Evaluating Improvements and Challenges in Affinity Chromatography for AAV Purification

by

Alexandra Dachenko

JHU Graduate Advisor: Dr. Michael Betenbaugh
Supervisor/Second Reader: Dr. Mahsa Hadidi
Manager: Catherine Grimm

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Project Purpose and Goals

1. To evaluate a prototype membrane device coupled with AVB ligand and determine whether it is a superior alternative to column resin for the affinity chromatography step.

   More specifically, to evaluate whether a “plug-and-play” implementation could be achieved with a simple transfer of current process parameters.

2. To investigate low recovery values observed for the affinity step.
Overview

01 Background

02 Materials & Methodology

03 Membrane Device Evaluation

04 Affinity Recovery Investigations
01 Background
AAV PURIFICATION AND AFFINITY CHROMATOGRAPHY
Gene Therapy Platforms: Adeno-Associated Viruses (AAV)

- **Package genetic material into a vector** that can enter specific cell types and provide therapeutic effects
- Different modalities exist to produce effect
- AAVs have been proven as effective vectors for in vivo therapies
- Non-pathogenic with one-time dosage
- **Require co-infection** with “helper” viruses to replicate in human cells
- Recombinant AAV (rAVV) → lacks viral protein coding sequences to increase packing of therapeutic genes
- Different serotypes → possess specific binding receptors and tissue tropism

A diagram of recombinant AAV (rAAV) typically used for in vivo gene therapy. 
Purification: Affinity Chromatography

- Polishing step utilizing a bio-specific ligand coupled to resin
- **Bind-and-elute** operation → impurities flow through unit
- Binding between molecule of interest (MOI) and ligand is *adsorption process*⁷

\[
[L] + [C] \rightleftharpoons [LC], \quad K_a = \frac{k_a}{k_d}
\]

- High \(K_a\) = strong binding affinity
- Elution conditions aim to lower \(K_a\) to cause MOI to unbind⁷

**Ligand unable to distinguish between empty and “full” capsids!⁸**
Membrane vs. Column Chromatography

**Membrane:**
- Usually, several layers of stacked porous membranes
- Larger pore size → faster flow rates
- Higher flow rates → no high pressure drops
- “Pre-packed” and disposable
- Large diameters of fibers limit binding capacity

**Packed Column:**
- Packed with porous resin beads
- Pressure limits restrict to using low flow rates
- Need long residence time for large molecules (i.e. AAVs)
- Require packing efficiency testing

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A representation of the mechanism of mass transfer in column resin and membrane chromatography.

A. membrane chromatography
B. packed bed chromatography
Dynamic Binding Capacity (DBC)

Represents amount of a target molecule that can bind to a chromatography matrix under given process conditions before a significant breakthrough of unbound molecule flows through.¹²

A depiction of a breakthrough curve based on absorbance readings over accumulated volume.

A depiction of a breakthrough curve where the ratio of breakthrough (C/Co) is plotted against effluent volume.¹³

Resin → Longer residence time = higher binding capacity¹¹
Membrane → Flow rate does not affect binding capacity¹¹
Prototype Affinity Membrane Device

**Device Properties**
- Prototype membrane device coupled with AVB ligand
- Electrospun cellulose fiber matrix
- Single-use
- Volume: 0.4 mL
- Compatible with existing resin buffers and chromatography systems

**Unique Attributes**
- Fast flow rates decreases overall time viruses are exposed to harsh elution conditions
- Shorter loading time → potential to eliminate TFF
- Cellulose has strong hydrophilicity and resistant to non-specific adsorption⁹
- Electrospinning allows for the pore sizes to be more easily controlled¹¹
- Smaller diameters increase available surface area for large molecules to bind → increases binding capacity
Based on the evaluations conducted, the prototype membrane device coupled with AVB ligand is not a superior alternative to column resin with the same affinity ligand.
02 Materials & Methodology
Load Material:
• Engineered capsid
• Material has undergone clarification, tangential flow filtration (TFF) and heat viral inactivation
Overview of Affinity Process

- **Conditioning**
- **Equilibration**
- **Load**
- **Wash**
- **Elution**
- **Cleaning-In Place (CIP)**
- **Unbound ligand**

- Target AAV particles
- Host cell contaminants (HCP, HC DNA)

**HCP**: host cell proteins
**HC DNA**: host cell DNA

*Image of a pre-packed column.*

¹⁴
## Buffers

### Main Process

<table>
<thead>
<tr>
<th>Type of Buffer</th>
<th>Main Components of Buffer</th>
<th>Key Characteristics of Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibration</td>
<td>Tris, Sodium Chloride</td>
<td>pH: ~7.0 - 9.0</td>
</tr>
<tr>
<td>Wash</td>
<td>Citrate, Sodium Chloride</td>
<td>pH: ~6.5 - 8.0</td>
</tr>
<tr>
<td>Elution</td>
<td>Citrate, Sodium Chloride</td>
<td>pH: ~2.0 - 3.0</td>
</tr>
<tr>
<td>Neutralization</td>
<td>Tris</td>
<td>pH: ~8.0 - 9.0</td>
</tr>
</tbody>
</table>

