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Separation of porcine parvovirus from bovine serum albumin using PEG–salt aqueous two-phase system

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ABSTRACT

Vaccine production faces a challenge in adopting conventional downstream processing steps that can efficiently purify large viral particles. Some major issues that plague vaccine purification are purity, potency, and quality. The industry currently considers 30% as an acceptable virus recovery for a vaccine purification process, including all downstream processes, whereas antibody recovery from CHO cell culture is generally around 80–85%. A platform technology with an improved virus recovery would revolutionize vaccine production. In a quest to fulfill this goal, we have been exploring aqueous two-phase systems (ATPSs) as an optional mechanism to purify virus. ATPS has been unable to gain wide implementation mainly due to loss of virus infectivity, co-purification of proteins, and difficulty of polymer recycling. Non-enveloped viruses are chemically resistant enough to withstand the high polymer and salt concentrations that are required for effective ATPS separations. We used infectious porcine parvovirus (PPV), a non-enveloped, DNA virus as a model virus to test and develop an ATPS separation method. We successfully tackled two of the three main disadvantages of ATPS previously stated; we achieved a high infectious yield of 64% in a PEG–citrate ATPS process while separating out the main contaminate protein, bovine serum albumin (BSA). The most dominant forces in the separation were biomolecule charge, virus surface hydrophobicity, and the ATPS surface tension. Highly hydrophobic viruses are likely to benefit from the discovered ATPS for high-purity vaccine production and ease of implementation.

1. Introduction

The goal of public sector immunization programs is to ensure that high-quality vaccines are produced in adequate quantity and at affordable prices. With industrialized countries producing original, higher profitable vaccines in lieu of traditional vaccines, vaccine manufacturing of older vaccines is steadily being outsourced to developing- and emerging-economy countries. This poses an overwhelming challenge of meeting the demand of vaccines for public-sector immunization programs while re-engineering the manufacturing steps to reduce the financial burdens [1]. With 70% of the overall vaccine production costs being spent on downstream processing, improving vaccine purification is an appropriate target to reduce manufacturing costs [2].

The purification strategies for the downstream processing of viral vaccines have included a variety of methods. Some of the typical unit operations are precipitation, centrifugation, ultrafiltration, and chromatography. Precipitation using ammonium sulfate and polyethylene glycol (PEG) has been used for virus precipitation based on particle–particle interaction and hydrophobicity [3]. However, the lack of process robustness and co-precipitation of impurities has limited the application of this method. Centrifugation has been predominantly used in lab-scale production of large biomolecules based on density gradients of cesium chloride or sucrose. Particle degradation from pressure and osmotic shock and lengthy processing times plague density gradient centrifugation, along with scale-up difficulties [4]. Tangential-flow filtration (TFF) has been used to purify viruses. However, the high transmembrane pressures in TFF can reduce virus infectivity and membrane fouling can reduce permeate flux [5,6].

Column chromatography using porous beads is the most routine method used for virus purification. Chromatography is designed for biomolecules and nanoparticulates <5 nm in diameter and virus particle diameters typically range between 20 and 200 nm. This creates issues pertaining to pore diffusion and pore plugging, which severely restricts virus adsorption within the inner surface area of the solid matrix [7,8]. Due to the reduced adsorption surface accessibility, monolith and membrane chromatography have gained considerable attention as adsorption matrices. However, membrane absorbers face a similar drawback as TFF, which is the

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degradation of liable virus particles due to shearing effects [8,9]. For each physically different stationary phase configuration, chromatography still requires a change in mobile phase. The change in salt concentration, pH, or addition of a solvent equally run the risk of inactivating the virus particles [10]. In vaccine manufacturing, chromatography also has a tendency to fail to discriminate between virus and protein contaminants [11], likely due to the different amount of surface area available to the proteins and virus particles that can alter the balance of the equilibrium.

Currently, the downstream processing of viral products combines several of the previously mentioned unit operations. The purification of viruses using clarification or filtration along with a chromatographic step have demonstrated recoveries of 32% for adenovirus [12], 25% for baculovirus [13], and 30–50% for adeno-associated virus [14]. Overall, virus recoveries of 30% are considered an acceptable standard for the entire vaccine purification train [15]. Our goal is to design an alternative unit operation that is capable of creating a high infectious yield. In a quest to fulfill this goal, we have been exploring aqueous two-phase systems (ATPSs) as an optional mechanism to purify virus. ATPS has been unable to gain widespread implementation mainly due to loss of virus infectivity, co-purification of proteins, and difficulty of polymer recycling [16]. Enveloped viruses have a sensitive lipid bilayer that makes them susceptible to inactivation in high ionic strength environments. The majority of non-enveloped virus families, reovirus, picornavirus, polyomavirus [17], and parvovirus [18], are known to withstand contact with high concentrations of polyethylene glycol (PEG).

