can efficiently generate high titer quantities of rAAV vectors with high purity and potency.

[0005] Though the effort to design efficient, large-scale methods to purify an AAV product suitable for human administration has been great, there still remains a need for better AAV purification methods. For example, current methods of generating AAV in cell culture result in the formation of "empty" capsids which have been shown to lead to T-cell-mediated immune responses against capsid antigen, leading to low-grade hepatotoxicity and partial loss of expression (Wright, *Molec Therapy* 22(1): 1-2 (2014)). AAV purification methods which include steps for removing empty AAV capsids from the final AAV product are therefore desired.

SUMMARY

[0006] A feature of AAV vector generation in cell culture is the formation of an excess of "empty" capsids, which lack the vector genome. Such empty capsids are unable to provide a therapeutic benefit associated with transgene production. The effect of the empty capsids on clinical outcome is not clear. However, there is a potential for increasing innate or adaptive immune responses to the vector, which then renders empty capsids a concern in gene therapy contexts. Wright, Molecular Therapy 22: 1–2 (2014).

[0007] ~~Provided~~ Described herein are methods of producing an adeno-associated virus (AAV) product, methods of purifying AAV, and methods of purifying full AAV capsids from a concentrated AAV fraction comprising empty AAV capsids and full AAV capsids. The methods of the present disclosure are advantageous over those known in the art, as the methods provided here are suitable for large-scale production of AAV and provide a highly pure, potent product suitable for clinical use. In one aspect, the present disclosure refers to A method of purifying full adeno-associated virus (AAV) capsids from a concentrated AAV fraction comprising empty AAV capsids and full AAV capsids, comprising: (i) loading into a zonal rotor a) the concentrated AAV fraction and b) at least two sugar solutions, each of which has a different sugar concentration and each of which comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 45% (w/w) to about 65% (w/w) sucrose, (ii) operating an

ultracentrifuge comprising the zonal rotor in batch mode, whereupon a sugar gradient is formed, (iii) obtaining a fraction of the sugar gradient to obtain an AAV fraction wherein at least or about 60% of the AAV particles in the AAV fraction are full AAV capsids. In another aspect, the present disclosure refers to a method of producing an AAV product comprising (i) transfecting host cells with gene or cDNA of interest, (ii) collecting a supernatant of a cell culture comprising the transfected host cells, and (iii) carrying out a method as disclosed herein. In ~~exemplary aspects~~some examples, the methods described herein provide an AAV product comprising AAV particles of a homogenous population and high purity. In ~~exemplary aspects~~some examples, the methods described herein provide an AAV product comprising full-length vector DNA. In ~~exemplary embodiments~~some examples, the methods described herein provide an AAV product that is substantially free of unwanted contaminants, including but not limited to, empty AAV particles (including containing truncated or incomplete vector DNA), AAV particles with incomplete protein composition and oligomerized structures, or contaminating viruses, e.g., non AAV, lipid

enveloped viruses. In ~~exemplary embodiments~~some examples, the methods described herein provide an AAV product containing a high amount of DNA (cDNA) encoding of the protein of interest.

[0008] In some examples~~exemplary embodiments~~, the methods of the present disclosure comprise an ultracentrifugation step to separate full AAV capsids from empty AAV capsids. In some examples~~exemplary aspects~~, the methods comprise (i) loading into a rotor a concentrated AAV fraction with at least two sugar solutions, each of which has a different sugar concentration, (ii) operating an ultracentrifuge comprising the loaded rotor in batch mode to form a sugar gradient, and (iii) obtaining a fraction of the sugar gradient to obtain an AAV fraction comprising full AAV capsids. In some examples~~exemplary aspects~~, the rotor is a zonal rotor.

[0009] In some examples~~exemplary aspects~~, the at least two sugar solutions each comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 45% (w/w) to about 65% (w/w) sucrose. In some examples~~exemplary aspects~~, one sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 52% (w/w) to about 58% (w/w) sucrose and another sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 57% (w/w) to about 63% (w/w) sucrose. Optionally, there is another sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 47% (w/w) to about 53% (w/w) sucrose. When loading to the bottom of the rotor or compartment, the order of loading sequence can be first the sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 47% (w/w) to about 53% (w/w) sucrose, if present, then the sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 52% (w/w) to about 58% (w/w) sucrose, and then the sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 57% (w/w) to about 63% (w/w) sucrose.

