A Generalized Purification Step for Viral Particles using Mannitol Flocculation

Caryn L. Heldt[1,2,*], Ashish Saksule[1], Pratik U. Joshi[1], and Majid Ghafarian[2]

[1]Department of Chemical Engineering and
[2]Department of Biological Sciences
Michigan Technological University
1400 Townsend Dr., Houghton MI 49931, USA

* Corresponding author: email: heldt@mtu.edu, phone: +1-906-487-1134,
fax: +1-906-487-3213

Biotechnology Progress
DOI: 10.1002/btpr.2651

Citation: Heldt, C. L., A. Saksule, P. U. Joshi and M. Ghafarian (2018). A Generalized Purification Step for Viral Particles using Mannitol Flocculation. Biotechnol. Prog.

Abstract

Vaccine manufacturing has conventionally been performed by the developed world using traditional unit operations like filtration and chromatography. There is currently a shift in the manufacturing of vaccines to the less developed world, requiring unit operations that reduce costs, increase recovery, and are amenable to continuous manufacturing. This work demonstrates that mannitol can be used as a flocculant for an enveloped and non-enveloped virus and can purify the virus from protein contaminants after microfiltration. The recovery of the virus ranges from 58-96% depending on virus, the filter pore size, and the starting concentration of the virus. Protein removal of 80% was achieved for the small non-enveloped virus using a 0.1 μm filter because proteins were not flocculated with the virus and flowed through the filter. It is hypothesized that mannitol dehydrates the viral surface by controlling the water structure surrounding the virus. Without the ability to become compact, as occurs with proteins, the virus aggregates in the presence of osmolytes and proteins do not. Osmolyte flocculation is a scalable processes using high flux microfilters. It has been applied to both an enveloped and non-enveloped virus, making this process friendly to a variety of vaccine and gene therapy products.

Key words: osmolyte, virus purification, protein, harvest

Introduction

In the past 15 years, the GAVI Vaccine Alliance has distributed half a billion vaccines to children and have averted seven million deaths in developing countries[1]. Vaccine manufacturers could facilitate an increase in distribution of vaccines by lowering manufacturing costs. With more manufacturing moving to India, China and Indonesia[2, 3], this is a prime opportunity to create new manufacturing methods for vaccines because these countries still need to qualify their products and are not hindered by current regulatory approvals. In addition, new viral products, for example gene therapy vectors, are being introduced into the market and the high amount of pre-clinical trials[4] leaves room for new virus manufacturing techniques to be adopted.

Several manufacturing methods have been used to produce viral products. The three most common are ultracentrifugation, ultrafiltration, and chromatography. Ultracentrifugation is a popular choice for the purification of adeno-associated virus (AAV) gene therapy vectors. A recent study showed a 60% yield for CsCl and a 65% infectious units recovery for an iodixanol based AAV purification method[5]. While yield and purity can be high with ultracentrifugation, the lack of scale-up options makes it unfeasible for long-term high volume production. A more scalable method of virus particle purification is ultrafiltration. Tangential flow filtration (TFF) methods have been used to concentrate and purify influenza virus, a retrovirus vector, and AAV using size exclusion as the main mechanism of separation[6-8] with recoveries up to 100%. Adsorption mechanisms, ranging in shape from chromatography beads[9-11] to monoliths[12, 13] are also often applied to viral particles. The recovery from adsorption methods is usually lower than filtration methods, with a typical range between 30-68%.

While there have been substantial improvements in virus particle purification methods, there still lacks a purification method that is applicable to many different virus particle types and could possibly be adapted into a platform process for multiple viral products. There is a need for a viral particle platform technology that mirrors the antibody revolution of the antibody platform[14, 15].

There are two categories of osmolytes, protecting and denaturing. Protecting osmolytes, including polyols, amino acids, and sugars, can stabilize protein structure[16] and have been shown to stabilize aggregation-prone peptides[17]. Denaturing osmolytes, like urea, are well-known for unfolding proteins. It is hypothesized that protecting osmolytes are depleted at the protein surface[18, 19], with a possible exception of trimethyl amine N-oxide (TMAO). Osmolytes in hydrophobic polymer solutions have been shown to stabilize the collapsed state[19, 20]. Denaturing osmolytes prefer the elongated polymer state [20] and denaturing osmolytes accumulate on the protein surface[18]. Osmolytes also have an interesting effect on surface charge. When silica particles were put into contact with the protecting osmolyte, glycerol, the surface charge was neutralized, even though glycerol is a neutral molecule[21]. Urea, on the other hand, greatly increased the charge[21]. The depletion and hydrophobic collapse of proteins in protecting osmolyte solutions has led to the theory that osmolytes control water structure around the protein. This leads to minor dehydration, hydrophobic collapse, and stabilization. However, in protecting osmolyte solutions, viruses have shown the preference to aggregate[22, 23], producing a very different response than proteins.

Novel flocculants have recently been discovered that flocculate both enveloped and non-enveloped virus particles[22, 23]. Porcine parvovirus (PPV) and Sindbis virus

(SINV), the model non-enveloped and enveloped viruses, respectively, were shown to be flocculated and over 80% of infectious virus removed with a 0.2 μm filter. This filter was 5-10 times greater in pore-size than the viruses filtered. The novel flocculating agents were amino acids and sugars, which belong to the category of compounds known as osmolytes. While the virus was removed with the microfilter, proteins such as bovine serum albumin (BSA) and lysozyme (LYS) passed through the filter without floccuation. The proof of concept experiments demonstrated that virus particles can be flocculated preferentially from proteins and purified using microfiltration. While alanine and mannitol were most effective at removing PPV[23], proline and mannitol removed SINV[22]. With mannitol being the common flocculant between the two viruses, it was desired to pursue this flocculant in a system that would demonstrate virus purification and recovery.

The most likely explanation for the flocculation of virus and the stabilization of proteins in protecting osmolyte solutions is the high hydrophobicity of viral particles. Two different groups have measured the hydrophobicity of viral particles and they have been found to be more hydrophobic than most of the proteins tested[24, 25]. This leads to the theory that protecting osmolytes dehydrate the protein or virus surface because they bind water more strongly than a protein or virus. In proteins, this dehydration leads to hydrophobic collapse, whereas viruses are rigid structures that cannot easily collapse. Therefore, the hydrophobic viruses aggregate when the hydrophobic patches are exposed on their surface. This is a weak interaction because the viruses can be separated by dilution of the osmolyte and detected with a cell viability assay that measure infectivity[22, 23]. If the virus particles were still aggregated in the infectivity

assay, the control titer would have been reduced upon addition of the osmolytes. However, this was not observed.

In this work, we have demonstrated that mannitol flocculation can be used to purify an enveloped and non-enveloped virus. A batch diafiltration set-up was configured and the recovery and purification of PPV and SINV was demonstrated. This is the first use of osmolyte flocculating agents in the purification of viral particles. The common flocculant mannitol has potential to become a platform precipitant for a large range of viral particle therapeutics. Since mannitol is an FDA approved molecule in final formulation, the inclusion in the purification does not require documented clearance in the final drug substance.