ATPS is formed by mixing water-soluble polymers or a water-soluble polymer and a salt, above a critical concentration that results in two immiscible aqueous phases [19,20]. It is a versatile method used for the separation and purification of biological molecules such as proteins [21], enzymes [22], nucleic acids [23], virus [24], antibodies [25], and cell organelles [20]. The partitioning is governed by many physicochemical properties, such as surface hydrophobicity, electrostatic interactions, van der Waal's forces, and hydrogen bonding [26]. ATPS combines clarification, concentration, and purification into a single, integrated step to obtain a high yield with a low financial burden. A monoclonal antibody was purified with ATPS and obtained a similar yield as protein A chromatography at 39% reduced operating costs [27]. In the recovery of penicillin acylase from recombinant *E. coli*, ATPS was able to reduce the number of unit operations from 7 to 4 and achieve a 97% yield with a gross cost reduction of 37% compared to ion-exchange chromatography [28]. ATPS also boasts other advantages: easy scale up, environmentally friendly, low cost, and high mass transfer [20]. Some of the disadvantages of ATPS include the removal of the PEG from the final biological product, dilution of the product, and large buffer volumes that would need to be recycled. As we pursue the purification of virus with this method, we will tackle many of these disadvantages.

Chemical cost is always a dominant factor in any biochemical process. Due to this, the inexpensive PEG–salt ATPS has been widely favored for commercial use instead of the PEG–dextran system. The drive to eliminate PEG is not so great as it is inexpensive when purchased in a large molecular weight (MW) range and forms two phases with most natural polymers and salts [29]. In order to achieve an extraction with high recovery of virus and purity from cell contaminants, the composition of ATPS needs to be carefully chosen. ATPS has been frequently used in the past for recovery of virus-like particles (VLPs). VLPs are multiprotein structures that contain the same or similar immunogenic features of infectious viruses, but lack the viral genome that is required for virus replication [30]. A VLP vaccine against the human papillomavirus was obtained with a 54% recovery in a PEG 1000–phosphate system [31]. A DNA plasmid vaccine was recovered from a PEG 400–phosphate system with a 37% yield [32]. However, appropriate technologies to obtain high yields for infectious virus particles are still being sought. Infectious bacteriophage T4 in a PEG 8000–phosphate system obtained a recovery of 30–38% [33]. The yields of <55% demonstrate a need to find an alternative purification method for large biomolecular vaccines.

The aim of this study is to recover infectious porcine parvovirus (PPV) using a PEG–salt system. PPV was chosen due to its small size, simplicity, and its structural similarity to adeno-associated virus (AAV), a commonly studied gene therapy vector. It is also used as a surrogate for Hepatitis A and poliovirus, both of which have vaccines. PPV is also a model non-enveloped virus for the human B19 parvovirus, a known blood-borne pathogen in humans. We successfully achieved 64% virus recovery in a PEG–citrate ATPS and eliminated the major contaminant protein, bovine serum albumin (BSA), which comes from the addition of serum to the media. Although many vaccines are now produced in serum-free media, we used this as a proof-of-concept study that a major protein contaminate could be separated from the virus with ATPS. The partition behavior of virus has been explained primarily on the basis of electrostatic interactions, surface hydrophobicity, and ATPS surface tension.

2. Materials and methods

2.1. Materials

PEG samples with MW of 3, 8, and 12 kDa were purchased from Sigma-Aldrich (St. Louis, MO). Sodium phosphate and sodium sulfate were purchased from VWR (Radnor, PA). For the phosphate buffer solution, sodium hypophosphite ($NaH_2PO_4.H_2O$) was purchased from VWR (Radnor, PA) and sodium triphosphate (Na_3PO_4) was purchased from Fisher Scientific (Pittsburgh, PA). Sodium hydroxide was purchased from Acros Organics (New Jersey, NY). Sodium chloride was purchased from Macron Chemicals (Center Valley, PA). Sodium citrate dihydrate and hydrochloric acid were purchased from EMD Chemicals (Billerica, MA).

Phosphate-buffered saline (PBS) pH 7.2, 0.25% trypsin/EDTA, penicillin/streptomycin (pen/strep), and minimum essential medium (MEM) for cell propagation were purchased from Life Technologies (Grand Island, NY). For virus titration, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (St. Louis, MO). High-pressure liquid chromatography (HPLC) grade acetonitrile and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO) and Alfa Aesar (Ward Hill, MA), respectively. BSA was purchased from Sigma-Aldrich (St. Louis, MO). All solutions were made with water from a NanoPure water system (Thermo Scientific, Waltham, MA) to a resistance of >18 MΩ.

2.2. Cells, virus, and titer assay

Porcine kidney cells (PK-13) were a gift from Dr. Ruben Carbonell at North Carolina State University. PK-13 cells were grown in MEM supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Oakwood, GA) and 1% pen/strep at 37 °C, 5% CO_2, and 100% humidity. The cells were propagated every 3–4 days at a split ratio of 1:5. PPV strain NADL-2, also a gift from Dr. Ruben Carbonell at North Carolina State University, was propagated in PK-13 cells, as described previously [34]. PPV was clarified with centrifugation prior to use.

PPV was titrated with a cell viability assay, the colorimetric MTT assay, as described previously [35]. Briefly, PK-13 cells were seeded in 96-well plates. Plates were infected with 25 μL of PPV and serially diluted across the 96-well plate. After 5 days of incubation, the