# Development and Optimization of a Hydrophobic Interaction Chromatography-Based Method of AAV Harvest, Capture, and Recovery

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**With many ongoing clinical trials utilizing adeno-associated virus (AAV) gene therapy, it is necessary to find scalable and serotype-independent primary capture and recovery methods to allow for efficient and robust manufacturing processes. Here, we demonstrate the ability of a hydrophobic interaction chromatography membrane to capture and recover AAV1, AAV5, AAV8, and AAV "Mutant C" (a novel serotype incorporating elements of AAV3B and AAV8) particles from cell culture media and cell lysate with recoveries of 76%–100% of loaded material, depending on serotype. A simple, novel technique that integrates release and recovery of cell-associated AAV capsids is demonstrated. We show that by the addition of lyotropic salts to AAV-containing cell suspensions, AAV is released at an equivalent efficiency to mechanical lysis. The addition of the lyotropic salt also promotes a phase separation, which allows physical removal of large amounts of DNA and insoluble cellular debris from the AAV-containing aqueous fraction. The AAV is then captured and eluted from a hydrophobic interaction chromatography membrane. This integrated lysis and primary capture and recovery technique facilitates substantial removal of host-cell DNA and host-cell protein impurities.**

## INTRODUCTION

Gene and cell therapies have been demonstrated to be potent and efficient biopharmaceuticals in multiple pre-clinical and clinical trials, with marketing authorization granted by regulatory agencies for three adeno-associated virus (AAV)-based products.[1,2] AAV is a powerful gene delivery vehicle capable of safely transducing a variety of tissues to provide long-term expression and is the vector of choice for nearly 8% of all gene therapy clinical trials since 1989, with 204 clinical trials open worldwide as of 2017.[3] However, production of clinical material often relies on inefficient (e.g., size exclusion chromatography) or expensive and serotype-specific (e.g., affinity) downstream primary capture processes.[4–6] Because primary capture and recovery of AAV particles is a major challenge in the large-scale manufacturing of AAV, scalable and serotype-independent primary capture and recovery methods based on single-use high-flow chromatography membranes offer an attractive alternative to conventional chromatography methods for efficient and robust AAV manufacturing.

Recombinant AAV vectors are produced through mammalian or insect cell culture utilizing transfection with plasmids or transduction with viral vectors, respectively, containing the DNA required for assembly and packaging of the AAV vector.[6–8] AAV is then either released into the cell culture media or retained within the cell, and the ratio of retained and released AAV is dependent upon time in culture and AAV serotype.[9,10] Whether an AAV particle is released or retained by its producer cell significantly impacts the techniques used to harvest the AAV from cell culture. In order to release cell-associated AAV, mechanical or chemical lysis methods are employed. At a small laboratory scale, repeated cycles of freezing and thawing are frequently used to release cell-associated AAV, while at a larger scale high-pressure microfluidization or homogenization can be employed.[10] Large-scale physical lysis methods require a significant capital investment and are not easily utilized when producer cells are grown within a solid support such as a perfusion bioreactor.[11] Chemical methods such as using detergent to release AAV from cells can be highly successful, however, the detergent then becomes an impurity that has to be removed to acceptable limits and poses a challenge to downstream processing. Furthermore, use of one of the most popular detergents, Triton X-100, was recently restricted in the European Union.[12,13] Inorganic salts such as NaCl have also been shown to be effective in releasing cell-associated AAV particles from producer cells.[9,14]

Capture and recovery of AAV particles from cell culture media or lysate follows standard techniques used for recombinant proteins including precipitation, centrifugation, chromatography, and tangential flow filtration.[6,10,15] Chromatographic methods such as ion exchange, affinity, and hydrophobic interaction have demonstrated an ability to bind and elute AAV capsids with high recoveries and varying degrees of selectivity.[4,5,16–21] Affinity chromatography allows for
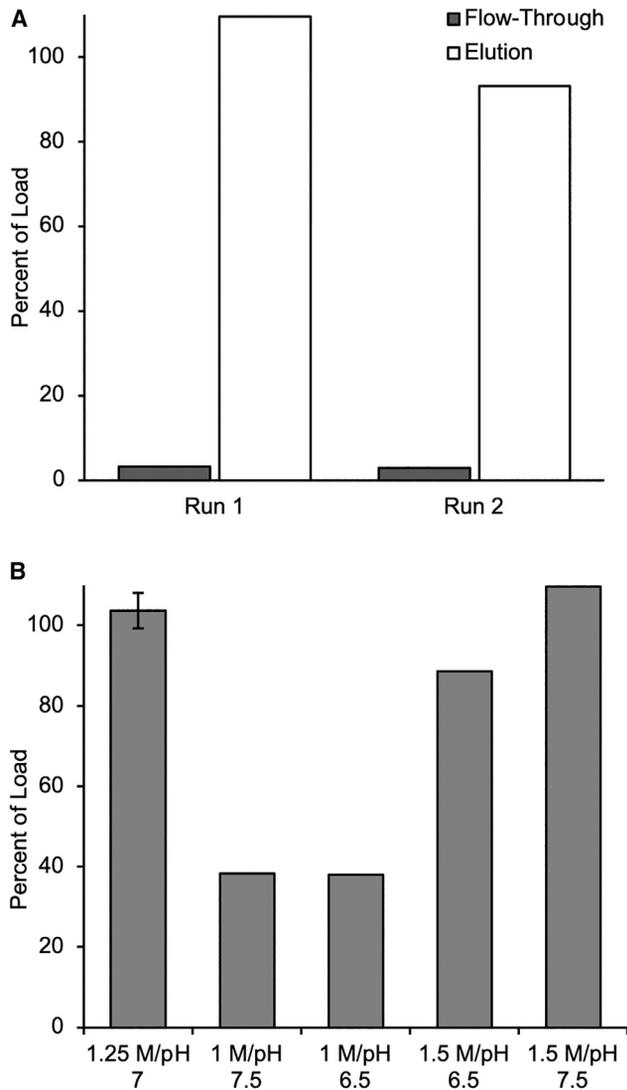
**Figure 1. Testing and Optimization of a Hydrophobic Interaction Membrane Adsorber for AAV8 Capture and Recovery**