Materials and Methods

Materials

Mannitol, thiazolyl blue tetrazolium bromide (MTT), sodium dodecyl sulphate (SDS), and hydrochloric acid (HCl) were purchased from Sigma Aldrich (St. Louis, MO). Reagents for cell culture, minimum essential medium (MEM), phosphate-buffered saline (PBS, pH 7.2), 0.25% trypsin/EDTA, and penicillin/streptomycin (pen/strep) were purchased from Life Technologies (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA). All the solutions were prepared with NanoPure water with resistivity of >18 MΩ-cm (Thermo Scientific, Waltham, MA) and were sterile filtered using a 0.2 μm filter. For the HPLC study, HPLC grade acetonitrile 99.93% and trifluoroacetic acid (TFA) ≥99.0% were purchased from Sigma Aldrich (St. Louis, MO). For TEM work, 50% glutaraldehyde and uranyl acetate were acquired from Sigma Aldrich (St. Louis, MO).

Methods

Cell Culture, Virus Preparation and Virus Titration

Porcine kidney (PK-13) cells were grown and cultured in (MEM) completed with 10% FBS and 1% pen/strep. Baby hamster kidney 21 (BHK) cells were grown and cultured in MEM supplemented with 10% FBS, 5% tryptose phosphate broth (TPB) (Thermo Scientific, Waltham, MA) and 1% gentamicin (Life Technologies, Carlsbad, CA). All cells were maintained at 37°C, 5% CO_2 and 100% humidity.

PPV virus was propagated in PK-13 cells as described earlier[26]. The clarified virus solution was stored at −80°C. Sindbis virus was propagated on BHK cells as described earlier[27] and also stored at −80°C. Virus quantification was done using the colorimetric cell viability assay, the MTT assay, as described earlier[27]. Briefly, appropriate cells were seeded in 96-well plates and incubated for 24 hours and then infected with samples to be analyzed. After incubation (5 days for PPV, 2 days for Sindbis virus), MTT solution in PBS was added to the plates and 4 hours after addition of MTT, the solubilizing agent (0.01 M HCl and 10% SDS in water) was added, followed by overnight incubation. The cell viability was quantified by measuring the absorbance of the solution at 550 nm using a Biotek Synergy Mx plate reader (Winooski, VT). The virus infectivity was calculated using the MTT_{50} 50% infectious dose value which can be determined based on absorbance values of infected cells[27].

Virus Flocculation and Diafiltration

Mannitol was prepared fresh at a concentration of 1 M in NanoPure water. For flocculation of virus particles with osmolytes, 9720 µl of mannitol and 405 µl of virus (PPV and Sindbis at 6 log MTT_{50}/ml in PBS, unless stated otherwise) were mixed and

kept for 2 hours at room temperature with manual rotation every 15 minutes. The concentration and ratio of osmolyte to virus was adapted from our previous studies[22, 23].

Flocculated virus with osmolyte solution was filtered using a batch diafiltration method as shown in **Fig. 1**. A 10 ml Amicon stirred cell filter, a gift from EMD Millipore, was equipped with different pore size membranes. The membranes were Durapore 0.2 and 0.1 µm and BioMax 500 kDa and 300 kDa MWCO, also gifts from EMD Millipore. Filtration cells were operated at 10 psi pressure, unless otherwise stated, from a compressed nitrogen tank and without stirring. Initially, 10 ml of mannitol and virus solution were added to the filtration cell and pressure was applied until 2-3 ml of filtrate was collected, with 2 ml being held up in the outlet tubing. This was the first fraction collected for filtrate (F1). A 300 µl sample was removed from the retentate (R1). After collection of the first fraction, 5 ml of 1 M mannitol was added to make a total of 10 ml solution in the filter unit. Pressure was applied to the filtration cell and the second fraction filtrate (F2) and retentate (R2) were collected. Similarly, for the third fraction, 5 ml of mannitol was added and filtrate (F3) and retentate (R3) were collected. All samples were analyzed by titration using an MTT assay mentioned earlier.

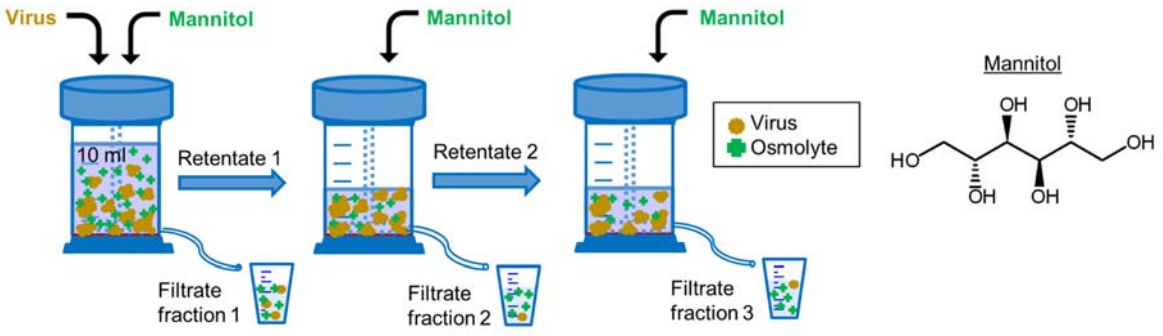

Figure 1: Schematic of batch diafiltration. Virus and mannitol were added to an Amicon Stirred Cell without agitation to a total volume of 10 ml. Pressure was applied until the volume was 5 ml. Additional mannitol was added and the process repeated.

Per unit recovery of infectious virus particles was calculated at each fraction of retentate with respect to input at each fraction as shown in the following equation,

$$PR_i = \left(\frac{Mass\ per\ MTT\ for\ retentate\ i}{Mass\ per\ MTT\ for\ input\ in\ i^{th}\ fraction} \right) \times 100 \quad (1)$$

where PR_i represents per unit percent recovery in the i^{th} fraction. Overall recovery was determined by multiplication of all previous fraction recoveries.

HPLC C-18 Chromatography

The samples collected from diafiltration using the 0.1 µm filters were analyzed for purification using reverse phase chromatography (RP-HPLC). A Waters XBridge BEH C18 Column 4.6 mm × 150 mm was used for samples analysis on a Waters Alliance HPLC equipped with a photo diode UV detector. A sample volume of 100 µl was injected onto the column and all the samples were run at the same conditions. The mobile phase started at 100% buffer A, comprised of 0.1% TFA in water, and an increasing buffer B, comprised of 0.1% TFA in acetonitrile. The flow rate for all samples was maintained at 0.500 ml/min, a sample temperature of 15°C and a column temperature of 25°C. The column was washed in between samples with 100% acetonitrile. The area under the peak was analyzed using Empower software to determine the reduction in protein content.