Manufacturer provided **2 new** prototypes to test

### Cleaning-in Place (CIP)

<table>
<thead>
<tr>
<th>Type of Solution</th>
<th>Main Components of Solution</th>
<th>Key Characteristics of Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strip</td>
<td>Citrate, Sodium Chloride</td>
<td>pH: 1.5 – 2.5</td>
</tr>
<tr>
<td>Flush</td>
<td>Phosphate-Buffered Saline (PBS)</td>
<td>pH: 7.2 – 7.4</td>
</tr>
<tr>
<td>Base Wash</td>
<td>Sodium Hydroxide</td>
<td>pH: 12</td>
</tr>
<tr>
<td>Acid Wash</td>
<td>PAB</td>
<td>pH: 1.5 – 1.6</td>
</tr>
<tr>
<td>Storage</td>
<td>20% ethanol (at 4°C)</td>
<td>N/A</td>
</tr>
</tbody>
</table>
### Process Conditions

**Resin Conditioning:**

- 3 wash cycles --> elution buffer + equilibration buffer

<table>
<thead>
<tr>
<th>Process Step</th>
<th>RT (min/CV)</th>
<th>RT (min/MV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Load</td>
<td>1.7</td>
<td>1</td>
</tr>
<tr>
<td>Elution</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

**Membrane Conditioning:**

- 1 wash cycle --> elution buffer + equilibration buffer

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*Sanofi*
**Chromatography Technology**

**AKTA Pure 25 systems (Cytiva)**

**UNICORN 7.6 Software**
- Software tool used with AKTA systems
- Allows to:
  - Control system
  - Edit chromatography methods
  - Evaluate chromatograms

**Analytical Technology**

**AAV DRP qPCR**
- Quantitative polymerase chain reaction
- Allows to measure titer of samples (vg/mL)

**Malvern Panalytical Zetasizer**
- Dynamic light scattering (DLS): measures particles size and distribution in sample

**Thermo Scientific™ Invitrogen™ Nanodrop™ One Spectrophotometer**
- Offline absorbance measurement to give approximate concentration of samples
- **Cannot** be used in place of AAV qPCR analytics
03 Prototype Membrane Device Evaluation
Control Runs

RESIN PERFORMANCE WITH LOAD MATERIAL & PROCESS PARAMETERS FOR AFFINITY LIGAND
## Control Run Results – 1 mL column

<table>
<thead>
<tr>
<th></th>
<th>Resin A</th>
<th>Resin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loading Density</td>
<td>2.22e14 vg/mL_resin</td>
<td>2.64e14 vg/mL_resin</td>
</tr>
<tr>
<td>Recovery – FT/Wash</td>
<td>8%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Recovery – Elution</td>
<td>60%</td>
<td>45%</td>
</tr>
</tbody>
</table>

**Chromatogram of control run using Resin A**
Dynamic Binding Capacity Experiments
Learnings from Initial DBC Study

- Manufacturer provided binding capacity data for a scaled-up device at a 1-minute residence time (RT)

<table>
<thead>
<tr>
<th>Loading Density</th>
<th>8.74e13 vg/mL\text{membrane}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Load RT</td>
<td>1.7 minute/MV</td>
</tr>
</tbody>
</table>

- No visible breakthrough in A254 profile
- Requires 7.5 MVs of transition, column only requires 1 CV

<table>
<thead>
<tr>
<th>Recovery – FT/Wash</th>
<th>&lt;2%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery - Eluate</td>
<td>17%</td>
</tr>
</tbody>
</table>

Chromatogram of main process
Learnings from Initial DBC Study

**Observations:**
- 10% breakthrough not reached!

**Lessons Learned:**
- Manufacturer said that they have observed in the past that if loading density is <50% capacity of unit, there can be re-binding events happening in the elution causing low product recovery.
- Incomplete mass balance might also suggest current resin elution buffer is not effective in recovering product in the membrane.
Approach of Subsequent DBC Studies

1. “Push” more material through the unit to ensure breakthrough and mitigate re-binding events

2. Lower binding RT from 1.7 minute to 1 minute

**Rationale:** The prototype membrane does not have the same mass transfer limitations as resin, so a shorter binding residence time should be as effective.