(A) AAV8 capsids recovered in the flow-through and elution fractions after application of cell culture media to a Sartobind Phenyl membrane. Media were adjusted to 1.5 M ammonium sulfate pH 7.5, applied to a phenyl membrane, and eluted with 20 mM Tris pH 7.5. These two runs demonstrated effective capture and recovery of AAV8 capsids, with an average 3.1% of loaded particles in the flow-through and 101% in the elution fraction. (B) AAV8 capsids recovered in the elution fraction after application of culture media to the phenyl membrane. Design of experiments was used to find the optimal pH and salt concentration for loading the phenyl membrane. Media were adjusted to either 1, 1.25, or 1.5 M ammonium sulfate at either pH 6.5, 7.0, or 7.5 and eluted with 20 mM Tris pH 6.5, 7.0, or 7.5. n = 1 for all groups except the midpoint, 1.25 M ammonium sulfate at pH 7, in which n = 3 and error bars represent the standard deviation. All capsid measurements were made using an AAV8 capsid ELISA.

highly efficient capture and elution of AAV particles, with high recoveries and purities.[22] Additionally, affinity ligands are composed of camelid antibodies, which are expensive and a potential source of impurities in the form of leeched ligand.

Ion exchange chromatography is frequently employed in downstream processes for viral gene therapy vectors, with particular interest in membrane-based chromatography sorbents due to their large pores, dependence on convective mass transfer, and high flow rates.[23,24] Both cation and, more commonly, anion exchange chromatography sorbents have been repeatedly demonstrated to be effective at binding and eluting AAV particles with high recoveries and have even demonstrated an ability to separate genome-containing and genome-free (empty) AAV particles.[6,25–28] While ion exchange can be considered serotype-independent, its utility can be limited by the buffer the AAV is in or the solubility of the serotype. For example, most ion exchange ligands cannot capture AAV directly from cell culture media due to the high conductivity of the solution. Additionally, AAV2 is highly insoluble in low conductivity buffers and therefore it is challenging to bind and elute AAV2 from an ion exchanger.[29]

Hydrophobic interaction chromatography (HIC) is often utilized in downstream processes for biopharmaceuticals.[30] However, there is only one report on the use of HIC in the purification of a single serotype of AAV.[19] HIC generally requires the use of high conductivity buffers for binding, e.g., ammonium sulfate, which is also often utilized to concentrate AAV by precipitation.[31,32]

Simple, robust, and scalable manufacturing processes are vital for successful production of drug product through pre-clinical testing and clinical trials, and into licensure. Here we demonstrate a novel, integrated method to chemically release AAV particles from cells that not only allows for significant removal of host cell impurities, including nucleic acids, but also provides the conditions for highly efficient binding and recovery from a hydrophobic interaction membrane. We evaluate the HIC membrane with AAV1, AAV5, and AAV8 serotypes. We also evaluate the HIC membrane with a novel, human hepatocyte-tropic serotype, AAV "Mutant C" (AAV-MutC), which is composed of sequences from AAV8 and AAV3B capsids.[33] We demonstrate efficient primary capture and recovery from both cell culture media and lysates for all serotypes tested. We demonstrate that this method is robust and potentially serotype-independent, provides significant capacity and impurity removal, and results in high vector potency, therefore making this process highly flexible, disposable, and amenable to scale-up.

## RESULTS

### Testing a Hydrophobic Interaction Membrane Adsorber for AAV8 Capture and Recovery

AAV8 was harvested from cell culture media, clarified, and adjusted to 1.5 M ammonium sulfate, 20 mM Tris-HCl pH 7.5. The adjusted cell culture media was applied to a phenyl membrane and eluted using 10 mM bis-tris propane (BTP), 10 mM Tris, pH 7.5. Over two runs, an average of 3.1% (3.0% in the first run, 3.2% in the second run) of loaded AAV8 capsids were detected in the flow-through while an average of 101% (110% in the first run, 93% in the second run) of loaded capsids were found in the eluate (Figure 1A). Capsid measurements were made using an AAV8 capsid ELISA (measurements showing greater than 100% recovery are due to inherent variability of the ELISA assay).
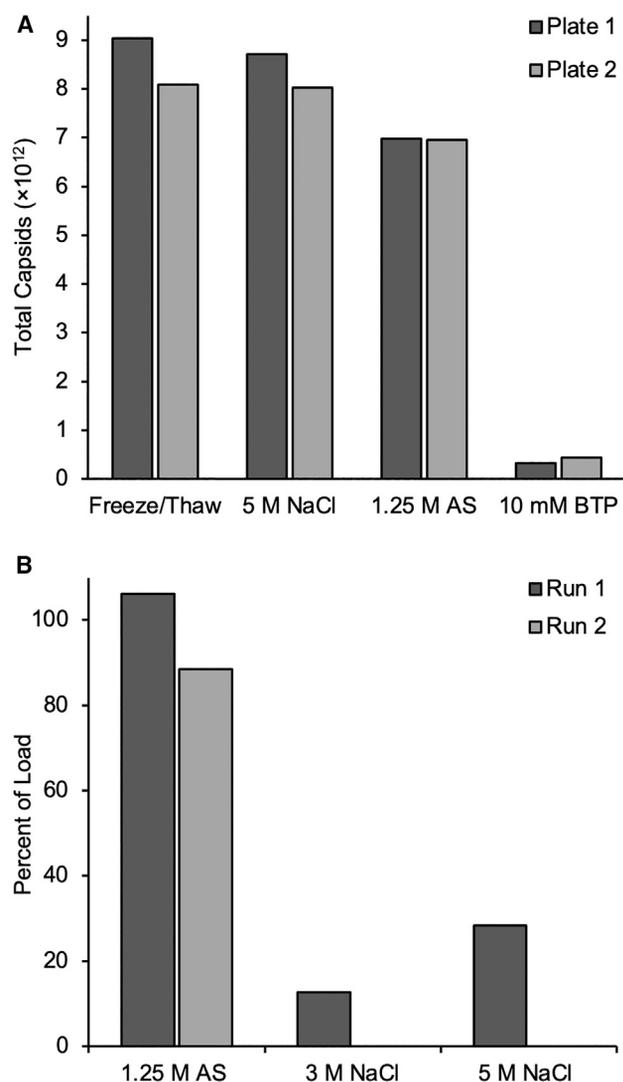
**Figure 2. Investigation into Release of Cell-Associated AAV Particles**