[0010] In certain ~~embodiments~~examples, the sample to be processed is added prior to the addition of the sugar solutions. In certain ~~embodiments~~examples, ultracentrifugation is conducted in a continuous flow manner and the sample is loaded after a density gradient is achieved during centrifugation.

[0011] In certain examplesembodiments, the methods of the present disclosure comprise an ultracentrifugation step to separate full AAV capsids from empty AAV capsids. In exemplary aspectscertain examples, the methods comprise (i) loading into a rotor a concentrated AAV fraction with at least two sugar solutions, each of which has a different sugar concentration and each of which comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 45% (w/w) to about 65% (w/w) sucrose, (ii) operating an ultracentrifuge comprising the loaded rotor in batch mode to form a sugar gradient, and (iii) obtaining a fraction of the sugar gradient to obtain an AAV fraction wherein at least or about 60% of the AAV particles in the AAV fraction are full AAV capsids. In exemplary aspectssome examples, the rotor is a zonal rotor. In certain examplesembodiments, the volume of the sugar solutions is greater than or equal to about 50% of the volume of the zonal rotor. In certain examplesembodiments, the total volume of the sugar solutions and the AAV fraction is less than or equal to the volume of the zonal rotor. In certain examplesembodiments, the ratio of the volume of the sugar solutions to the volume of the AAV fraction is less than or equal to one.

[0012] In certain examplesembodiments, each sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 50% (w/w) to about 60% (w/w) sucrose. In certain examplesembodiments, each sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 55% (w/w) to about 60% (w/w) sucrose. In certain examplesembodiments, at least one of the sugar solutions comprises sugar at a concentration equivalent to a sucrose concentration greater than about 50% (w/w) sucrose. In certain examplesembodiments, at least one of the sugar solutions comprises sugar at a concentration equivalent to a sucrose concentration greater than about 55% (w/w) sucrose. In certain examplesembodiments, at least one of the sugar solutions comprises sugar at a concentration equivalent to a sucrose concentration ranging from about 60% (w/w) to about 65% (w/w) sucrose.

[0013] In certain examplesembodiments, one sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 52% (w/w) to about 58% (w/w) sucrose, wherein a second sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 57% (w/w) to about 63% (w/w) sucrose, and optionally wherein a third sugar solution comprises a

sugar at a concentration equivalent to a sucrose concentration ranging from about 47% (w/w) to about 53% (w/w) sucrose.

[0014] In certain examplesaspects, two sugar solutions are loaded into the zonal rotor. In certain examplesembodiments, two sugar solutions are loaded into the zonal rotor and wherein one sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 52% (w/w) to about 58% (w/w) sucrose and a second sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 57% (w/w) to about 63% (w/w) sucrose.

[0015] In certain examplesaspects, at least three sugar solutions are loaded into the zonal rotor. In certain examplesembodiments, three sugar solutions are loaded into the zonal rotor. In certain examplesembodiments, three sugar solutions are loaded into the zonal rotor and wherein one sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 47% (w/w) to about 53% (w/w) sucrose, a second sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 52% (w/w) to about 58% (w/w) sucrose, and a third sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 57% (w/w) to about 63% (w/w) sucrose.

[0016] In certain examplesembodiments, the concentrated AAV fraction is loaded before a sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 52% (w/w) to about 58% (w/w) sucrose, and wherein the sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 52% (w/w) to about 58% (w/w) sucrose is loaded before a sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 57% (w/w) to about 63% (w/w) sucrose.

[0017] In certain examplesembodiments, the concentrated AAV fraction is loaded before a sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 47% (w/w) to about 53% (w/w) sucrose, wherein the sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 47% (w/w) to about 53% (w/w) sucrose is loaded before a sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 52% (w/w) to about 58% (w/w) sucrose, and wherein

the sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 52% (w/w) to about 58% (w/w) sucrose is loaded before a sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 57% (w/w) to about 63% (w/w) sucrose.

