Dynamic High Capacity Mustang® Q Membrane Units for Scaleable Anion Exchange Chromatography Purification of Adenoviral Vectors

Introduction

Adenovirus vectors (rAd) are widely used in gene therapy to deliver genes of interest. It is therefore essential to develop a purification process that clears the adenovirus of undesired contaminants while purifying high titre stock adenovirus. The current standard method of purification uses a CsCl density gradient, which is formed during centrifugation. The limitations of this procedure include the speed of the process, the cost of trying to scale up to pharmaceutically relevant levels and the potential carryover of high molecular weight proteins. A chromatographic method, which includes a matrix that has a high dynamic binding capacity for the rAd and is able to withstand high flow rates to render faster purification, will enable researchers to purify concentrated quantities of rAd and scale the process from bench to production. Mustang Q membrane is such a chromatography matrix and has the additional benefits of being available in a sterilizable, disposable format so that cleaning validation protocols and all costs associated with that process are eliminated. Other benefits of Mustang Q chromatography include maintaining resolution at faster flow rates than traditional ion exchange resins, eliminating column packing, and easily adapting to bench-scale work. All of these factors will speed up the drug development process.

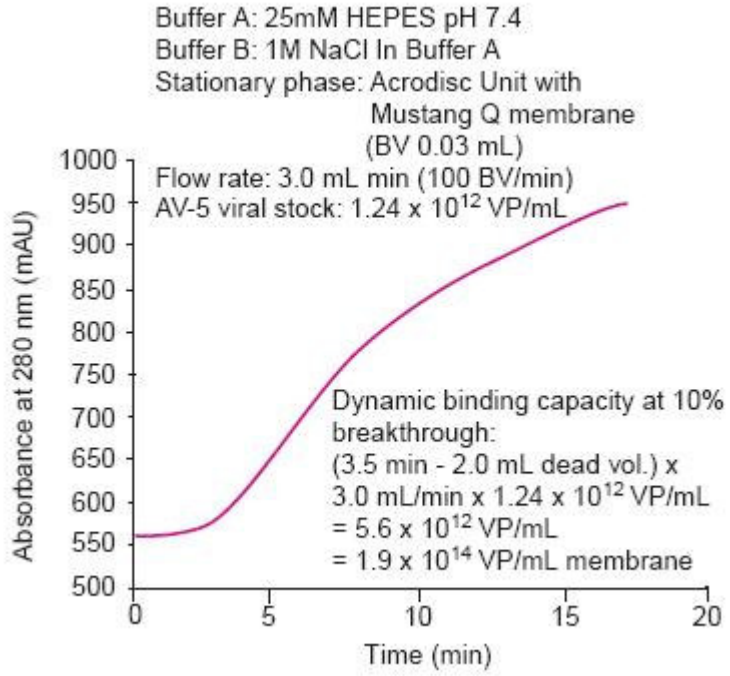
Materials and Methods

Adenovirus Preparation
Harvest infected cells by pelleting and resuspending the pellet in PBS. Lyse host cells by three freeze-thaw cycles and separate the cell debris by spinning down at 20,000 x g for 60 minutes at 4 ºC. The crude cell lysate can then be filtered through a 25 mm Acrodisc® syringe filter with 1.2 µm Supor® membrane followed by a 25 mm Acrodisc syringe filter with 0.2 µm Supor membrane. This two-step filtration process may require more then just a single Acrodisc filter depending on the effectiveness of the pelleting procedure. At this stage, the filtered rAd containing lysate should be clear.

Incubate rAd at room temperature for 30 minutes with either 100 units of Benzonase or 100U DNAase/RNAse cocktail per mL of cell lysate. Filter with 0.2 µm filtration (25 mm Acrodisc PF syringe filter with Supor membrane, PN 4187) to ensure that the lysate is free of particulate matter. Adjust suspension to a final concentration of 0.3 M NaCl. This crude lysate sample can be applied directly onto a Mustang membrane for isolation of high titre rAd. The Mustang Q membrane column (PN MSTG25Q6 or coin) should be preconditioned with 0.3 M NaCl in 25 mM HEPES, pH 7.4 (Figure 2) for a quick purification process.