(A) Capsid titer of cells transfected to produce AAV-MutC after lysis via five repeated freeze/thaw cycles or contact with 5 M NaCl, 1.25 M ammonium sulfate, or 10 mM BTP, 10 mM Tris pH 9, showing that salt can be used to effect AAV particle release from cells (n = 2 per group; 10 mM BTP group is statistically significantly different as measured by one-way ANOVA with Tukey post hoc analysis). (B) Recovery of AAV-MutC particles after application to a phenyl membrane after release with 1.25 M ammonium sulfate or 5 M NaCl. The material released through contact with 5 M NaCl was also diluted to 3 M NaCl and applied to the phenyl membrane. Elution from the membrane was performed with the application of 20 mM Tris pH 7. This indicates that the more lyotropic salt, ammonium sulfate, leads to a greater recovery of AAV. Capsid titer was via AAV8 capsid ELISA.

## Optimization of AAV8 Recovery from a Hydrophobic Interaction Membrane Adsorber via Design of Experiments Methodology

A two-factor, two-level full factorial design was generated using MODDE to examine the optimal pH, from 6.5–7.5, and optimal ammonium sulfate concentration, from 1.0–1.5 M, for recovering AAV in culture media from a phenyl membrane. At 1.5 M ammo-

nium sulfate, pH 7.5, 110% of loaded capsids were recovered in the elution fraction. This fell to 89% for 1.5 M, pH 6.5. By contrast, 1 M with pH 6.5 and 7.5 led to recoveries of only 38% in the elution fraction for each condition, with 41%–57% of capsids in the flow-through. However, a midpoint of 1.25 M and pH 7 led to a recovery of 100% ± 4.4% of loaded AAV8 capsids in the elution fraction (n = 3 for this group, n = 1 for all others; Figure 1B).

## Release of Cell-Associated AAV Particles

Four duplicate sets of 10 cm tissue culture plates of HEK293T/17 cells were cultured to produce AAV-MutC particles, which are predominantly found in lysate at the time of harvest, in contrast to AAV8 (see Figure S1 for distribution of AAV-MutC particles; the phenomenon of serotype-dependent release into culture medium, including AAV8, has been described previously[10,34]). Cells were harvested 3 days post-transfection and lysed via repeated cycles of freeze/thaw or via addition of salt solutions directly to cell culture plates after removal of culture media (stated as final concentrations; additional details in Materials and Methods): 5 M NaCl; 1.25 M ammonium sulfate; or 10 mM BTP, 10 mM Tris, pH 9. The total quantity of released capsids was assessed by AAV8 capsid ELISA (because AAV-MutC includes capsid sequences from AAV8, it contains the conformational epitope required to bind the ADK8 antibody). Figure 2A shows that statistically similar quantities of AAV are released by freeze/thaw ($8.1–9.0 \times 10^{12}$ particles), 5 M NaCl ($8.0–8.7 \times 10^{12}$ particles), and 1.25 M ammonium sulfate ($6.9–7.0 \times 10^{12}$ particles), as measured by ANOVA, with the 10 mM BTP groups releasing significantly fewer capsids ($3.3–4.3 \times 10^{11}$ particles; n = 2 per group; $F_{3,4} = 172.13$, p < 0.001).

## Direct Application of AAV-Containing Lysate to a Hydrophobic Interaction Membrane

The lysate produced using ammonium sulfate and sodium chloride from the previously described section was filtered and directly loaded onto a phenyl membrane or, in the case of the 5 M NaCl lysate, directly loaded or diluted to 3 M NaCl and then loaded to assess binding of AAV in different concentrations of NaCl. The material lysed in 1.25 M ammonium sulfate and loaded directly onto the membrane showed recoveries of 88% and 106% (n = 2), while the 5 M NaCl lysate yielded only 28% and the sample diluted to 3 M NaCl yielded only 13% of loaded particles, with the remaining particles in the flow-through (n = 1 each; Figure 2B). Therefore, 1.25 M ammonium sulfate was used for all subsequent experiments with the phenyl membrane (with the exception of AAV5; see below).