[0018] In certain examplesembodiments, each sugar solution is loaded in the zonal rotor at equal volumes. In certain examplesembodiments, the sugar solution with the smallest sugar concentration is loaded in the zonal rotor at a volume which is twice the volume of at least one of the other sugar solutions in the zonal rotor. In certain examplesembodiments, wherein the sugar solution with the smallest sugar concentration is loaded in the zonal rotor at a volume which is at least the volume of all other sugar solutions combined in the zonal rotor. In certain examplesembodiments, the sugar solution with the smallest sugar concentration is loaded in the zonal rotor at a volume which is at least twice the volume of the sugar solution with the largest sugar concentration, optionally, wherein the volume of the sugar solution with the largest sugar concentration is equal to the volume of the sugar solution with the intermediate sugar concentration. In certain examplesembodiments, at least two sugar solutions are loaded into the zonal rotor, wherein the sugar solution with the smallest sugar concentration is loaded in the zonal rotor at a volume which is at least twice the volume of at least one other sugar solution in the zonal rotor. In certain examplesembodiments, at least two sugar solutions are loaded into the zonal rotor, wherein the sugar solution with the smallest sugar concentration is loaded in the zonal rotor at a volume which is the same volume of at least one other sugar solution in the zonal rotor. In certain examplesembodiments, the sugar solution with the smallest sugar concentration is loaded in the zonal rotor at a volume which is half the volume of the concentrated AAV fraction. In certain examplesembodiments, where in the ratio of the volume of the total sugar gradient to the volume of the AAV fraction loaded in the zonal rotor is from about 1:1 to about 1:5.

[0019] In certain examplesaspects, the AAV fraction comprises a buffered solution. In certain examplesembodiments, the buffered solution includes, without limitation, phosphate buffers, histidine (e.g., L-histidine), sodium citrate, HEPES, Tris, Bicine, glycine, N-glycylglycine, sodium acetate, sodium carbonate, glycyl glycine, lysine, arginine, sodium phosphate, and mixtures thereof. In certain examplesembodiments, the AAV fraction comprises TrisHCl and NaCl. In certain examplesembodiments, the TrisHCl is at a concentration of about 20 to about 50

mM and the NaCl is at a concentration of about 150 mM to about 900 mM. In certain examplesembodiments, the NaCl is at a concentration of about 500 mM to about 750 mM. In certain examplesembodiments, the buffered solution has a pH of about 7.4 to about 9.0. In certain examplesembodiments, the buffer comprises about 50 mM TrisHCl and about 500 mM NaCl with a pH of 8.5. In certain examplesembodiments, the buffer comprises about 50 mM TrisHCl and about 750 mM NaCl with a pH of 8.0. In certain examplesembodiments, the buffered solution comprises 45-55% (w/w) ethylene glycol.

[0020] In certain examplesembodiments, each sugar solution comprises a disaccharide or trisaccharide. In certain examplesembodiments, the disaccharide comprises sucrose, maltose, lactose, and combinations thereof. In certain examplesembodiments, each sugar solution comprises sucrose. In certain examplesembodiments, each of the sugar solutions further comprises TrisHCl and NaCl. In certain examplesembodiments, the TrisHCl is at a concentration of about 20 to about 50 mM and the NaCl is at a concentration of about 150 mM to about 500 mM. In certain examplesembodiments, the buffered solution has a pH of about 7.4 to about 8.5. In certain examplesembodiments, the buffer comprises 20 mM TrisHCl and 8 g/L NaCl with a pH of 7.4.

[0021] In certain examplesaspects, the methods comprise operating the ultracentrifuge at a first rotational speed of less than 10,000 rpm for less than 60 minutes, and at a second rotational speed within the range of about 30,000 to about 40,000 rpm for at least 4 hours.

[0022] In certain examplesaspects, the methods comprise operating the ultracentrifuge at a first rotational speed of less than 10,000 rpm for less than 60 minutes, and at a second rotational speed within the range of about 30,000 to about 40,000 rpm for at least 12 hours.

