- Peak tailing at elution pH≥ 4.8
- Material eluted low pH wash 5.3
Fc fusion screening with Praesto® Jetted A50 HipH

- Eluting at milder pH levels significantly improved clearance of rDNA without impacting recovery and purity
- Equivalent quality using elution pH 4-4.5
- Larger elution volume at pH 4.8
- Wide window of operation for c fusion
- Further scope to optimise wash v DoE
Praesto Jetted HipH Elution vs MabSelect Sure and PrismA SEC analysis

<table>
<thead>
<tr>
<th></th>
<th>MabSelect PrismA</th>
<th>MabSelect Sure</th>
<th>Praesto Jetted A50 HipH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rel Area</td>
<td>83.20</td>
<td>73.56</td>
<td>99.59</td>
</tr>
<tr>
<td>% monomer</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Jetted A50: polyclonal IgG Capacity

<table>
<thead>
<tr>
<th>Phase</th>
<th>Buffer</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibration</td>
<td>PBS, pH 7.2</td>
<td>5</td>
</tr>
<tr>
<td>Sample load</td>
<td>Polyclonal hIgG - Loaded until 50% mAU breakthrough</td>
<td>N/A</td>
</tr>
<tr>
<td>Chase</td>
<td>PBS, pH 7.2</td>
<td>5</td>
</tr>
<tr>
<td>Elution</td>
<td>0.1 M acetic acid</td>
<td>5</td>
</tr>
<tr>
<td>CIP</td>
<td>0.1 M NaOH (15 min contact time)</td>
<td>3</td>
</tr>
<tr>
<td>Equilibration</td>
<td>PBS, pH 7.2</td>
<td>5</td>
</tr>
</tbody>
</table>
Jetted A50: Smart Cycling

<table>
<thead>
<tr>
<th>Resin</th>
<th>Residence Time (mins)</th>
<th>DBC (g/L)*</th>
<th>Cycles</th>
<th>Column Dimension (ID x BH cm)</th>
<th>Buffer Volume (L)</th>
<th>Total Processing Time (h)</th>
<th>Total Resin Cost ($)</th>
<th>Productivity (g/L/H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Praesto Jetted A50</td>
<td>2</td>
<td>50.4 (40.3)</td>
<td>8</td>
<td>60 x 10</td>
<td>3845</td>
<td>7</td>
<td>$465,034</td>
<td>51.3</td>
</tr>
<tr>
<td>MabSelect™ SuRe™ LX*</td>
<td>4</td>
<td>50.2 (40.2)</td>
<td>4</td>
<td>60 x 20</td>
<td>3845</td>
<td>7</td>
<td>$1,252,023*</td>
<td>25.7</td>
</tr>
<tr>
<td>MabSelect™ SuRe™*</td>
<td>4</td>
<td>45.9 (36.7)</td>
<td>4</td>
<td>60 x 20</td>
<td>3845</td>
<td>7</td>
<td>$1,252,023*</td>
<td>25.7</td>
</tr>
</tbody>
</table>

* Pricing sourced from cytiva.com 02/08/22

- 2x increase in productivity
- 65% reduction in resin costs
- Fits directly into existing batch protocol
Affinity Chromatography Cleaning and Reuse
Cleaning and sanitisation

Cleaning: defined as the physical removal of soil, organic debris and particulars from surfaces.

Sanitisation: Defined as removal or elimination of micro-organisms.

Cleaning for resins:

Re-Equilibration – returns resin to starting conditions for next cycle, after each separation, e.g. 3-4 CV start buffer – check UV PH and conductivity signals return to steady baseline levels. Important for reproducibly.

CIP – eliminates material not removed by regen, prevents progressive build up of contaminants – after every 1-10 cycles, protocol designed according to feed steam, resin and wetted material stability (reverse flow and low flow rate give best results.