DNA Quantification

DNA was quantified with the Quant-iT PicoGreen dsDNA kit (Thermo Scientific, Waltham, MA). All the samples collected from diafiltration were analyzed for active dsDNA contents. For PPV and SINV infectious particles recovery, diafiltration was done

using a 0.1 μm pore size filter. Before filtration and after filtration samples were analyzed to see whether flocculation with osmolytes removed the host cell DNA. Samples were analyzed for fluorescence activity using a Synergy Mx microplate reader at an excitation of 480 nm and emission at 520 nm. Removal of DNA content per unit in retentate samples was calculated using equation 2.

$$Removal\ of\ DNA\ = \left(1 - \left(\frac{DNA\ in\ retentate\ (\mu g)}{DNA\ in\ input\ (\mu g)}\right)\right) \times 100 \qquad (2)$$

Transmission Electron Microscopy

For studying flocculation of PPV with mannitol, TEM imaging of flocculated and non-flocculated samples were taken. After flocculation, virus samples were inactivated using 0.5% glutaraldehyde[28]. Samples were mounted directly on copper grids and air-dried overnight. Grids were washed in NanoPure water and then stained in 2% uranyl acetate for 2 minutes. Grids were washed again after staining with pure water to prevent contamination. TEM images were captured on a JEOL JEM-2010 (Peabody, MA) at 80 kV.

Results

It has been discovered that PPV and SINV can be removed by flocculation with osmolytes[22, 23]. The original work described the proof of concept that viruses can be flocculated with osmolytes and the flocs can be removed from solution with a 0.2 μm filter. It was desired to translate this proof of concept removal into a method for purification. In order to do this, batch diafiltration experiments were carried out in a Millipore Amicon Stirred Cell (see **Fig. 1**). Mannitol was chosen as the common

flocculant that would aggregate both of the model viruses. Mannitol is a linear sugar molecule with neutral charge, as show in **Fig. 1**.

One of the unique aspects of this work is that the physical properties of the viruses are quite distinct, as shown in **Table 1**. The most notable features are the size difference between the viruses and the presence of an envelope for SINV. The size difference affects the filter pore size chosen for the purification of each virus. However, the presence of the envelope does not change the ability of mannitol to flocculate virus.

PPV Purification

The flocculation and recovery of PPV in mannitol was compared to PPV diluted in water as a negative control. As the pore size is decreased (**Fig. 2A**), the recovery of PPV increases. This indicates that there is likely a range of virus aggregate sizes in solution and some are larger and some are smaller than 0.1 μm, the largest filter tested for PPV. The 0.1 μm filter was able to recover 58% of infectious virus particles, which is well within the typically accepted recoveries for traditional viral particle separations[33], although newer unit operations are reaching 80% or more[34]. An increase in the starting material concentration also increased recovery (**Fig. 2B**). The transmembrane pressure increased with the highest titer tested and this could indicate cake formation and pore plugging, which would increase recovery but may decrease purity.

Table 1: Physical properties of model viruses

Virus	Enveloped	Family	Related Human Viruses	Size (nm)	pI	References
Porcine parvovirus (PPV)	N	Parvovirus	B19, AAV	18-26	5.3	29,30
Sindbis virus (SINV)	Y	Alphatoga	Equine encephalitis	48-52	4.2	31,32

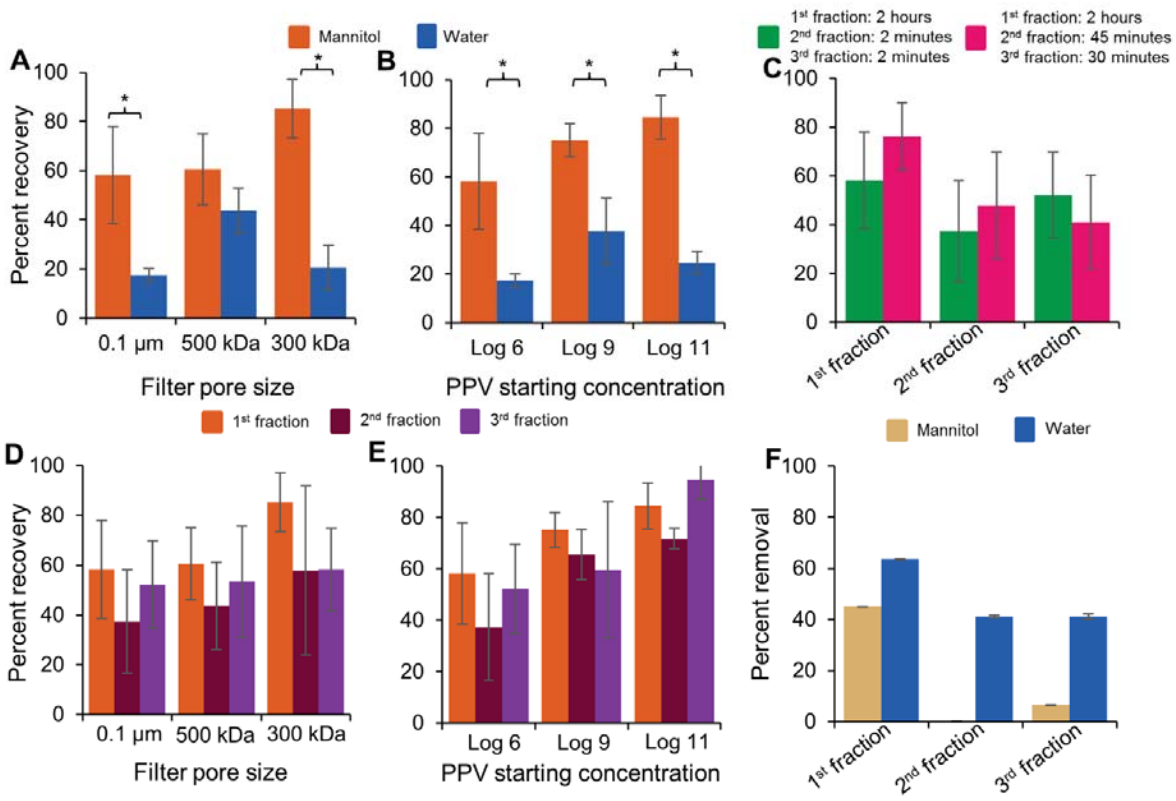

Figure 2: Non-enveloped PPV purification. The recovery of infectious PPV in the retentate was compared to the negative control of water at different (A) filter pore sizes and (B) starting concentrations. The recovery was measured for three rounds of diafiltration at different (C) flocculation times, (D) filter pore sizes and (E) starting concentrations. The removal of (F) DNA was determined by PicoGreen assay. * $p<0.05$.

To determine if the batch Amicon cell could simulate a diafiltration system, different fractions of the system were sampled, as detailed in **Fig. 1**. Earlier work indicated that the flocculation required at least 30 minutes to occur. Therefore, the effect of time was determined between each fraction of the diafiltration for PPV using a 0.1 µm filter. There was a slight, but not significant, reduction in recovery when only a two minute hold time was permitted, as compared to 45 and 30 minutes. The results are shown in **Fig. 2C**. The longer hold times were used for all of the subsequent PPV

studies because increased recovery was desired. However, the shorter hold times would be feasible and would be needed to make this process continuous.