[0023] In certain examplesembodiments, the first rotational speed is about 3,000 rpm to about 6,000 rpm, optionally, about 4,000 rpm. In certain examplesembodiments, the second rotational speed is about 35,000 rpm and optionally is maintained for about 4 to about 6 hours. In certain examplesembodiments, the second rotational speed is maintained for at least about 16 hours or at least 20 hours. In certain examplesembodiments, the second rotational speed is about 35,000 rpm and optionally is maintained for about 16 to about 20 hours.

[0024] In certain examples~~embodiments~~, the concentrated AAV fraction loaded into the zonal rotor comprises at least 1 x 10^{12} AAV capsids per mL. In certain examples~~embodiments~~, the methods comprise harvesting a supernatant from a cell culture comprising HEK293 cells transfected with a triple plasmid system. In certain examples~~embodiments~~, the methods comprise (i) harvesting the supernatant about 3 to about 5 days after transfection of the HEK293 cells or (ii) when the cell culture has a cell density of greater than or about $5x10^6$ cells/mL and has a cell viability of greater than 50%. In certain examples~~embodiments~~, the methods comprise filtering the harvested supernatant via depth filtration. In certain examples~~embodiments~~, the methods comprise filtering the harvested supernatant through a filter comprising cellulose and perlites and having a minimum permeability of about 500 L/m^2. In certain examples~~embodiments~~, the methods comprise filtering the harvested supernatant through a filter with a minimum pore size of about 0.2 µm. In certain examples~~embodiments~~, the methods comprise concentrating an AAV fraction using an ultra/diafiltration system. In certain examples~~embodiments~~, the methods comprise concentrating an AAV fraction using an ultra/diafiltration system before, after, or before and after a step comprising applying an AAV fraction to an anion exchange (AEX) chromatography column under conditions that allow for the AAV to flow through the AEX chromatography column. In certain examples~~embodiments~~, the methods comprise inactivating lipid enveloped viruses of an AAV fraction with a solvent detergent. In certain examples~~embodiments~~, the methods comprise nanofiltration of an AAV fraction to remove viruses greater than 35 nm. In certain examples~~embodiments~~, the methods comprise a polish step comprising performing AEX chromatography with a column comprising tentacle gel.

[0025] In certain examples~~embodiments~~, the methods comprise (i) applying an AAV fraction to an anion exchange (AEX) chromatography column under conditions that allow for the AAV to flow through the AEX chromatography column and (ii) collecting the flow-through comprising the AAV. In certain examples~~embodiments~~, the AAV fraction is applied to the AEX chromatography column with a loading buffer comprising about 100 mM to about 150 mM NaCl, optionally, wherein the pH of the loading buffer is about 8 to about 9. In certain examples~~embodiments~~, the loading buffer comprises about 115 mM to about 130 mM NaCl, optionally, the loading buffer comprises about 120 mM to about 125 mM NaCl.

[0026] In certain examples~~embodiments~~, host cell proteins are removed. In certain examples~~embodiments~~, host cell proteins are HSP70 and/or LDH.

[0027] In ~~exemplary aspects~~some examples, at least or about 55% of the AAV particles in the fraction obtained from the sugar gradient are full AAV capsids. In ~~exemplary aspects~~some examples, greater than or about 60% of the AAV particles in the fraction obtained from the sugar gradient are full AAV capsids. In ~~exemplary aspects~~some examples, greater than or about 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the AAV particles in the fraction obtained from the sugar gradient are full AAV capsids.

[0028] In certain examples~~embodiments~~, the methods comprise testing an AAV fraction via an AAV-specific ELISA. In certain examples~~embodiments~~, the AAV specific ELISA is a sandwich ELISA specific for AAV. In certain examples~~embodiments~~, the methods do not include a step of measuring potency via quantitative PCR.

[0029] In certain examples~~embodiments~~, the AAV is AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, or AAV10. In certain examples~~embodiments~~, the AAV is AAV8.

[0030] Provided herein are also AAV products produced by the methods described above and herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] Figure 1 is a schematic of an exemplary method of the present disclosure.