## Determination of the Dynamic Binding Capacity of a Hydrophobic Interaction Membrane for AAV Particles

AAV8-containing cell culture media with 1% serum and AAV-MutC-containing cell lysate were generated through culture and transient transfection of HEK293T/17 cells. These fractions were then applied to a phenyl membrane and the concentration of AAV capsids in the flow-through was measured using AAV8 capsid ELISA, which

**A**



**B**
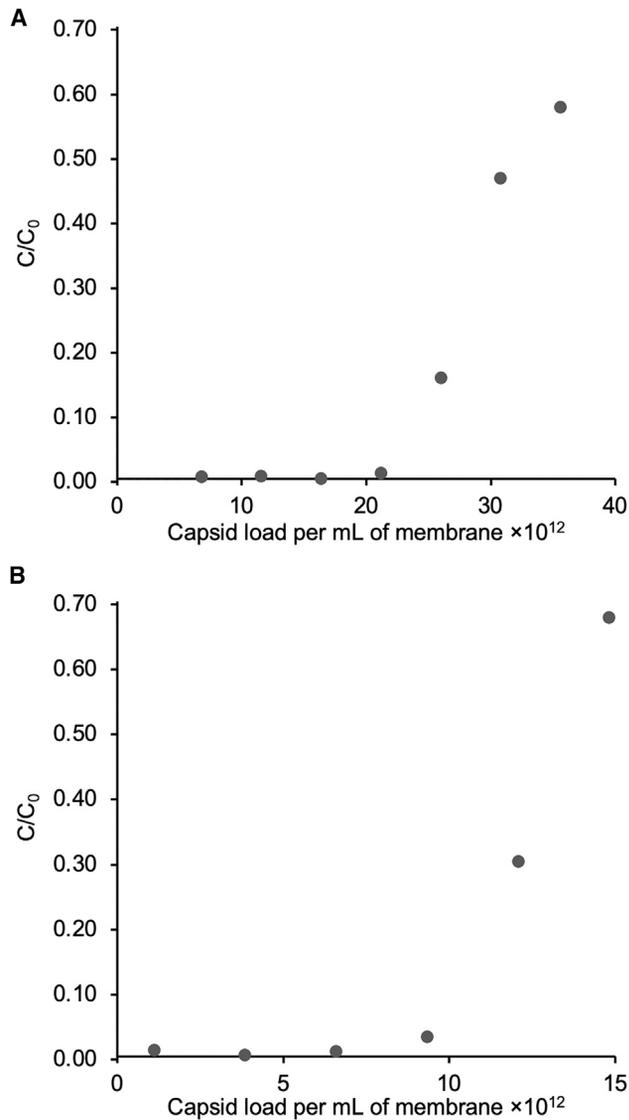


**Figure 3. Determination of the Dynamic Binding Capacity of a Hydrophobic Interaction Membrane for AAV Particles**

(A and B) Scatterplots show the dynamic binding capacity of the phenyl membrane with (A) AAV8 particles in culture media with 1% serum, and (B) AAV-MutC particles from cell lysate. Capsid concentration in the flow-through was compared to concentration in the load ($C/C_0$). AAV8 media were adjusted to 1.25 M ammonium sulfate, pH 7 prior to loading at $7.7 \times 10^{11}$ capsids/mL and applied at 5 membrane volumes per min, and demonstrate a dynamic capacity at 10% breakthrough of approximately $2 \times 10^{13}$ capsids per milliliter of membrane. AAV-MutC capsids were released from cells by lysis in 1.25 M ammonium sulfate, 20 mM Tris pH 7, filtered, and applied to the phenyl membrane at a concentration of $4.4 \times 10^{11}$ capsids/mL at 5 membrane volumes per minute, demonstrating a dynamic capacity at 10% breakthrough of approximately $1 \times 10^{13}$ capsids per milliliter of membrane. All capsid measurements were made by AAV8 capsid ELISA.

allowed the dynamic capacity of the membrane for AAV capsids to be assessed. Figure 3A shows the breakthrough curve for AAV8 in cell culture media with 1% serum. This demonstrates a dynamic capacity

at 10% breakthrough of approximately $2 \times 10^{13}$ capsids/mL of membrane with a load concentration of approximately $7.7 \times 10^{11}$ capsids/mL. Figure 3B shows the breakthrough curve of lysate containing AAV-MutC, demonstrating a dynamic capacity of $1 \times 10^{13}$ capsids/mL of membrane with a load concentration of $4.4 \times 10^{11}$ capsids/mL.

### Investigation into Phase Separation of Nucleic Acids from AAV Capsids During Lysis

A 10-layer cell stack containing HEK293T/17 cells was cultured and transfected with plasmids required for AAV-MutC production. 3 days post-transfection, the cell culture media was removed and the cells were harvested using a PBS-EDTA solution. A 3.5 M ammonium sulfate solution was added to the PBS-EDTA/cell mixture to a final concentration of 1.25 M ammonium sulfate and mixed thoroughly. This mixture was incubated at 4°C for 15 to 19 h (i.e., overnight) without agitation. During incubation, a phase separation occurred and a white non-aqueous layer formed at the top of the lysate/ammonium sulfate mixture. The non-aqueous, low density material was subsequently physically removed. Figure S2 shows this insoluble layer and demonstrates the dispersion of this non-aqueous material when treated with Benzonase nuclease for 1 h at 37°C, compared to a nuclease-free control. PicoGreen analysis also reveals high concentrations of double-stranded DNA (dsDNA) in the non-aqueous fraction and a reduction of greater than 90% of total dsDNA when this layer is removed (data not shown). Note that, due to the high concentration of ammonium sulfate prior to HIC, nucleases were generally not used in this process until after capture and recovery over the HIC membrane.