The effect of pore size and starting concentration did not change the result and the recovery stayed consistent across the different fractions, as shown in **Fig. 2D&E**, except for the 300 kDa MWCO membrane. There was a drop in the average recovery in fractions 2 and 3, but they were not statistically significant due to the large error bars that accompany infectivity results, the chosen method to analyze virus concentration.

To determine purity, the amount of DNA and host cell proteins were determined. Unfortunately, DNA removal was lower with mannitol flocculation than with the negative control of water (**Fig. 2F**). It is likely that mannitol interacts with highly charged DNA through hydrogen bonding. Although mannitol wasn't studied directly, other protecting osmolytes, and sugars in particular, have been shown to disrupt the hydrogen bonding network of water[35]. Benzonase® treatment was able to reduce the original DNA content in PPV preparations to below detectable limits (data not shown). Therefore, removal of DNA is possible with other methods and should be performed upstream of this flocculation step. Flocculation of PPV with mannitol reduced the amount of protein in solution by 80%. This was determined by reverse-phase chromatography, as shown in **Fig. 3**, due to the low concentration of proteins used in the experiments. A full analysis of the recovery and purification of PPV can be found in the Supplementary Information in **Table S1**.

TEM of Mannitol Flocculation

TEM images were taken of PPV flocculated with 1 M mannitol (**Fig. 4A&B**) and with the negative water control (**Fig. 4C)**. The virus particles are of the expected size

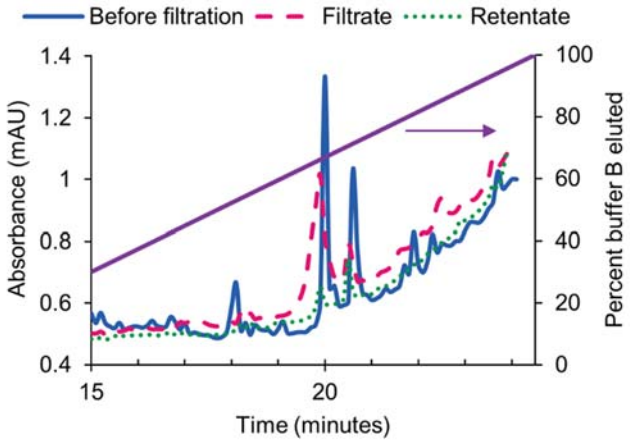

Figure 3: Removal of contaminating proteins in PPV. Contaminant proteins were detected with RPC and were found in the filtrate.

and show a web-like structure when mannitol is present and individual particles when mannitol is not present. The web-like structure could be protein or mannitol. It is not likely protein because it did not stain like protein. Most likely, the web-like structure is mannitol that aggregated during the drying process required to prepare TEM images. It is not likely that mannitol is binding to the surface or within the aggregate, as described in more detail in the Discussion section.

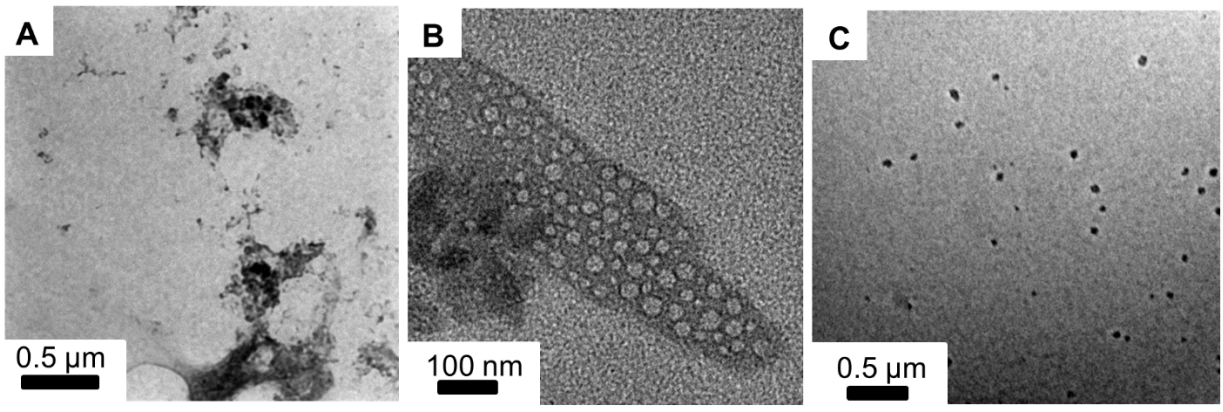

Figure 4: TEM of PPV flocs. (A & B) show PPV in contact with mannitol at two different magnifications. (C) shows PPV in contact with water.

SINV Purification

The results of the flocculation and purification of SINV were very similar to PPV. Larger pore size filters were chosen for SINV since the virus is larger. **Figs. 5A&D** show a clear increase in recovery as the filter pore size decreases, reaching as high as 96% recovery with a 500 kDa MWCO membrane. For the 0.1 µm pore size filter, SINV recovery was 77% compared to 58% for PPV. For starting concentration, there was a similar trend as PPV, with higher starting concentration providing higher recover, shown in **Figs. 5B&E**, albeit, SINV had higher overall recoveries and was less sensitive to starting concentration. It is not clear if size, charge, presence of a membrane or other