[0032] Figure 2 is a graph of the amount of virus particles (viral grams) per ml of supernatant of a transfected cell culture plotted over a time course (days). Propagation of AAV8 in HEK293 cell cultures under established standard conditions (e.g. pH, Temp, agitation rate) in 2L scale (4 parallel runs with two different PEI lots): increase of titer in cell culture supernatant over time measured by FIX specific qPCR (T01=transfection day 1, T02=transfection 2, etc.). The titer was measured by FIX specific qPCR in 3 x 2L scale bioreactors.

[0033] Figure 3 is a graph of the amount of virus particles (viral grams) per ml of supernatant of a transfected cell culture plotted over a time course (days). Propagation of AAV8 in HEK293 cell cultures under established standard conditions (e.g. pH, Temp, agitation rate) in 2L scale (4 parallel runs with two different PEI lots): increase of titer in cell culture supernatant over time measured by an AAV-specific ELISA

WHAT IS CLAIMED IS:

1. A method of purifying full adeno-associated virus (AAV) capsids from a concentrated AAV fraction comprising empty AAV capsids and full AAV capsids, comprising:

(i) loading into a zonal rotor a) the concentrated AAV fraction and b) at least two sugar solutions, each of which has a different sugar concentration and each of which comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 45% (w/w) to about 65% (w/w) sucrose,

(ii) operating an ultracentrifuge comprising the zonal rotor in batch mode, whereupon a sugar gradient is formed,

(iii) obtaining a fraction of the sugar gradient to obtain an AAV fraction wherein at least or about 60% of the AAV particles in the AAV fraction are full AAV capsids.~~,~~

~~wherein (A) the volume of the sugar solutions is greater than or equal to about 50% of the volume of the zonal rotor, (B) the total volume of the sugar solutions and the AAV fraction is less than or equal to the volume of the zonal rotor, (C) the ratio of the volume of the sugar solutions to the volume of the AAV fraction is less than or equal to one, or (D) a combination thereof.~~

2. The method of claim 1, wherein (A) the volume of the sugar solutions is greater than or equal to about 50% of the volume of the zonal rotor, (B) the total volume of the sugar solutions and the AAV fraction is less than or equal to the volume of the zonal rotor, (C) the ratio of the volume of the sugar solutions to the volume of the AAV fraction is less than or equal to one, or (D) a combination thereof.

~~2.~~3. The method of claim 1 or claim 2, wherein each sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 50% (w/w) to about 60% (w/w) sucrose.

~~3.~~4. The method of claim 1 or claim 2, wherein each sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 55% (w/w) to about 60% (w/w) sucrose.

~~4. The method of claim 1 or claim 2, wherein at least one of the sugar solutions comprises sugar at a concentration equivalent to a sucrose concentration greater than about 50% (w/w) sucrose.~~

~~5. The method of any one of claims 1-4, wherein at least one of the sugar solutions comprises sugar at a concentration equivalent to a sucrose concentration greater than about 55% (w/w) sucrose.~~

~~6. The method of any one of claims 1-5, wherein at least one of the sugar solutions comprises sugar at a concentration equivalent to a sucrose concentration ranging from about 60% (w/w) to about 65% (w/w) sucrose.~~

~~7.~~5. The method of any one of claims 1~~, 2, or 4-6~~-3, wherein one sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 52% (w/w) to about 58% (w/w) sucrose, wherein a second sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 57% (w/w) to about 63% (w/w) sucrose, and optionally wherein a third sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 47% (w/w) to about 53% (w/w) sucrose.

~~8.~~6. The method of any one of claims 1-~~7~~5, wherein two sugar solutions are loaded into the zonal rotor.

~~9. The method of any one of claims 1, 2, or 4-8, wherein two sugar solutions are loaded into the zonal rotor and wherein one sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 52% (w/w) to about 58% (w/w) sucrose and a second sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 57% (w/w) to about 63% (w/w) sucrose.~~

~~10.~~7. The method of any one of claims 1-~~7~~5, wherein at least three sugar solutions are loaded into the zonal rotor.

~~11.~~8. The method of any one of claims 1-~~7~~ 5 or ~~10~~7, wherein three sugar solutions are loaded into the zonal rotor.