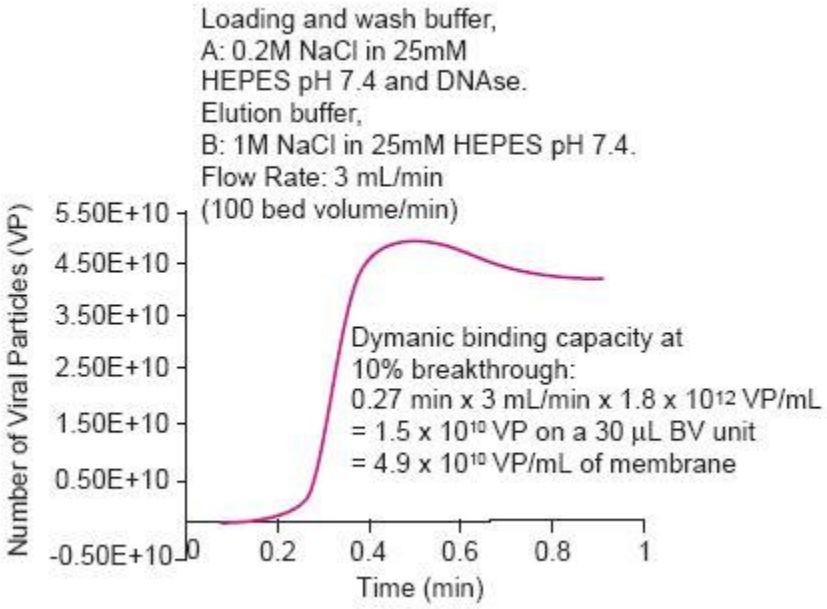
Binding Capacity Determination
To determine binding capacity of Mustang Q membrane for rAd, a CsCl purified adenovirus preparation (1.24 E4 VP/mL) was loaded onto an Acrodisc unit with Mustang E membrane (0.03 mL bed volume). Optical density was determined at 280 nm for real time monitoring of breakthrough (Figure 1).

Figure 1
Determination of Dynamic Binding Capacity of Mustang Q Membranes for Purified rAd



The binding capacity for adenovirus in crude lysate was determined (Figure 2) by isolating the adenovirus on an Acrodisc device, quantifying the material isolated (data not shown) then reloading the virus (1.8E12 VP/mL) onto an Acrodisc unit with Mustang membrane and monitoring for the breakthrough point. The curve in Figure 2 indicates that the dynamic binding capacity of Mustang membrane for crude lysate purified virus at 10% breakthrough is 4.9 x 10^{13} VP/mL of membrane. This shows that the membrane is capable of binding significant amounts of virus for both concentrating purified viral samples or purifying virus out of crude lysate, which could save time and money, and decrease the exposure of a researcher to hazardous chemicals by eliminating the use of CsCl.

Figure 2
Determination of Dynamic Binding Capacity of Mustang Q Membranes for rAd from Lysate