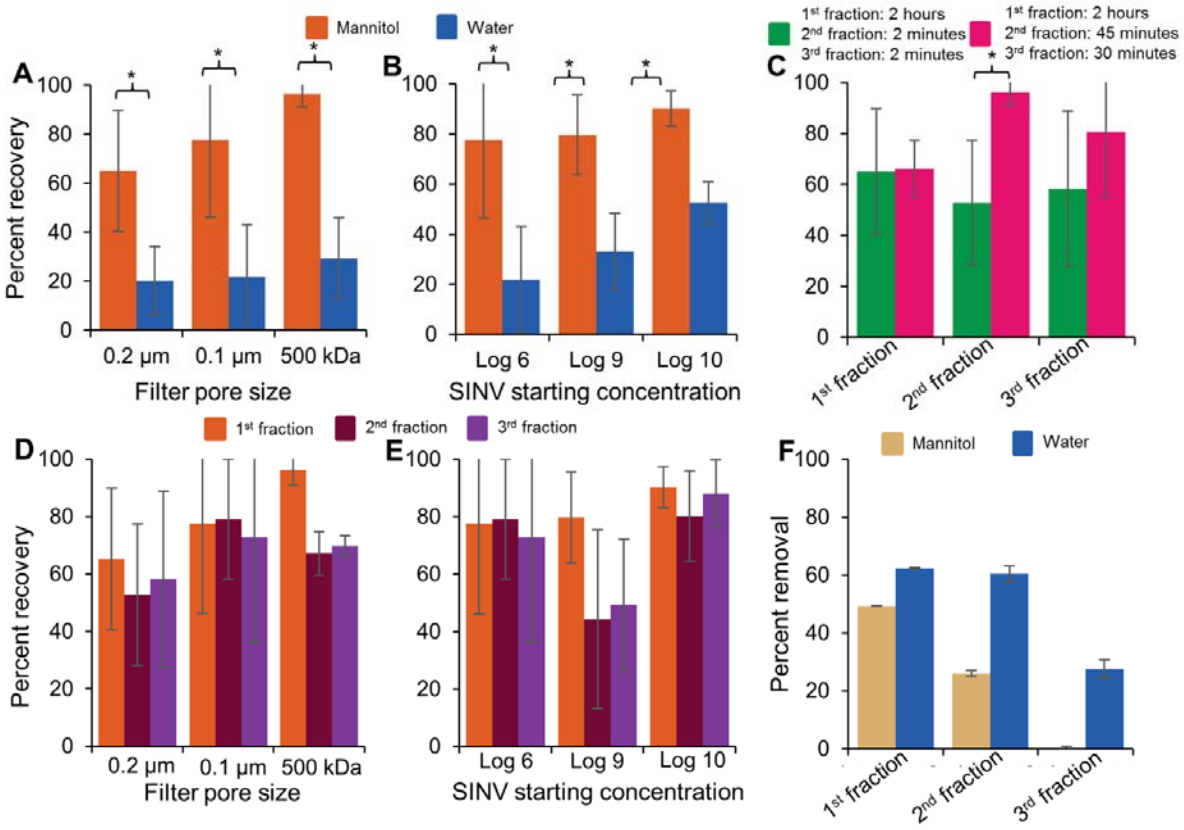

Figure 5: Enveloped SINV purification. The recovery of infectious SINV in the retentate was compared to the negative control of water at different (A) filter pore sizes and (B) starting concentrations. The recovery was measured for three rounds of diafiltration at different (C) flocculation times, (D) filter pore sizes and (E) starting concentrations. The removal of (F) DNA was determined by PicoGreen assay. * $p<0.05$.

physical properties were responsible for the improved recoveries of SINV over PPV. The change in flocculation time, shown in **Fig. 5C**, appears to have more of an effect for SINV than PPV. This may suggest an interaction of mannitol with the envelope of the virus or a change in how mannitol structures water in the presence of a lipid bilayer. Detailed SINV purification information can be found in **Table S2**.

Similar to PPV, DNA was flocculated by mannitol and was removed to a lower degree than the control water, shown in **Fig. 5F**. Also similar to PPV, a large amount of protein was removed, leaving a retentate with few peaks, as detected by RPC and shown in **Fig. 6**.

Discussion

The function of naturally occurring osmolytes is to control the location of water. By having a strong affinity for water, osmolytes can maintain cell volume by strongly binding to water in a cell. This is observed in deep sea fish as a method to combat high salt and high pressure environments[36]. It has also been determined that osmolytes can stabilize proteins and are often used in protein formulations[16]. Influenza virus formulations can also be stabilized with concentrations of osmolytes around 0.5 M[37]. However, it was found that between 0.3 – 1.0 M osmolytes have the ability to cause virus flocculation[22, 23]. Yet, the flocculation system does not change the infectivity of the virus, which was the measure of stability for the influenza virus study[37], therefore the study of influenza virus stability and this work are not necessarily contradictory to one another.

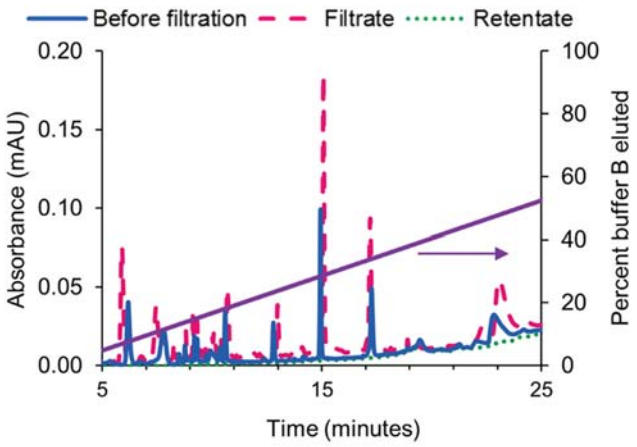

Figure 6: Removal of contaminating proteins in SINV. Contaminant proteins were detected with RPC and were found in the filtrate.

The proposed mechanism of virus flocculation in osmolyte solutions can be found in **Fig. 7**. Osmolyte induced protein stability is brought about by unfavorable interactions with the protein backbone causing an osmophobic effect[38, 39]. The depletion of the osmolyte at the surface of the protein and the strong water binding capability of osmolytes creates a minor dehydration of the protein surface. This occurs because the water molecules that are near hydrophobic amino acids on the surface of the protein are not as tightly bound as water that is near hydrophilic amino acids[40]. This makes it more energetically favorable for the water to bind to the osmolyte than the hydrophobic protein surface. When the water around the more hydrophobic amino acids is removed, the protein collapses into a more compact shape, which is often more stable against heat[16] and chemical denaturation[41].

The dehydration of a virus surface is more drastic than for proteins, causing virus aggregation. This is due to two things, (1) proteins can collapse and refold to account for the dehydrated surface, whereas viral surfaces are more rigid and less apt to

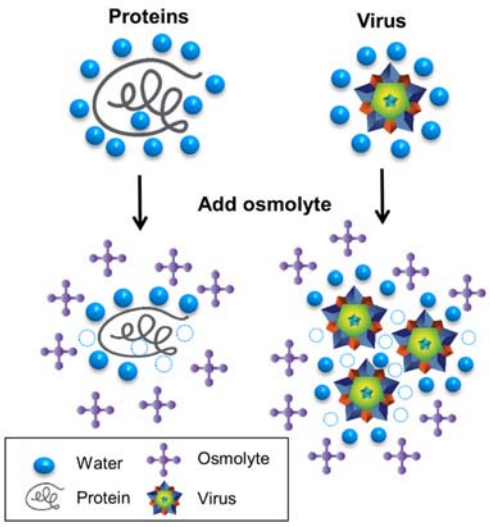

Figure 7: Cartoon of flocculation of proteins and virus in contact with osmolytes. Osmolytes dehydrate protein and virus surfaces. Proteins become more compact and stable. Viruses are too rigid to compact and this causes the particles to aggregate.

collapse, and (2) viral surfaces have more exposed hydrophobic amino acids than proteins, leading to more water molecules that can be removed from the surface. Viral hydrophobicity has been experimentally determined by two different groups[24, 25]. The hydrophobic interaction has biological significance, as viruses desire to bind to cell surface receptors. This interaction is likely a key mechanism for this interaction and has been selected for in the evolution of viral particles. In osmolyte solutions, the more hydrophobic virus is not able to collapse during surface dehydration and therefore is likely to aggregate to satisfy the thermodynamic water changes caused by the osmolyte. The aggregation is easily reversed by dilution of the osmolyte for most osmolytes and was demonstrated by a retention of infectivity of the virus in osmolyte solutions[23]. It was shown that for the enveloped SINV, some of the osmolytes caused infectivity loss of >1 log, in particular glycine, arginine and serine[22].