~~12. The method of any one of claims 1, 2, 4-7, 10, or 11, wherein three sugar solutions are loaded into the zonal rotor and wherein one sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 47% (w/w) to about 53% (w/w) sucrose, a second sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 52% (w/w) to about 58% (w/w) sucrose, and a third sugar solution comprises a sugar at a concentration equivalent to a sucrose concentration ranging from about 57% (w/w) to about 63% (w/w) sucrose.~~

~~13.~~9. The method of any one of claims 1~~, 2, or 4~~-~~12~~8, wherein in step (i), the concentrated AAV fraction is loaded before a sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 52% (w/w) to about 58~~% -~~ % (w/w) sucrose, and wherein the sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 52% (w/w) to about 58% (w/w) sucrose is loaded before a sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 57% (w/w) to about 63% (w/w) sucrose.

~~14.~~10. The method of any one of claims ~~1, 2, 4-7, or 10-13~~1-3, 5, 7, or 8, wherein in step (i), the concentrated AAV fraction is loaded before a sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 47% (w/w) to about 53% (w/w) sucrose, wherein the sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 47% (w/w) to about 53% (w/w) sucrose is loaded before a sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 52% (w/w) to about 58% (w/w) sucrose, and wherein the sugar solution comprising a

sugar at a concentration equivalent to a sucrose concentration ranging from about 52% (w/w) to about 58% (w/w) sucrose is loaded before a sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration ranging from about 57% (w/w) to about 63% (w/w) sucrose.

~~15.~~11. The method of any one of claims 1-~~14~~10, wherein each sugar solution is loaded: (i) in the zonal rotor at equal volumes; (ii) with the smallest sugar concentration loaded in the zonal rotor at a volume which is twice the volume of at least one of the other sugar solutions in the zonal rotor; (iii) with the smallest sugar concentration loaded in the zonal rotor at a volume which is at least the volume of all other sugar solutions combined in the zonal rotor; (iv) with the smallest sugar concentration loaded in the zonal rotor at a volume which is at least twice the volume of the sugar solution with the largest sugar concentration, optionally, wherein the volume of the sugar solution with the largest sugar concentration is equal to the volume of the sugar solution with the intermediate sugar concentration; (v) with the smallest sugar concentration loaded in the zonal rotor at a volume which is at least twice the volume of at least one other sugar solution in the zonal rotor; (vi) with the smallest sugar concentration at a volume which is the same volume of at least one other sugar solution in the zonal rotor; and/or (vii) with the smallest sugar concentration loaded in the zonal rotor at a volume which is half the volume of the concentrated AAV fraction.

~~16. The method of any one of claims 1-14, wherein the sugar solution with the smallest sugar concentration is loaded in the zonal rotor at a volume which is twice the volume of at least one of the other sugar solutions in the zonal rotor.~~

~~17. The method of any one of claims 1-14 or 16, wherein the sugar solution with the smallest sugar concentration is loaded in the zonal rotor at a volume which is at least the volume of all other sugar solutions combined in the zonal rotor.~~

~~18. The method of claim 1-14, 16, or 17, wherein the sugar solution with the smallest sugar concentration is loaded in the zonal rotor at a volume which is at least twice the~~

~~volume of the sugar solution with the largest sugar concentration, optionally, wherein the volume of the sugar solution with the largest sugar concentration is equal to the volume of the sugar solution with the intermediate sugar concentration.~~

~~19. The method of any one of claims 1-14 or 16-18, wherein at least two sugar solutions are loaded into the zonal rotor, wherein the sugar solution with the smallest sugar concentration is loaded in the zonal rotor at a volume which is at least twice the volume of at least one other sugar solution in the zonal rotor.~~

~~20. The method of any one of claims 1-19, wherein at least two sugar solutions are loaded into the zonal rotor, wherein the sugar solution with the smallest sugar concentration is loaded in the zonal rotor at a volume which is the same volume of at least one other sugar solution in the zonal rotor.~~

~~21. The method of any one of claim 1-20 wherein the sugar solution with the smallest sugar concentration is loaded in the zonal rotor at a volume which is half the volume of the concentrated AAV fraction.~~

~~22.~~12. The method of any one of claims 1-~~21~~11, where in the ratio of the volume of the total sugar gradient to the volume of the AAV fraction loaded in the zonal rotor is from about 1:1 to about 1:5.