### Investigation into Impurity Removal Using a Hydrophobic Interaction Membrane

AAV-MutC-containing material in 1.25 M ammonium sulfate was loaded onto a 150 mL phenyl membrane and eluted using a step gradient to 40% and 100% 20 mM Tris pH 7.0 (Buffer B). The load, flow-through, wash, 40% eluate, and 100% eluate fractions were then assayed for capsid ELISA, dsDNA via picogreen, and host-cell protein (HCP) via ELISA. The 40% elution fraction contained an average of 90% of loaded particles, while the 100% elution fraction contained less than 10% of loaded particles (Figure 4A). Figure 4B shows a greater than 90% reduction in total HCP from the load to the 40% elution fraction, and a greater than 80% reduction in dsDNA from the load to the 40% elution fraction (Figure 4C, n = 2), not including the intact dsDNA physically removed from the lysate prior to loading.

### Protein Analysis

A representative AAV-MutC sample in 1.25 M ammonium sulfate was loaded over a 3 mL phenyl membrane and eluted as described above. The protein concentrations in the load, flow-through, wash, 40% eluate, and 100% eluate fractions were assessed by Micro BCA (Figure 4D) and samples were normalized, where possible, and analyzed by silver stained gel (Figure 4E) and SDS capillary gel

## Capture and Recovery of Different Serotypes of AAV Using a Hydrophobic Interaction Membrane

AAV1 and AAV5 was produced in 10-layer cell stacks and harvested at 3 days after transfection. Culture media was decanted and mixed with 3.5 M ammonium sulfate, 60 mM Tris, pH 7.0 to a final concentration of 1.25 M ammonium sulfate for AAV1 and 1 M ammonium sulfate for AAV5. Cells were detached using PBS+EDTA and mixed with concentrated ammonium sulfate to a final concentration of 1.25 M for AAV1 and 1 M for AAV5. A lower concentration of ammonium sulfate was used for AAV5 after it was discovered that AAV5 particles in 1.25 M ammonium sulfate were overwhelmingly lost in filtration prior to HIC (see Discussion). AAV1- or AAV5-containing cell culture media or cell lysate was applied to a 3 mL phenyl membrane and step-eluted using 20 mM Tris-HCl pH 7.0. The concentration of AAV capsids in the load, flow-through, and elution fractions were measured using AAV1 or AAV5 capsid ELISA. AAV1 was efficiently captured and eluted, with an average recovery of over 90% from lysate and over 85% for clarified cell culture media. AAV5 was bound and recovered slightly less effectively, with an average of over 80% recovered in lysate and over 76% recovered in media. See

electrophoresis (CGE; Figure 4F). Micro BCA demonstrated an 80% decrease in total protein from the load to the 40% elution fraction, with over half of the loaded protein in the 100% elution fraction. SDS CGE shows each phenyl membrane fraction in comparison to an AAV-MutC sample that was captured and recovered over HIC and further purified via AVB affinity chromatography, in which only the VP1, VP2, and VP3 peaks are present (the ratio of VP1, 2, and 3, respectively, measured as a function of peak area is 1.0 to 0.9 to 6.9, though this may not be directly proportional to the number of molecules of the VP proteins since they may have different extinction coefficients). The VP3 peaks are further evident in the signals for the load, 40% eluate, and the 100% eluate fractions, while VP2 and VP1 are also evident in the signal for the 40% elution fraction.

**Table 1. Recovery of AAV Serotypes Over 3 mL Phenyl Membrane**

| | Media | | Lysate | |
|---|---|---|---|---|
| Serotype | Run 1 | Run 2 | Run 1 | Run 2 |
| AAV1 | 86.4 ($1.58 \times 10^{13}$) | 88.6 ($2.03 \times 10^{13}$) | 98.3 ($6.15 \times 10^{13}$) | 88.8 ($5.23 \times 10^{13}$) |
| AAV-MutC | 84.3 ($5.62 \times 10^{11}$) | 94.8 ($5.69 \times 10^{11}$) | 106.2 ($6.48 \times 10^{12}$) | 88.4 ($6.70 \times 10^{12}$) |
| AAV5 | 72.2 ($1.70 \times 10^{13}$) | 81.4 ($1.66 \times 10^{13}$) | 75.5 ($5.50 \times 10^{13}$) | 86.9 ($6.61 \times 10^{13}$) |
| AAV8 | 109.5 ($7.08 \times 10^{13}$) | 93.2 ($7.31 \times 10^{13}$) | 91.3 ($2.73 \times 10^{13}$) | 92.0 ($2.75 \times 10^{13}$) |

Percent recoveries are listed first, followed by eluted capsids in parentheses.

Table 1 for a summary of recoveries from all serotypes and fractions tested, and see Table S1 for a summary of AAV5 recovery during filtration in different concentrations of ammonium sulfate.

### Assessment of Vector Potency

AAV-MutC particles carrying a Factor IX (FIX) transgene were used to transduce HuH7 cells, after which AAV vector potency was measured. AAV-MutC particles were recovered either over the phenyl membrane as described above; harvested by freeze-thaw of cells and purified over AVB affinity resin (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA), as described previously,[5] with no contact with ammonium sulfate or the phenyl ligand; or recovered over the phenyl membrane and then purified using AVB affinity resin. Vector potency (expressed as the normalized ratio of FIX activity over the amount of FIX antigen produced in HuH7 cells) was comparable between the groups, at $5.71 \pm 0.27$ for the freeze-thaw-AVB group, $5.65 \pm 0.14$ for the phenyl group, and $5.39 \pm 0.12$ for the phenyl-AVB group (Figure 5A; all transductions were performed in triplicate). One sample from each group was assessed by isolating DNA from HuH7 cells and quantifying viral genomes using qPCR. Figure 5B shows this data as viral genomes per cell for the above-mentioned groups, and shows comparable transduction efficiency with each method, with 8,900 vg/cell for the freeze-thaw-AVB group, 7,200 vg/cell for the phenyl group, and 8,600 vg/cell for the phenyl-AVB group.