Sanitisation – inactivates vegetative cells – between batches, 0.5-1M NaOH contact time 30-60 min, prevents microbial growth and build up of endotoxins.
200 purification cycles were performed using a CHO cell culture at 3.3 g/L of a IgG1 monoclonal antibody

The load was 46.1 g/L resin throughout the study, corresponding to 73% of the initial dynamic binding capacity at 10% breakthrough

Loading and operational flow at a 4 minutes residence time

The antibody was eluted with 0.1 M acetic acid pH 4.0

The column was cleaned with 0.1 M NaOH every cycle, with 0.5 M NaOH every 10th cycle. Feed dependant (higher concentration may not be needed)

<table>
<thead>
<tr>
<th>Phase</th>
<th>Buffer</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibration</td>
<td>PBS</td>
<td>5</td>
</tr>
<tr>
<td>Load</td>
<td>3.3 g/L IgG1 feed</td>
<td>N/A</td>
</tr>
<tr>
<td>Chase</td>
<td>PBS</td>
<td>3</td>
</tr>
<tr>
<td>Elution</td>
<td>0.1 acetic acid, pH 4.0</td>
<td>4</td>
</tr>
<tr>
<td>Clean in Place</td>
<td>0.1 M NaOH cycles 1-9, 0.5 M NaOH every 10th cycle.</td>
<td>15 minute contact time</td>
</tr>
<tr>
<td>Re-equilibration</td>
<td>PBS</td>
<td>5</td>
</tr>
</tbody>
</table>
Repligen Lifetime – Praesto Jetted A50 HipH

![Graph showing yield (%) and % of initial DBC (%) over cycle number.](image-url)
Repligen Lifetime – Praesto Jetted A50 HipH

Log removal (HCP)

Cycle number

Monomer content (%)

Cycle number
Affinity Chromatography
Tool box
# Purolite Protein A Resin Portfolio

<table>
<thead>
<tr>
<th>Product</th>
<th>Antibody Type</th>
<th>Capacity</th>
<th>Base Matrix</th>
<th>Customer Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Praesto® Jetted A50</strong></td>
<td></td>
<td>Up to 80 g/l</td>
<td>50 μm Jetted base matrix</td>
<td>Improve productivity and costs of goods</td>
</tr>
<tr>
<td>NGL-Impact™ ligand with very high capacity and alkaline stability (CIP 0.1 to 0.5 M NaOH, 15min R.T)</td>
<td></td>
<td></td>
<td>Uniform beads with a narrow particle size distribution</td>
<td></td>
</tr>
<tr>
<td>Good binding to FAbs of the VH3 family</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Praesto® Jetted A50 HipH</strong></td>
<td></td>
<td>Up to 60 g/l</td>
<td>50 μm Jetted base matrix</td>
<td>High yield at high elution pH (3.5-5.0) Ideal for labile fc containing mabs, fragment and fusion proteins</td>
</tr>
<tr>
<td>very high capacity and alkaline stability (CIP 0.1 M NaOH, 15min R.T)</td>
<td></td>
<td></td>
<td>Uniform beads with a narrow particle size distribution</td>
<td></td>
</tr>
<tr>
<td>Designed to purify 3rd generation molecules, that would precipitate at normal PrA elution conditions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fc-binding</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
OPUS® Pre-packaged Columns

Brodest range of pre-packed columns from bench to production scale
Toolbox of resin formats

Drug Discovery research

Lead Identification/ process development

Phase 0

Phase 1

Phase 2

Phase 3

Filing

Phase 4 Manufacture for patients

OPUS® Robocolumn
OPUS® PipetColumn
OPUS® CentriColumn

OPUS® MiniChrom
OPUS® ValiChrom
OPUS® 5cm
OPUS® 8cm
OPUS® 10cm
OPUS® 14cm
OPUS® 20cm
OPUS® 25cm
OPUS® 30cm
OPUS® 45cm
OPUS® 60cm

0.05mL - 0.6mL
0.2mL - 10mL
2mL – 300mL
0.1L – 1.75L
0.5L – 85L
Summary: Affinity chromatography is:

- Simple to do

- Fast

- Concentrating

- Capture and purification in one column, minimizing subsequent steps needed

- Jetted beads give higher resolution, easier column packing, improved mass transfer, sustainability
Thank you for listening