Osmolyte flocculation has only been tested with icosahedral, symmetric viral particles. It is unknown if asymmetric viruses, especially plant viruses or bacteriophages, exhibit the same flocculation behavior. Most testing of virus hydrophobicity[24, 25] and bacteriophage hydrophobicity[42] have been done with icosahedral viruses, making it difficult to predict the ability of other viral particles to flocculate in osmolyte solutions.

An unusual observation during this work is that DNA appears to also aggregate in the presence of mannitol because it is removed less than what should have been the negative control of water (see **Figs. 2F** & **5F**). The more compact shape of DNA[43] and RNA[44] appears to be favored in high TMAO concentrations. It is also possible that the neutral mannitol could cause DNA to lose its charge, similar to the study of silica particles being neutralized in the presence of the neutral, protecting osmolyte, glycerol[21]. While not many studies have been conducted in other osmolyte solutions, it appears that 1 M mannitol also stabilizes not only a more compact DNA structure, but it might also induce aggregation due to the ability of a 0.1 μm filter to withhold DNA in our purification system. Osmolytes should be explored further for their ability to purify DNA for future DNA vaccines or plasmid applications.

Conclusions

This work demonstrates the feasibility of using osmolyte flocculation to purify viral particles for vaccine applications. Mannitol was chosen because it was a common flocculant that was found when flocculants were screened for an enveloped and non-enveloped virus[22, 23]. A system was developed using batch diafiltration for the recovery

of infectious virus particles after mannitol flocculation. The recovery ranged from 58-96% depending on virus and filter pore size. Recovery could also be improved by increasing the titer of the virus used. This is common, as virus can stick to processing equipment and the loss of virus is more noticeable when low titers are used than with high titer systems.

It was observed that DNA was not removed in the system. There appeared to be flocculation of the DNA along with the virus in the mannitol system. TMAO has been shown to enhance compact conformations of DNA[43] and RNA[44], but little is known on how mannitol affects nucleic acids. The mechanism of osmolyte flocculation of nucleic acids should be explored and may be a future method to purify plasmid and other DNA or RNA therapeutic products.

While the mechanism of DNA flocculation with osmolytes is not well understood, there is a plethora of information on the flocculation of proteins with osmolytes. This theoretical knowledge can be applied to the flocculation of viral particles in the presence of proteins. Osmolytes have a strong affinity for water molecules. Due to this affinity, osmolytes bind stronger to water molecules than do the hydrophobic amino acids on the surface of proteins and viral particles. Experimental evidence has shown that viral particles tend to be more hydrophobic than proteins[24, 25]. The dehydration of the hydrophobic surface and the larger amount of hydrophobic surface exposed on viral surfaces makes the virus aggregate. This aggregation is reversible by dilution of the osmolyte and therefore makes this a potential virus purification process for vaccine and gene therapy manufacturing.

Acknowledgements

This work was funded by NSF CBET 1159425 and 1451959, and the James and Lorna Mack Chair in Bioengineering. The authors are grateful to EMD Millipore for supplying filters, Benzonase® and other equipment used in this work. The authors thank Jessica Brassard in the Michigan Tech Research Development Office for assistance with the artwork.

The authors declare no conflict of interest.

References

1. GAVI Half a billion children vaccinated and seven million lives saved thanks to Gavi partners. http://www.gavi.org/library/news/press-releases/2015/half-a-billion-children-vaccinated-and-seven-million-lives-saved-thanks-to-gavi-partners/ (July 17, 2016).

2. Chandrasekharan, S.; Amin, T.; Kim, J.; Furrer, E.; Matterson, A. C.; Schwalbe, N.; Nguyen, A., Intellectual property rights and challenges for development of affordable human papillomavirus, rotavirus and pneumococcal vaccines: Patent landscaping and perspectives of developing country vaccine manufacturers. *Vaccine* **2015,** 33, (46), 6366-70.

3. Pagliusi, S.; Makhoana, M.; Datla, M.; Leite, L.; Hendriks, J.; Gholami, A.; Huang, W.; Gao, Y.; Jadhav, S.; Harshavardhan, G. V.; Wu, Y.; Suhardono, M.; Homma, A., Developing Countries Vaccine Manufacturers Network (DCVMN): engaging to step up for vaccine discovery and access. Meeting report 2012. *Vaccine* **2013,** 31, (31), 3111-5.

4. Hastie, E.; Samulski, R. J., Adeno-Associated Virus at 50: A Golden Anniversary of Discovery, Research, and Gene Therapy Success-A Personal Perspective. *Hum Gene Ther* **2015,** 26, (5), 257-265.

5. Strobel, B.; Miller, F. D.; Rist, W.; Lamla, T., Comparative Analysis of Cesium Chloride-and Iodixanol-Based Purification of Recombinant Adeno-Associated Viral Vectors for Preclinical Applications. *Human gene therapy methods* **2015,** 26, (4), 147-157.

6. Wickramasinghe, S. R.; Kalbfuss, B.; Zimmermann, A.; Thom, V.; Reichl, U., Tangential flow microfiltration and ultrafiltration for human influenza A virus concentration and purification. *Biotechnology and bioengineering* **2005,** 92, (2), 199-208.

7. Geraerts, M.; Michiels, M.; Baekelandt, V.; Debyser, Z.; Gijsbers, R., Upscaling of lentiviral vector production by tangential flow filtration. *The journal of gene medicine* **2005,** 7, (10), 1299-310.

8. Nestola, P.; Martins, D. L.; Peixoto, C.; Roederstein, S.; Schleuss, T.; Alves, P. M.; Mota, J. P.; Carrondo, M. J., Evaluation of novel large cut-off ultrafiltration membranes for adenovirus serotype 5 (ad5) concentration. *PLoS One* **2014,** 9, (12), e115802.

9. Riske, F.; Berard, N.; Albee, K.; Pan, P.; Henderson, M.; Adams, K.; Godwin, S.; Spear, S., Development of a platform process for adenovirus purification that removes human set and nucleolin and provides high purity vector for gene delivery. *Biotechnol. Bioeng.* **2013,** 110, (3), 848-856.

10. Vajda, J.; Weber, D.; Stefaniak, S.; Hundt, B.; Rathfelder, T.; Muller, E., Mono- and polyprotic buffer systems in anion exchange chromatography of influenza virus particles. *J Chromatogr A* **2016,** 1448, 73-80.

11. Yu, M.; Li, Y.; Zhang, S.; Li, X.; Yang, Y.; Chen, Y.; Ma, G.; Su, Z., Improving stability of virus-like particles by ion-exchange chromatographic supports with large pore size: advantages of gigaporous media beyond enhanced binding capacity. *J Chromatogr A* **2014,** 1331, 69-79.