~~23.~~13. The method of any one of claims 1-~~22~~12, wherein the AAV fraction comprises a buffered solution.

~~24. The method of claim 23, wherein the buffered solution comprises TrisHCl and NaCl.~~

~~25. The method of claim 24, wherein the TrisHCl is at a concentration of about 20 to about 50 mM and the NaCl is at a concentration of about 150 mM to about 900 mM.~~

~~26. The method of any one of claims 23-25, wherein the buffered solution has a pH of about 7.4 to about 9.0.~~

~~27. The method of any one of claims 23-26, wherein the buffered solution comprises 45-55% (w/w) ethylene glycol.~~

~~28.~~14. The method of any one of the previous claims, wherein each sugar solution comprises: (i) a disaccharide or trisaccharide; (ii) sucrose, maltose, lactose, or combinations thereof; and/or (iii) sucrose.

~~29. The method of claim 28, wherein the disaccharide comprises sucrose, maltose, lactose, and combinations thereof.~~

~~30. The method of any one of the previous claims, wherein each sugar solution comprises sucrose.~~

~~31. The method of any one of the previous claims, wherein each of the sugar solutions further comprises TrisHCl and NaCl.~~

~~32.~~15. The method of ~~claim 31~~any one of the previous claims, wherein each of the sugar solutions and/or AAV fraction: ~~, wherein the~~(i) comprises TrisHCl ~~is~~ at a concentration of about ~~20~~ 10 to about ~~50~~ 100 mM and the NaCl ~~is~~ at a concentration of about ~~150~~ 100 mM to about ~~500 mM~~1 M; and/or (ii) has a pH of about 7.0 to about 9.0.

16. The method of claim 15, wherein the TrisHCl is at a concentration of 20 mM to 50 mM.

17. The method of claim 15 or claim 16, wherein the NaCl is at a concentration of 150 mM to 750 mM or 150 mM to 500 mM.

~~33.~~18. The method of any one of claims 13 or 15-17~~claim 31 or claim 32~~, wherein the ~~buffered solution has a~~ pH ~~of about~~is 7.4 to about 8.5.

19. The method of any one of claims 13 or 15-17 wherein the AAV fraction comprises 45-55% (w/w) ethylene glycol.

20. The method of any one of claims 13 or 15-17, wherein the AAV fraction comprises 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, or 60% (w/w) ethylene glycol.

21. The method of any one of claims 13 or 15-17, wherein the buffered solution comprises 45-55% (w/w) ethylene glycol.

22. The method of any one of claims 13 or 15-17, wherein the buffered solution comprises 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, or 60% (w/w) ethylene glycol.

~~34.~~23. The method of any one of the previous claims, comprising (i) operating the ultracentrifuge at a first rotational speed of less than 10,000 rpm for less than 60 minutes, and at a second rotational speed within the range of about 30,000 to about 40,000 rpm for at least 4 hours; or (ii) operating the ultracentrifuge at a first rotational speed of less than 10,000 rpm for less than 60 minutes, and at a second rotational speed within the range of about 30,000 to about 40,000 rpm for at least 12 hours.

~~35. The method of any one of the previous claims, comprising operating the ultracentrifuge at a first rotational speed of less than 10,000 rpm for less than 60 minutes, and at a second rotational speed within the range of about 30,000 to about 40,000 rpm for at least 12 hours.~~

~~36.~~24. The method of claim ~~34 or claim 35~~23, wherein the first rotational speed is about 3,000 rpm to about 6,000 rpm, optionally, about 4,000 rpm.

~~37.~~25. The method of claim ~~34~~ 23 or claim ~~36~~24, wherein the second rotational speed is (i-) about 35,000 rpm and optionally is maintained for about 4 to about 6 hours; or (ii) about 35,000 rpm and optionally is maintained for about 16 to about 20 hours.

~~38.~~26. The method of any one of claims ~~34-36~~23-25, wherein the second rotational speed is maintained for at least about 16 hours or at least 20 hours.