### DISCUSSION

We demonstrated here highly efficient direct primary capture and recovery of AAV particles from cell culture media and cell lysates using a HIC phenyl ligand, with up to 99.9% of loaded AAV vectors being bound to the adsorber. Furthermore, we demonstrated that with a phenyl HIC membrane, it is possible to capture and elute multiple serotypes across several clades of AAV with high recoveries, allowing for a potentially serotype-independent method of AAV harvest and primary capture and recovery.[35] Binding and elution of AAV1, AAV-MutC, and AAV8 particles in culture media and cell lysates from the phenyl membrane resulted in average recoveries of greater than 87% of loaded AAV particles. Interestingly, AAV5 binding and recovery was not as high as the other serotypes, though it was

consistently above 70%. It was found that for AAV5 samples in 1.25 M ammonium sulfate, the concentration used for all other serotypes, particles were overwhelmingly lost in filtration prior to loading the membrane (Table S2). Therefore, a lower concentration of 1 M ammonium sulfate was used to load AAV5 particles over the phenyl adsorber. It may have been possible to increase the concentration of ammonium sulfate to 1.25 M after filtration of AAV5, but this was not attempted. Whether this could work would depend on whether 1.25 M ammonium sulfate causes precipitation of AAV5 or simply alters the interaction between AAV5 and the filter. It is also possible that increasing the concentration of ammonium sulfate after filtration would precipitate other proteins, increasing the burden on the HIC membrane. While 1 M ammonium sulfate led to lower recoveries for AAV8, as demonstrated in Figure 1B, it was sufficient for adequate recovery of AAV5. It should be noted that other studies have described several unique features of AAV5 relative to other serotypes, including its protein sequence.[36–38] These features may lead to a unique interaction between the AAV5 capsid and the phenyl ligand.

We further demonstrated binding capacities for the phenyl adsorber, which to our knowledge is the first published report of the dynamic binding capacity of any non-affinity adsorber for AAV. The phenyl membrane demonstrated a capacity at 10% breakthrough of up to $2 \times 10^{13}$ capsids per milliliter of membrane for AAV8, and $1 \times 10^{13}$ capsids per milliliter of membrane for AAV-MutC. The capacity of the phenyl membrane for AAV8 is within the range of the published capacity of available AAV8 affinity resins, which claim a capacity of greater than $1 \times 10^{13}$ viral genomes per mL of resin.[39] However, the capacity reported is lower than that extrapolated from currently available literature. Chahal et al.[19] reported the ability to bind approximately $2.7 \times 10^{14}$ capsids per milliliter of Butyl-650M resin, potentially demonstrating that traditional resin-based chromatography sorbents have greater capacity for AAV capsids. However, membrane adsorbers are available in a variety of sizes to process at scale and allow for much faster flow rates. With the knowledge of these binding capacities, effective and robust downstream processes based on these membrane adsorbers can be designed.

The use of high salt concentration to promote release of cell-associated AAV in the absence of cell lysis has been previously demonstrated with AAV6 and AAV9.[9] However, we show here that lyotropic salts can be used to effect cellular lysis and AAV release, resulting in an integrated process that not only allows for effective capture and recovery of AAV particles on a phenyl adsorber, but also provides phase separation of dsDNA-containing insoluble material from the AAV-containing fraction, reducing total dsDNA by more than 90%. Furthermore, the phenyl membrane was shown to be very effective at enriching AAV vectors from clinically relevant impurities in the media and cell lysate, such as HCP and additional dsDNA, while maintaining vector potency. Using an AAV-MutC capsid that was bound and eluted from the phenyl membrane with a recovery of 90%, HCP levels were reduced by 90% and dsDNA by 80%. The extent to which AAV was enriched over the phenyl membrane is evident from the total protein results and, especially, SDS CGE
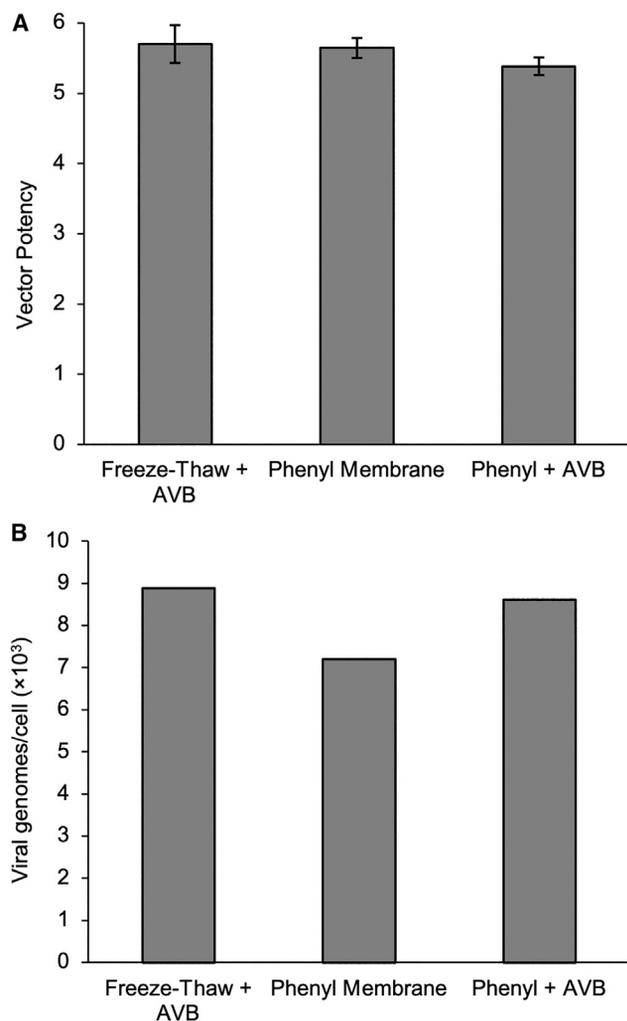
**Figure 5. Assessment of Vector Potency After Phenyl Membrane Purification**