12. Etzel, M. R.; Riordan, W. T., Viral clearance using monoliths. *J. Chromatogr. A* **2009,** 1216, (13), 2621-2624.

13. Leuchs, B.; Frehtman, V.; Riese, M.; Muller, M.; Rommelaere, J., A novel scalable, robust downstream process for oncolytic rat parvovirus: isoelectric point-based elimination of empty particles. *Appl. Microbio. Biotechnol.* **2017,** 101, 3143-3152.

14. Baumann, P.; Hubbuch, J., Downstream process development strategies for effective bioprocesses: Trends, progress, and combinatorial approaches. *Eng. Life Sci.* **2017,** 17, (11), 1142-1158.

15. Shukla, A. A.; Hubbard, B.; Tressel, T.; Guhan, S.; Low, D., Downstream processing of monoclonal antibodies--Application of platform approaches. *J. Chromatogr. B* **2007,** 848, (1), 28-39.

16. Roberts, C. J.; Nesta, D. P.; Kim, N., Effects of Temperature and Osmolytes on Competing Degradation Routes for an IgG1 Antibody. *J Pharm Sci* **2013,** 102, (10), 3556-66.

17. Nayak, A.; Lee, C. C.; McRae, G.; Belfort, G., Osmolyte controlled fibrillation kinetics of insulin: New insight into fibrillation using the preferential exclusion principle. *Biotechnol. Prog.* **2009,** 25, (5), 1508-1514.

18. Street, T. O.; Bolen, D. W.; Rose, G. D., A molecular mechanism for osmolyte-induced protein stability. *Proc. Natl. Acad. Sci.* **2006,** 103, (45), 17064-17064.

19. Liao, Y. T.; Manson, A. C.; DeLyser, M. R.; Noid, W. G.; Cremer, P. S., Trimethylamine N-oxide stabilizes proteins via a distinct mechanism compared with betaine and glycine. *Proc. Natl. Acad. Sci.* **2017,** 114, (10), 2479-2484.

20. Mondal, J.; Halverson, D.; Li, I. T. S.; Stirnemann, G.; Walker, G. C.; Berne, B. J., How osmolytes influence hydrophobic polymer conformations: A unified view from experiment and theory. *Proc. Natl. Acad. Sci.* **2015,** 112, (30), 9270-9275.

21. Govrin, R.; Schlesinger, I.; Tcherner, S.; Sivan, U., Regulation of Surface Charge by Biological Osmolytes. *J Am Chem Soc* **2017,** 139, (42), 15013-15021.

22. Gencoglu, M. F.; Heldt, C. L., Enveloped virus flocculation and removal in osmolyte solutions. *J. Biotechnol.* **2015,** 206, 8-11.

23. Gencoglu, M. F.; Pearson, E.; Heldt, C. L., Porcine Parvovirus Flocculation and Removal in the Presence of Protecting Osmolytes. *J. Biotechnol.* **2014,** 186, 83-90.

24. Heldt, C. L.; Zahid, A.; Vijayaragavan, K. S.; Mi, X., Experimental and computational surface hydrophobicity analysis of a non-enveloped virus and proteins. *Colloids Surfaces B: Biointerfaces* **2017,** 153, 77-84.

25. Johnson, S. A.; Walsh, A.; Brown, M. R.; Lute, S. C.; Roush, D. J.; Burnham, M. S.; Brorson, K. A., The step-wise framework to design a chromatography-based hydrophobicity assay for viral particles. *J Chromatogr B* **2017,** 1061-1062, 430-437.

26. Tafur, M. F.; Vijayaragavan, K. S.; Heldt, C. L., Reduction of Porcine Parvovirus Infectivity in the Presence of Protecting Osmolytes. *Antivir. Res.* **2013,** 99, (1), 27-33.

27. Heldt, C. L.; Hernandez, R.; Mudiganti, U.; Gurgel, P. V.; Brown, D. T.; Carbonell, R. G., A colorimetric assay for viral agents that produce cytopathic effects. *J. Virol. Methods* **2006,** 135, (1), 56-65.

28. Moller, L.; Schunadel, L.; Nitsche, A.; Schwebke, I.; Hanisch, M.; Laue, M., Evaluation of virus inactivation by formaldehyde to enhance biosafety of diagnostic electron microscopy. *Viruses* **2015,** 7, (2), 666-79.

29. Mengling, W. L., *Diseases of Swine*. 1999.

30. Weichert, W. S.; Parker, J. S. L.; Wahid, A. T. M.; Chang, S.-F.; Meier, E.; Parrish, C. R., Assaying for Structural Variation in the Parvovirus Capsid and Its Role in Infection. *Virology* **1998,** 250, (1), 106.

31. He, L.; Piper, A.; Meilleur, F.; Myles, D. A. A.; Hernandez, R.; Brown, D. T.; Heller, W. T., The Structure of Sindbis Virus Produced from Vertebrate and Invertebrate Hosts as Determined by Small-Angle Neutron Scattering. *J. Virol.* **2010,** 84, (10), 5270-5276.

32. Dalrymple, J. M.; Schlesinger, S.; Russell, P. K., Antigenic characterization of two Sindbis envelope glycoproteins separated by isoelectric focusing. *Virology* **1976,** 69, (1), 93-103.

33. Wolff, M. W.; Reichl, U., Downstream processing of cell culture-derived virus particles. *Expert Rev. Vaccines* **2011,** 10, (10), 1451-1475.

34. Nestola, P.; Peixoto, C.; Silva, R. R.; Alves, P. M.; Mota, J. P.; Carrondo, M. J., Improved virus purification processes for vaccines and gene therapy. *Biotechnol Bioeng* **2015**.

35. Verma, P. K.; Lee, H.; Park, J. Y.; Lim, J. H.; Maj, M.; Choi, J. H.; Kwak, K. W.; Cho, M., Modulation of the Hydrogen Bonding Structure of Water by Renal Osmolytes. *J Phys Chem Lett* **2015,** 6, (14), 2773-9.

36. Zerbst-Boroffka, I.; Kamaltynow, R. M.; Harjes, S.; Kinne-Saffran, E.; Gross, J., TMAO and other organic osmolytes in the muscles of amphipods (Crustacea) from shallow and deep water of Lake Baikal. *Comp Biochem Physiol A Mol Integr Physiol* **2005,** 142, (1), 58-64.

37. White, J. A.; Estrada, M.; Flood, E. A.; Mahmood, K.; Dhere, R.; Chen, D., Development of a stable liquid formulation of live attenuated influenza vaccine. *Vaccine* **2016,** 34, (32), 3676-83.

38. Hu, C. Y.; Pettitt, B. M.; Roesgen, J., Osmolyte solutions and protein folding. *F1000 Biol Rep* **2009,** 1, 41.

39. Bolen, D. W., Effects of naturally occurring osmolytes on protein stability and solubility: issues important in protein crystallization. *Methods* **2004,** 34, (3), 312-22.

