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Porcine parvovirus flocculation and removal in the presence of osmolytes

Maria F. Gencoglu, Eric Pearson, Caryn. L. Heldt*

Department of Chemical Engineering, Michigan Technological University, 1400 Townsend Dr. Houghton, MI 49931, USA

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ABSTRACT

Viruses can be modified into viral vaccines or gene therapy vectors in order to treat acquired or genetic diseases. To satisfy the current market demand, an improvement in current vaccine manufacturing is needed. Chromatography and nanofiltration are not suitable for all types of viruses. In this study, we propose to use virus flocculation with osmolytes, followed by microfiltration, as a potential virus purification process. We hypothesize that osmolytes strongly bind to water, thus leading to the formation of a hydration layer around the virus particles and stimulation of aggregation. We have discovered that osmolytes, including sugars, sugar alcohols and amino acids, preferentially flocculate porcine parvovirus (PPV), and demonstrate a >80% removal with a 0.2 μm filter while leaving model proteins in solution. This large pore size filter increases the flux and decreases the transmembrane pressure of typical virus filters. The best flocculants were tested for their ability to aggregate PPV at different concentrations, shear stress, pH and ionic strength. We were able to remove 96% of PPV in 3.0 M glycine at a pH of 5. Glycine is also an excipient, and therefore may not require removal later in the process. Virus flocculation using osmolytes, followed by microfiltration could be used as an integrated process for virus purification.

1. Introduction

More than 90% of all human diseases are caused by viral infections (Norkin, 2010). However, viruses do not only cause disease; they can be modified into viral vaccines or viral vectors to prevent or treat diseases (Sharma et al., 2004). Many viral diseases, like yellow fever and polio, are under control due to the development of effective vaccines (Plotkin and Plotkin, 2004). Treatment of a wide variety of diseases, such as cancer and Alzheimer's disease, will benefit from future viral gene therapy vectors (Ausubel et al., 2010; Dullaers et al., 2005; Tuszynski et al., 2005). The World Health Organization (WHO) reported in 2010 that the vaccine market growth rate is 10–15% per year, higher than the growth rate of other pharmaceuticals at 5–7% per year (WHO, 2012). Since there is a large market for viral vaccines, a more efficient method to manufacture and purify viral particles is needed. Lyddiatt and Sullivan estimated that between 10^{11} and 10^{14} viral vectors will be required for a single dose of gene therapy vectors (Lyddiatt and O'Sullivan, 1998). Due to this large dosage, an efficient, cost-effective, and quick process will be required to satisfy the viral vector market demand.

Conventional virus purification techniques, like ultracentrifugation and density gradient centrifugation, have been used to isolate viruses at the laboratory scale (Burova and Ioffe, 2005; de las Mercedes Segura et al., 2005). However, due to the size of the equipment, the difficult scale-up, long processing times, and low purity, ultracentrifugation remains cost-ineffective for large-scale vaccine production (Andreadis et al., 1999; Braas et al., 1996; Peixoto et al., 2007).

Alternatives such as filtration and chromatography have been used to overcome the disadvantages of ultracentrifugation. Tangential flow filtration (TFF) has effectively recovered and purified virus particles (Grzenia et al., 2008; Wickramasinghe et al., 2005). TFF is successful due to the large size difference of virus particles and typical protein contaminants. The main advantages of TFF are easy scalability, high throughput, high viral titer and high viral recoveries (Geraerts et al., 2005; Kuiper et al., 2002; Rodrigues et al., 2007). However, the capacity of the membranes can be affected by fouling and this can lead to longer filtration times, low flux through the membrane and high transmembrane pressures (Morenweiser, 2005; Segura et al., 2013). Additionally, a platform approach for virus filtration does not exist, since the success of current processes depend on the properties of individual viral therapeutics (Grein et al., 2013).

Chromatography is the predominant technique for large-scale virus purification (Burova and Ioffe, 2005; de las Mercedes Segura

* Corresponding author. Tel.: +1 906 487 1134; fax: +1 906 487 3213.
E-mail address: heldt@mtu.edu (Caryn.L. Heldt).

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Nomenclature

A. SUL	ammonium sulfate
ALA	D-alanine
ARG	D-arginine
BET	betaine
BSA	bovine serum albumin
DSP	downstream processes
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
FBS	fetal bovine serum
GLY	glycine
H_2O	water
HCl	hydrochloric acid
IgG	immunoglobulin G
LRV	log reduction value
M. CHL	magnesium chloride
M. SUL	magnesium sulfate
MAN	D-mannitol
MEM	minimum essential medium
MTT	thiazolyl blue tetrazolium bromide
MTT_{50}	the 50% infectious dose
MW	molecular weight
NaCl	sodium chloride (in text)
NaOH	sodium hydroxide
PBS	phosphate-buffered saline
PEG	poly(ethylene) glycol
Pen/Strep	penicillin/streptomycin
pI	isoelectric point
PK-13	porcine kidney
PRO	L-proline
PPV	porcine parvovirus
RAF	D-(+)-raffinose pentahydrate
S. CHL	sodium chloride
SDS	sodium dodecyl sulfate
SER	L-serine
SUC	sucrose
TFF	tangential flow filtration
TMAO	trimethylamine N-oxide
TRE	(+)-trehalose dehydrate
TRIS	Tris(hydroxymethyl)aminomethane hydrochloride
WHO	World Health Organization