~~39. The method of any one of claims 34-36 or 38, wherein the second rotational speed is about 35,000 rpm and optionally is maintained for about 16 to about 20 hours.~~

~~40.~~27. The method of any one of the previous claims, wherein the concentrated AAV fraction loaded into the zonal rotor comprises at least 1 x 10^{12} AAV capsids per mL.

~~41. The method of any one of the previous claims, comprising harvesting a supernatant from a cell culture comprising HEK293 cells transfected with a triple plasmid system.~~

~~42. The method of claim 41, comprising harvesting the supernatant about 3 to about 5 days after transfection of the HEK293 cells or when the cell culture has a cell density of greater than or about 5x10^{6} cells/mL and has a cell viability of greater than 50%.~~

~~43. The method of claim 41 or claim 42, comprising filtering the harvested supernatant via depth filtration.~~

~~44. The method of claim 43, comprising filtering the harvested supernatant through a filter comprising cellulose and perlites and having a minimum permeability of about 500 L/m^{2}.~~

~~45. The method of claim 43, comprising filtering the harvested supernatant through a filter with a minimum pore size of about 0.2 µm.~~

~~46. The method of any one of the previous claims, comprising (i) applying an AAV fraction to an anion exchange (AEX) chromatography column under conditions that allow for the AAV to flow through the AEX chromatography column and (ii) collecting the flow-through comprising the AAV.~~

~~47. The method of claim 46, wherein the AAV fraction is applied to the AEX chromatography column with a loading buffer comprising about 100 mM to about 150 mM NaCl, optionally, wherein the pH of the loading buffer is about 8 to about 9.~~

~~48. The method of claim 47, wherein the loading buffer comprises about 115 mM to about 130 mM NaCl, optionally, wherein the loading buffer comprises about 120 mM to about 125 mM NaCl.~~

~~49. The method of any one of the previous claims, comprising concentrating an AAV fraction using an ultra/diafiltration system.~~

~~50. The method of claim 49, comprising concentrating an AAV fraction using an ultra/diafiltration system before, after, or before and after a step comprising applying an AAV fraction to an anion exchange (AEX) chromatography column under conditions that allow for the AAV to flow through the AEX chromatography column.~~

~~51. The method of any one of the previous claims, wherein host cell proteins are removed.~~

~~52. The method of claim 51, wherein the host cell proteins are HSP70 and/or LDH.~~

~~53. The method of any one of the previous claims, further comprising inactivating lipid enveloped viruses of an AAV fraction with a solvent and/or detergent.~~

~~54. The method of any one of the previous claims, further comprising nanofiltration of an AAV fraction to remove viruses greater than 35 nm.~~

~~55. The method of any one of the previous claims, further comprising a polish step comprising performing AEX chromatography with a column comprising tentacle gel.~~

~~56.~~28. A method of producing an AAV product comprising (i) transfecting host cells with ~~three plasmids~~gene or cDNA of interest, (ii) collecting a supernatant of a cell culture comprising the transfected host cells, and (iii) carrying out a method in accordance with any one of claims 1-~~55~~25.

~~57. The method of claim 56, wherein the steps of the method occur in the order as shown in Figure 1.~~

~~58. The method of any one of the previous claims, comprising testing an AAV fraction via an AAV-specific ELISA.~~

~~59. The method of claim 58, wherein the method does not include a step of measuring potency via quantitative PCR.~~

~~60. The method of claim 58 or claim 59, wherein the AAV specific ELISA is a sandwich ELISA specific for AAV.~~

~~61.~~29. The method of any one of the previous claims, wherein the method comprises a first sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration of about 55%, a second sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration of about 60%, and optionally a third sugar solution comprising a sugar at a concentration equivalent to a sucrose concentration of about 50%.

~~62.~~30. The method of any one of the previous claims, wherein AAV is AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, or AAV10.

~~63. The method of any one of the previous claims, wherein AAV is AAV8.~~

~~64. An AAV product produced by a method according to any one of claims 1-63.~~

~~65. A pharmaceutical composition comprising an AAV product produced by a method according to any one of claims 1-63.~~