AAV-MutC carrying a Factor IX (FIX) transgene were used to transduce HuH7 cells, after which AAV vector potency was measured. AAV-MutC particles were recovered from the phenyl membrane, harvested by freeze-thaw of cells and purified over AVB affinity resin, with no contact with ammonium sulfate or the phenyl ligand, or recovered from the phenyl membrande and further purified using AVB affinity resin. (A) Vector potency (expressed as the fold change in FIX activity normalized by FIX antigen expression in HuH7 cells) was comparable between all groups. Transductions were performed in triplicate. Error bars represent standard deviation. (B) DNA was isolated from HuH7 cells and AAV genomes were quantified using qPCR and normalized by cell number to measure transduction efficiency, which is comparable between all groups (n = 1 per group).

results, in which the AAV proteins VP1, VP2, and VP3 are visible on the electropherogram from the 40% elution fraction. This demonstrates the potential of HIC for highly effective primary capture and recovery of AAV-derived gene therapy vectors. In the future, it may be possible to further optimize loading conditions to increase impurities in the flow-through, and elution conditions to further reduce impurities in the main AAV-containing fraction.

Multiple scale-up scenarios can be envisioned for this process. Most obviously from the work described herein is scale-out using adherent cells in a large number of cell stacks. A more elegant method would employ a fixed-bed bioreactor such as an iCELLis or scale-X and, especially where the AAV product is primarily associated with the cells, it would be a simple matter to pump out the media and replace it with a buffer containing ammonium sulfate to effect lysis. Transitioning to suspension cells would also be possible. To concentrate cells within the media for simultaneous processing, tangential flow filtration with hollow fiber membranes could be utilized. This would allow for lysis in a lower volume of ammonium sulfate and reduce the total protein burden prior to addition of ammonium sulfate. Alternatively, continuous centrifugation could be employed to collect the cells for lysis via ammonium sulfate, followed by recombining with media or separate fraction processing, as desired.

In conclusion, we demonstrate a highly effective chromatography membrane technique for primary capture and recovery of AAV vectors from cell culture media and lysate. We also demonstrate that high concentrations of ammonium sulfate will release cell-associated AAV from producer cells and lead to phase separation of dsDNA-containing insoluble impurities from the aqueous AAV-containing fraction. The use of a high-salt buffer provides for effective capture and recovery of multiple serotypes of AAV from a phenyl adsorber. This has important implications for large-scale production, as this novel technique is amenable to either suspension or fixed bed adherent cell culture. This simple, robust, and scalable process integrates release and primary capture and recovery of AAV vectors and is compatible with multiple industrially relevant serotypes of AAV.

## MATERIALS AND METHODS
### Chemicals and Reagents
All chemicals were purchased from Sigma Aldrich and were of molecular biology grade or higher.

### Cell Culture and Transfection
Adherent HEK293T/17 cells[40] were cultured in 10 cm plates or 10-layer cell stacks (Corning, Corning, NY, USA) and transfected using serum-free DMEM or DMEM supplemented with 1% FBS and 2 mM GlutaMAX (Life Technologies, Grand Island, NY, USA). AAV was produced by two-plasmid transfection using PEIpro (Polyplus-transfection SA, Illkirch, France) 1 day after seeding cells at a density of $7.26 \times 10^4$ cells/cm$^2$. Cell stacks were actively gassed with incubator air (37°C, 5% $CO_2$, and 95% relative humidity) at 300–500 mL/min. Viral genomes flanked by AAV2 inverted terminal repeats were packaged into AAV1, AAV-MutC, AAV5, or AAV8 capsids. Cell cultures were maintained from between 3 and 6 days post-transfection.

### AAV Harvest
At the time of harvest, culture media were pipetted off plates or decanted from cell stacks, after which cells were detached using PBS with 5 mM EDTA (PBS-EDTA). Cells were lysed via freeze/thaw or chemically. For freeze/thaw, the cells were pelleted, resuspended in lysis buffer (140 mM NaCl, 25 mM Tris-HCl, 6.5 mM MgCl$_2$, 5 mM

KCl, and 0.7 mM potassium phosphate dibasic), and subjected to 5 freeze/thaw cycles. For chemical lysis, the cells were incubated with high concentrations of salt. This was performed in initial experiments directly on a 10 cm plate by adding salt solution (e.g., 1.25 M ammonium sulfate) to the cell culture plate and incubating at 37°C for 30 min, then pipetting contents off the plate and vortexing. In subsequent experiments, cells were detached using PBS-EDTA and combined with concentrated 3.5 M ammonium sulfate (to a final concentration of 1.25 M ammonium sulfate), and incubated overnight at 4°C. This method allowed for the formation of an impurity-containing non-aqueous phase. Following harvest, media were filtered with polyethersulfone Stericup filters with a 0.22 μm cutoff (MilliporeSigma, Burlington, MA, USA) or cell lysates were filtered with a minimum pore size of 1.2 μm polypropylene (Pall, Port Washington, NY, USA). If a non-aqueous phase developed, it was removed via physical separation, e.g., pipetting.

### AAV Capsid ELISA
Assembled AAV particles were detected using the AAV1, AAV5, or AAV8 Titration ELISA Kit (PRAAV1, PRAAV5, and PRAAV8; Progen Biotechnik GmbH, Heidelberg, Germany). Briefly, samples were diluted in kit-provided sample buffer and incubated on the provided ELISA plate for 1 h. Plates were then incubated with biotin-conjugated anti-AAV1, 5, or 8, followed by a streptavidin peroxidase conjugate, and a substrate containing tetramethylbenzidine. Absorbance was measured on a Spectramax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 450 nm. A linear or quadratic fit was used to construct a standard curve, as per kit instructions.