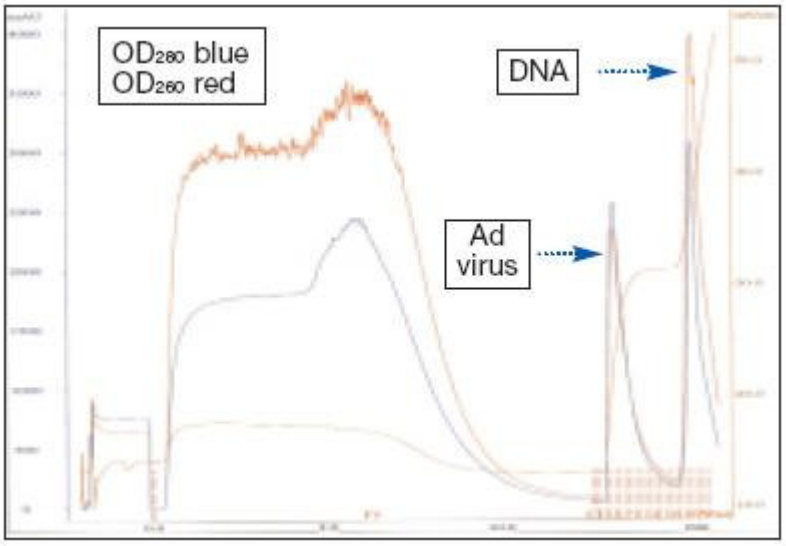


Elution of Adenovirus from Mustang Q Membrane
To determine the elution conditions for rAd from Mustang membrane, an ÄKTA Explorer* 10 LC system was setup in a 4 ºC refrigerator and ~3^{11} infective adenovirus particles in 0.2M NaCl were loaded onto an 0.18 mL bed volume (BV) Mustang Q column. The viral particles were eluted at 20 column volumes (CV)/min (3.5 mL/min) using buffer A (0.2M NaCl in 20mM HEPES pH 7.5, 1mM $MgCl_2$) and buffer B (1M NaCl in 20mM HEPES pH 7.5, 1mM $MgCl_2$). The gradient was 0 to 40% B in 0.5 CV, then held at 40% B for 20 CV and a final elution gradient of 40 to 100% B in 10 CV 0.5 mL fractions was collected.

An aliquot of each fraction was run on an SDS-PAGE gel to determine which fractions contained adenovirus. Sucrose was added to a final concentration of 8% to each fraction containing adenovirus. These fractions were pooled for further processing. This pooled sample was then concentrated and desalted on a Microsep™ centrifugal device with 300K Omega™ membrane (PN OD300C41 or OD300C46).

The viral peak eluted at 30 mS/cm in the 0 to 40% B gradient (Figure 3). The cellular DNA eluted in the 40 to 100% B gradient (based on the OD_{280}/OD_{260} intensities). These two peaks were completely resolved when the viral peak was washed with 20 CV of 40% B. This extended hold at 40% B was necessary to ensure the viral peak reached baseline.

Figure 3



Flow Rate Affect on Capacity

To determine whether the flow rate of Mustang membrane would be a significant barrier in using this technology for viral purification, 3.7×10^{11} adenoviral plaque forming units from a CsCl purified stock were loaded at flow rates of 20 and 40 column volumes per minute. These flow rates are 200 and 400 times faster than traditional resin-based chromatography, which would not be able to capture adenovirus at these speeds. Biological plaque forming units (pfu) were assayed by agar overlay of infected cells in 60 mm^2 (Table 1).

Table 1
Adenovirus Particle Counts and Plaque Forming Units (pfu)

1.1	Adenovirus Particles	% Recovery
Loading capacity		
Start	1.0×10^{13}	
Final	8.0×10^{12}	80

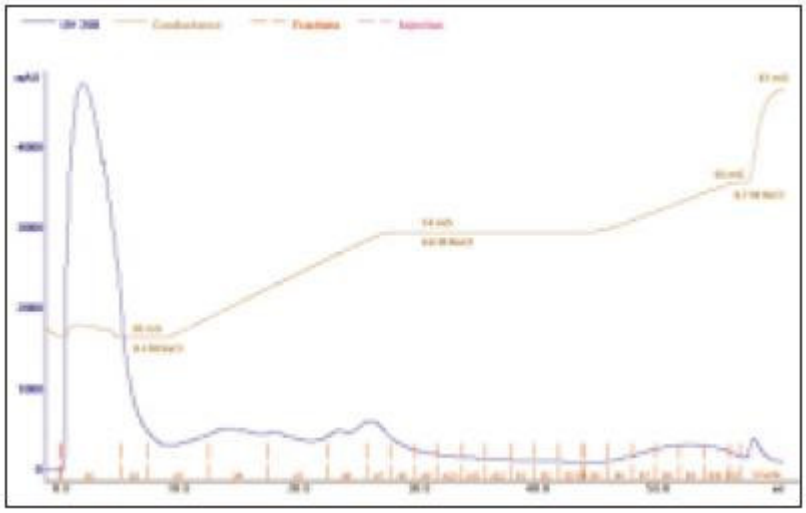
1.2	Adenovirus pfu	% Recovery
Flow rate capacity		
Start	3.7×10^{11}	
At 20 cv/min	2.4×10^{11}	65
At 40 cv/min	1.7×10^{11}	46