3. Attempt at least 3 cycles of use to assess capacity drop
Key Takeaway:

- Although designed to be single-use, the prototype membrane device can be cycled at least 3x without a major capacity drop.
Elution Investigations
Arginine is an osmolyte often used in monoclonal antibody (mAb) manufacturing

- Can increase stability and help reduce aggregation of AAVs\(^\text{18}\)
- Higher salt can prevent aggregation
- Ionic strength of the solution disrupts the electrostatic interactions between the virus particles and increases vector solubility\(^\text{19}\)

**Evaluating a New Elution Buffer**

- 50 mM Citrate, 500 mM Sodium Chloride, 500 mM Arginine, pH 2.5

**Current Elution Buffer:** Sodium Citrate, Sodium Chloride, pH ~2.0 – 3.0

Utilized an “old” device to preserve new device → expected some product would be lost to FT
Key Takeaways:
- New buffer more effective for prototype membrane device than elution buffer used for resin with same ligand
- Elution peak has a shoulder
Evaluating Capsid Binding Affinity to Ligand

**Key Takeaways:**
- Eluted at beginning at 464 mM NaCl (condo = 22.5 mS/cm)
- Indicates our capsid may have weaker affinity to the ligand

**Approach:**
- Elution with linear gradient of NaCl
- Shallow gradient with slope of 23 mM/mL NaCl
- If weakly bound, expected to elute off right at beginning of gradient

*Used “old” device*

<table>
<thead>
<tr>
<th>Process Phase</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT</td>
<td>56.6%</td>
</tr>
<tr>
<td>Washes</td>
<td>5.7%</td>
</tr>
<tr>
<td>Eluate (main peak)</td>
<td>12%</td>
</tr>
<tr>
<td>Strip</td>
<td>5.7%</td>
</tr>
</tbody>
</table>
Non-Specific Binding Evaluation
Is the AAV binding to cellulose?

- Manufacturer was unable to provide a prototype membrane device stripped of AVB ligand
- Alternative → use affinity device with similar base matrix but with ligand used for mAbs
  - Designed for mAbs → should not bind AAV!

**Goal:** To test if the membrane has any non-specific binding phenomena, contributing to lower recovery in elution than observed in column resin.
**Key Takeaway:**

- Likely no non-specific binding of AAV to cellulose matrix
- Potential binding of impurities to base matrix based on CIP chromatogram (can also be other uncharacterized species, i.e. leached ligand)

### Process Phase Table

<table>
<thead>
<tr>
<th>Process Phase</th>
<th>Recovery @ pH &gt; 2</th>
<th>Recovery @ pH 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT &amp; Wash</td>
<td>141%</td>
<td>99%</td>
</tr>
<tr>
<td>Elution</td>
<td>6%</td>
<td>0.05%</td>
</tr>
</tbody>
</table>

### Loading Density

<table>
<thead>
<tr>
<th>Loading Density</th>
<th>Elution @ pH &gt; 2</th>
<th>Elution @ pH 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.63e13 vg/mL&lt;sub&gt;membrane&lt;/sub&gt;</td>
<td>9.45e13 vg/mL&lt;sub&gt;membrane&lt;/sub&gt;</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** CIP not collected because mAb affinity device uses 0.5 M NaOH cleaning solution
Prototype Affinity Membrane Device: Performance
DISCUSSION AND FUTURE WORK
Why is the device not a superior alternative (right now)?

**Advantages:**
- Single-use → does not need to packed and evaluated for column efficiency like resin
- Fast flow rates and low residence times
- Similar binding capacity to resin at lower RT

**Disadvantages:**
- Requires additional process development efforts to match performance to resin with same ligand
- Serotype-dependence for ligand in different base matrices

**Future Work: *after device commercialization***
- Further DBC studies with more flowthrough fractions
- Analyze impurity clearance
- Investigate optimal elution buffer
- Investigate different residence times (loading and elution)
- Test different serotypes
- Test pre-TFF material
04 Recovery Investigations
UNDERSTANDING WHERE THE AAV “GOES” DURING THE AFFINITY STEP
Non-Specific Binding Evaluation Using Resin

Approach:
- Evaluate if there is non-specific binding to resin using an affinity resin with a ligand designed for mAbs
- Has similar base matrix to affinity resin evaluated

<table>
<thead>
<tr>
<th>Process Phase</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT &amp; Wash</td>
<td>99%</td>
</tr>
<tr>
<td>Elution</td>
<td>0%</td>
</tr>
</tbody>
</table>

Key Takeaways:
- No non-specific binding to resin bead matrix which can cause product loss!

A254 profiles of load and flowthrough lined up (as expected!)

No elution peak!
Using Pre-Purified Load Material

Resin B (2.5 mL)

Diluted to match titer of load material

Diluted with Equilibration Buffer

Resin B (1 mL)

>90% recovery

Diluted with FT of 2.5 mL

Resin B (1 mL)

>90% recovery
Key Takeaways:
- Pre-purified material does yield higher recovery
- Adding back in impurities yields same recovery
- Uncharacterized binding interaction in initial feed stream leading to lower recovery
Thank you!

Huge thank you to my manager, Catherine Grimm, for her mentorship over the past 6 months. Also, thank you to Mahsa Hadidi for all her guidance and advice. Lastly, thank you to Sanofi’s GMU CMC Purification Process Development group for the opportunity to learn from incredible scientists!

Thank you Dr. Betenbaugh for being my advisor for the past 5 years and for all of your support!
Literature Cited


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