et al., 2007). All of the typical chromatography modes have been utilized for virus particle purification (Wolf and Reichl, 2011). The more successful chromatographic purification processes for viruses are combined with size-based separation methods (Burova and Ioffe, 2005; Morenweiser, 2005). However, a universal chromatography process cannot be applied to all the viruses, since the operating conditions depend on the charge, size, and specificity of the target virus (Coroadinha et al., 2010; Rodrigues et al., 2007). Moreover, conventional chromatography resins are designed for the purification of proteins, but not for large biomolecules, such as virus particles. Virus particles have difficulties accessing the high internal surface area of the resins (Flickinger, 2013; Trilisky and Lenhoff, 2007), making the resins highly inefficient. Membrane chromatography has shown promise to overcome many of the disadvantages of pore accessibility in resin chromatography (Opitz et al., 2007; Vicente et al., 2011). However, there still remains the difficulty of optimizing the binding and elution conditions for each viral product without causing virus inactivation.

Precipitation and flocculation followed by separation using centrifugation or filtration have been used as an alternative for virus purification (Morenweiser, 2005). Commonly, the additives are compounds that promote aggregation, like salts or polymeric agents (Braas et al., 1996). Salts and polymers often precipitate all proteins in solution, although there have been reports of selective precipitation with these agents. Ammonium sulfate at specific conditions has been shown to selectively precipitate immunoglobulin G (IgG) over bovine serum albumin (BSA) (Venkiteshwaran et al., 2008) likely due to the hydrophobicity difference of the two proteins (Tessier et al., 2003). Salts have been used to precipitate viruses from protein-free solutions (Fiksdal and Leiknes, 2006; John et al., 2011; Zhu et al., 2005). Polyethylene glycol (PEG) is well-known to precipitate all proteins (Atha and Ingham, 1981), although specific conditions have been found to selectively precipitate IgG4 from cell culture (Knevelman et al., 2010) and viruses from DNA and protein contaminants (Branston et al., 2012) using PEG. However, PEG precipitation requires extended periods of incubation time (Hartmann and Halden, 2012) and polymer additives may interact with virus particles and form complexes that are difficult to dissociate (Landázuri et al., 2006; Le Doux et al., 2001).

The aim in virus flocculation is to improve process robustness; a selective, global, non-toxic, and economical flocculant is desired (Wolf and Reichl, 2011). In this study we propose to use osmolytes to overcome these challenges. Osmolytes are found in the cells of many organisms, and their main function is to stabilize intracellular proteins against environmental stresses, such as extreme temperatures or high osmotic pressures, by changing the water content of the cells (Bolen, 2004; Rose et al., 2006; Yancey, 2005). There are two types of osmolytes, protecting and denaturing. Protecting osmolytes have the ability to fold proteins by binding to the water molecules and changing the water contents around the protein backbone. Denaturing osmolytes have the ability to unfold the proteins, by binding directly to the protein backbone (Street, 2007). One advantage of osmolytes, such as glycine, alanine and mannitol as compared to salt or polymer flocculants, is that they are often used as an excipient to stabilize the final formulation of biotherapeutics (Minne et al., 2008; Roberts et al., 2013), making them an ideal addition to a biotherapeutic manufacturing process.

Porcine parvovirus (PPV), a non-enveloped, single-strand DNA, icosahedral virus, with a diameter of 18–26 nm (Halder et al., 2012; Norkin, 2010) was used to demonstrate the ability of osmolytes to preferentially flocculate virus particles. We propose that osmolytes bind to water, thus leading to the reduction of a hydration shell around the virus and causing PPV particle aggregation. Using high-throughput screening methods, we have discovered that osmolytes flocculate PPV and demonstrate a >80% removal with a 0.2 μm filter with <5% removal of model host cell proteins. This micropore filter is usually used to retain bacteria, and therefore this is a unique application of microfiltration for small virus particle removal and future purification.

2. Materials and methods

2.1. Materials

The osmolytes, trimethylamine N-oxide (TMAO) dihydrate, glycine, betaine, D-alanine, D-arginine, L-proline, L-serine, D-mannitol, sucrose, D-(+)-trehalose dihydrate, D-(+)-raffinose pentahydrate, and urea, and the salts, ammonium sulfate, sodium chloride (NaCl), magnesium sulfate, and magnesium chloride were purchased from Sigma-Aldrich (St. Louis, MO) at a minimum purity of 98%. Sodium hydroxide (NaOH), PEG, with a molecular weight (MW) of 12,000 Da, and albumin from bovine serum (BSA) were also purchased from Sigma-Aldrich (St. Louis, MO). Tris (hydroxymethyl) aminomethane hydrochloride (Tris) was purchased from Thermo Scientific (Waltham, MA). MTT Assay reagents, thiazolyl blue tetrazolium bromide (MTT), sodium dodecyl sulfate (SDS)