1.3	Adenovirus pfu/mL	Adenovirus pfu
Cell lysate recovery		
Fraction A6	1.6×10^{10}	5.0×10^{10}
Fraction A7	2.4×10^{11}	4.8×10^{11}
Fraction B9	1.0×10^{9}	2.0×10^{9}

From this information, it appears that speed does affect binding capacity but only when the speed is already 400 times faster than other resin-based chromatographic protocols.

Adenovirus PFU Recovery from Cell Lysate
Adenovirus-containing supernatant from centrifuged and filtered cell lysate was adjusted to a final concentration of 0.4M NaCl and loaded at 2 mL per minute onto the 180 µL Pall Mustang Q Acrodisc filter unit. Elution of adenovirus was achieved with a linear gradient from 0.4M to 0.6M NaCl (Figure 4). Fractions were buffer exchanged with a standard HEPES buffer and assayed for biological plaque forming units (Table 1.3).

Figure 4



CsCl Purification Method Versus Mustang Q Purification

Adenovirus preparations which started with the same cellular lysate material filtered to 0.2 µm were then purified using either Mustang Q membrane or ultra-centrifugation CsCl gradient. Side-by-side analysis such as particle count recovery, infectious units, cytotoxicity, and purity on SDS-PAGE were performed.

Both methods gave comparably high titers and transgene expression, and the total amount of virus recovered from each method was equivalent (Table 2). One clear advantage of the Mustang Q membrane process was that purified virus could be obtained within a single day, compared to the multi-day process needed for the conventional method. However, the Mustang Q membrane chromatography purified material contained higher total protein and thus a lower final specific activity. Regardless, the Mustang Q membrane chromatography purified virus demonstrated far less cytotoxicity in primary neurons then normally experienced with CsCl purified virus.

Table 2
Biological Assay Data on Adenovirus Serotype 5 Activity in Crude Prep, Mustang Q Purified Adenovirus and CsCl Purified Adenovirus

Crude Prep	Mustang Q Purification Method	CsCl Purification Method
Total volume	3.5 mL	3 mL

	(remaining volume)	(remaining volume)
Number of infected cells for each method	2.5E+08 of 293S cells	2.5E+08 of 293S cells
Total infectious particles (TCID50%)	1.15E+10	1.98E+10
Infectious particles/mL (TCID50%/mL)	3.3E+09 (28.7% recovery)	6.6E+09 (33.3% recovery)
OD (OPU/mL)	3.65E+12	3.85E+11
Total OD (OPU)	1.27E+13	1.15E+12
Total protein mg (BCA)	82.31	14.38
Protein mg/mL (BCA)	23.51	4.79

Conclusions

Adenoviral vectors are widely used as highly efficient gene transfer vehicles in a variety of biological research strategies including human gene therapy. However, issues regarding purification processes still present a challenge. In that regard, any method that renders the purification process that is faster, cheaper and safer than what is currently available, may represent a major advance in adenoviral technology. In the present work, we report the development of a chromatographic process incorporating the new Mustang Q membrane as an alternative to CsCl gradient purification. Mustang Q membrane yields high recoveries of infectious viral particles and processes rAd particles from lysate in a fraction of the time required using the traditional CsCl method. One separation can be performed in less than 30 minutes compared to three days. In addition, Pall Mustang Q Acrodisc filter units are ideal devices to purify viral particles for a range for any lab-scale process and are scalable in both disposable or reusable formats for large-scale purification requirements. Overall, this novel purification method proved to be faster and more cost effective than the CsCl gradient purification.