### dsDNA and Host Cell Protein Analysis
dsDNA concentration was measured with the Quant-iT PicoGreen double-stranded DNA (dsDNA) Assay Kit, according to kit instructions (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, PicoGreen reagent, standards, and unknown samples are diluted in $1 \times$ TE buffer. 100 μL of standards and samples are loaded in triplicate in a 96-well plate and 100 μL of PicoGreen reagent is added to each well and mixed by pipetting. The plate is incubated for 2–5 min at room temperature, protected from light, and then fluorescence data at 480/520 nm is captured on a Spectramax M2. AAV samples measured with this kit were not pre-treated prior to dilution and contained single-stranded genomes, which did not contribute to the fluorescence signal. HEK293T/17 host cell protein was determined using a HEK293 Host Cell Protein ELISA kit (Cygnus Technologies, Southport, NC, USA) per manufacturer's instruction.

### Chromatography
Filtered cell culture media or lysate was applied to Sartobind Phenylligand membranes (3 mL: cat. no. 96HICP42EUC11–A; and 150 mL: cat. no. 96HICP42E9BFF; Sartorius Stedim North America, Bohemia, NY, USA); using an AKTAexplorer 100, AKTA Avant, or AKTA Pilot chromatography system (GE Healthcare Life Sciences, Little Chalfont, UK).

### Hydrophobic Interaction Membrane
AAV-containing samples titrated to 1, 1.25, or 1.5 M ammonium sulfate and 20 mM Tris pH 6.5, 7.0, or 7.5 were applied to the phenyl membranes and run at a flow rate between 1.7 and 5 MV/min. Buffer A was either 1, 1.25, or 1.5 M ammonium sulfate, 20 mM Tris pH 6.5, 7.0, or 7.5; and Buffer B was either 20 mM Tris pH 6.5, 7.0, or 7.5. Following loading of material, the membranes were washed and eluted using a gradient of the A and B buffers. A two-factor, two-level full factorial interaction model DoE was generated and analyzed using MODDE 10 (Umetrics AB, Umea, Sweden). Table S2 shows conditions generated by MODDE.

### Protein Analysis
Samples from the phenyl membrane were normalized by protein concentration and applied to a 4%–12% gradient SDS-PAGE Criterion Gel (Bio-Rad Laboratories, Hercules, CA, USA), after which the gel was stained with a Pierce Silver Stain Kit (Thermo Fisher Scientific, Waltham, MA, USA). Additionally, samples were dialyzed into PBS using low molecular weight 3 kDa-cutoff Slide-A-Lyzer Dialysis Cassettes (Thermo Fisher Scientific) and sample stacking SDS capillary gel electrophoresis was performed as previously described.[41]

### Vector Potency
AAV-MutC particles expressing a FIX transgene were prepared for transduction into HuH7 cells by dialysis into serum-free DMEM, followed by 0.22 μm filtration. HuH7 cells were seeded at a density of $2 \times 10^5$ cells/well in a 12-well plate and incubated overnight in HuH7 growth medium (DMEM containing 10% FBS, 1% Glutamax, and 10 μg/mL Vitamin $K_1$). 1 day after seeding, the cells were transduced with virus at an MOI of $1 \times 10^5$ in serum-free DMEM containing 10 μg/mL Vitamin $K_1$. Viral preps were normalized by vector genome titer and transductions were performed in triplicate. Growth medium was added to each well 6 h post-transduction and, 42 h later, media was exchanged to 0.5 mL of serum-free DMEM per well. Culture media were harvested 24 h later and the Asserachrom IX:Ag Enzyme Immunoassay kit was used to detect FIX antigen levels according to kit instructions (Diagnostica Stago, Parsippany, NJ, USA; Ref 00943). FIX enzymatic activity was analyzed using the BIOPHEN FIX chromogenic assay according to kit instructions (HY-PHEN BioMed, SAS, Neuville-sur-Oise, France; Ref. 221806-RUO). Both of these assays were quantitated on a SpectraMax i3 plate reader using Softmax Pro version 6.5.1 software (Molecular Devices, Sunnyvale, CA, USA). Vector potency is displayed as the normalized ratio of FIX activity over the amount of FIX antigen produced. Transduction efficiency was determined by isolating DNA from HuH7 cells and quantifying viral genomes using qPCR via an ABI 7500 Fast Real-Time PCR System with attached AccuSEQ software (Thermo Fisher Scientific).

### Statistics
Data are expressed as the average plus or minus the standard deviation, where appropriate. Analysis was performed in Excel (Microsoft, Redmond, WA, USA) with one-way ANOVA with a Tukey

comparison where appropriate. $p < 0.05$ was considered significant in all comparisons.

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.09.015.

## AUTHOR CONTRIBUTIONS

Conceptualization: D.J.M. and B.A.P.; Methodology: D.J.M., B.A.P., C.M.W., and M.M.M.; Investigation: D.J.M., B.A.P., and C.M.W.; Resources: T.D.L. and M.M.M.; Writing – Original Draft: D.J.M. and B.A.P.; Writing – Review and Editing: D.J.M., B.A.P., C.M.W., T.D.L., and M.M.M.; Supervision: T.D.L. and M.M.M.; Funding Acquisition: M.M.M.

## CONFLICTS OF INTEREST

D.J.M., B.A.P., and M.M.M. are inventors on a patent that includes the work in this manuscript.

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