40. Patel, A. J.; Varilly, P.; Jamadagni, S. N.; Hagan, M. F.; Chandler, D.; Garde, S., Sitting at the edge: how biomolecules use hydrophobicity to tune their interactions and function. *J. Phys. Chem. B* **2012,** 116, (8), 2498-503.

41. Rahman, S.; Ali, S. A.; Islam, A.; Hassan, M. I.; Ahmad, F., Testing the dependence of stabilizing effect of osmolytes on the fractional increase in the accessible surface area on thermal and chemical denaturations of proteins. *Arch Biochem Biophys* **2016,** 591, 7-17.

42. Dika, C.; Ly-Chatain, M. H.; Francius, G.; Duval, J. F. L.; Gantzer, C., Non-DLVO adhesion of F-specific RNA bacteriophages to abiotic surfaces: Importance of surface roughness, hydrophobic and electrostatic interactions. *Colloid Surf. A-Physicochem. Eng. Asp.* **2013,** 435, 178-187.

43. Patra, S.; Anders, C.; Erwin, N.; Winter, R., Osmolyte Effects on the Conformational Dynamics of a DNA Hairpin at Ambient and Extreme Environmental Conditions. *Angew Chem Int Ed Engl* **2017,** 56, (18), 5045-5049.

44. Lambert, D.; Leipply, D.; Draper, D. E., The osmolyte TMAO stabilizes native RNA tertiary structures in the absence of Mg2+: evidence for a large barrier to folding from phosphate dehydration. *J Mol Biol* **2010,** 404, (1), 138-57.

Supplementary Information

A Generalized Purification Step for Viral Particles using Mannitol Flocculation

Caryn L. Heldt[1,2,*], Ashish Saksule[1], Pratik U. Joshi[1], and Majid Ghafarian[2]

[1]Department of Chemical Engineering and
[2]Department of Biological Sciences
Michigan Technological University
1400 Townsend Dr., Houghton MI 49931, USA
* Corresponding author: email: heldt@mtu.edu

Table S1: PPV recovery and purification with different filter pore sizes and diafiltration steps.

PPV			**Flocculation with mannitol**				
Filter pore size	DNA removal[a] (%)	Protein removal[b] (%)	Starting concentration $log_{10}(MTT_{50}/ml)$	Final retentate concentration $log_{10}(MTT_{50}/ml)$ by fraction		Step yield[c] (%) per fraction	Overall recovery[d] (%)
0.1 µm	45	85	6.63 ± 0.1	1st	6.35 ± 0.2	58 ± 19	58 ± 19
				2nd	5.75 ± 0.3	37 ± 20	21 ± 15
				3rd	5.47 ± 0.4	52 ± 17	10 ± 9
500 kDa	-	-	6.93 ± 0.5	1st	6.70 ± 0.5	60 ± 14	60 ± 14
				2nd	5.29 ± 0.4	43 ± 17	25 ± 8
				3rd	5.94 ± 0.6	53 ± 22	11 ± 1
300 kDa	-	-	6.47 ± 0.2	1st	6.55 ± 0.2	85 ± 11	85 ± 11
				2nd	6.06 ± 0.1	57 ± 33	53 ± 31
				3rd	5.86 ± 0.1	58 ± 16	36 ± 22
			Negative control flocculation with water				
0.1 µm	63	0	6.60 ± 0.2	1st	5.84 ± 0.2	17 ± 2	17 ± 2
				2nd	5.43 ± 0.3	44 ± 22	7 ± 2
				3rd	5.29 ± 0.3	77 ± 27	7 ± 4
500 kDa	-	-	7.04 ± 0.7	1st	6.68 ± 0.8	43 ± 9	43 ± 9
				2nd	6.01 ± 0.7	23 ± 14	9 ± 3
				3rd	5.09 ± 0.5	29 ± 3	2 ± 1
300 kDa	-	-	6.61 ± 0.4	1st	5.96 ± 0.3	20 ± 9	20 ± 9
				2nd	5.64 ± 0.3	55 ± 31	12 ± 11
				3rd	5.44 ± 0.3	81 ± 24	8 ± 3

Errors are the standard deviation from three trials.
a: Based on Quant-iT PicoGreen dsDNA quantification method
b: Based on area under the curve calculations from reverse phase chromatography (RPC)
c: Based on infectious particles titer calculations using the MTT assay per unit recovery calculations from equation 1
d: Based on infectious particles titer calculations using the MTT assay overall recovery

Table S2: SINV recovery and purification with different filter pore sizes and diafiltration steps.

SINV	**Flocculation with mannitol**						
Filter pore size	DNA removal[a] (%)	Protein removal[b] (%)	Starting concentration $log_{10}(MTT_{50}/ml)$	Final retentate concentration $log_{10}(MTT_{50}/ml)$ by fraction		Step yield[c] (%) per fraction	Overall recovery[d] (%)
0.2 µm	49	na	6.43 ± 0.2	1st	6.3 ± 0.5	65 ± 24	65 ± 24
				2nd	6.0 ± 0.5	52 ± 24	45 ± 25
				3rd	5.8 ± 0.5	58 ± 30	35 ± 20
0.1 µm	-	-	7.03 ± 0.8	1st	7.1 ± 0.5	77 ± 31	77 ± 31
				2nd	7.0 ± 1.0	79 ± 20	62 ± 24
				3rd	6.6 ± 0.6	72 ± 36	45 ± 18
500 kDa	-	-	6.07 ± 0.7	1st	6.1 ± 0.7	96 ± 5	96 ± 5
				2nd	5.9 ± 0.7	67 ± 7	71 ± 8
				3rd	5.4 ± 0.7	69 ± 3	46 ± 2
	Negative control flocculation with water						
0.2 µm	62	na	6.69 ± 0.2	1st	6.0 ± 0.5	20 ± 13	20 ± 13
				2nd	6.2 ± 0.5	86 ± 22	32 ± 24
				3rd	5.8 ± 0.8	48 ± 27	20 ± 18
0.1 µm	-	-	7.55 ± 1	1st	6.6 ± 1.0	21 ± 31	21 ± 21
				2nd	6.5 ± 1.0	65 ± 26	22 ± 18
				3rd	5.3 ± 1.0	76 ± 39	21 ± 24
500 kDa	-	-	6.95 ± 1	1st	6.4 ± 1.0	29 ± 16	29 ± 16
				2nd	5.7 ± 0.7	36 ± 28	11 ± 7
				3rd	5.6 ± 0.9	74 ± 23	7 ± 4

Error bar are standard deviation from three trials.
na: not available
a: Based on Quant-iT PicoGreen dsDNA quantification method
b: Based on area under the curve calculations from reverse phase chromatography (RPC)
c: Based on infectious particles titer calculations using the MTT assay per unit recovery calculations from equation 1
d: Based on infectious particles titer calculations using the MTT assay overall recovery