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Short communication

Enveloped virus flocculation and removal in osmolyte solutions

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ABSTRACT

Our ability to reduce infectious disease burden throughout the world has been greatly improved by the creation of vaccines. However, worldwide immunization rates are low. The two most likely reasons are the lack of sufficient distribution in underdeveloped countries and the high cost of vaccine products. The high costs are due to the difficulties of manufacturing individual vaccine products with specialized purification trains. In this study, we propose to use virus flocculation in osmolytes, followed by microfiltration, as an alternative vaccine purification operation. In our previous work, we demonstrated that osmolytes preferentially flocculate a non-enveloped virus, porcine parvovirus (PPV). In this work we show that osmolytes flocculate the enveloped virus, Sindbis virus heat resistant strain (SVHR), and demonstrate a >80% removal with a 0.2 μm microfilter membrane while leaving proteins in solution. The best osmolytes were tested for their ability to flocculate SVHR at different concentrations, pH and ionic strengths. Our best removal was 98% of SVHR in 0.3 M mannitol at a pH of 5. We propose that osmolytes are able to flocculate hydrophobic non-enveloped and enveloped virus particles by the reduction of the hydration layer around the particles, which stimulates virus aggregation. Now that we have demonstrated that protecting osmolytes flocculate viruses, it has the potential to be a future platform purification process for vaccines.

Viral vaccines are the most effective method to prevent and control viral infections (Adida et al., 2013). Vaccines prevent around 2.5 millions deaths per year (WHO, 2013). However, vaccines are not able to offer global coverage, due to manufacturing limitations on the supply side (Adida et al., 2013). Once a vaccine is ready at lab scale, the manufacturing development time and the overall cost of the process needs to decrease in order to have sufficient supply and broad distribution. The ultimate goal would be a platform approach to vaccine manufacturing that would parallel the advances made in antibody purification and manufacturing (Shukla and Thommes, 2010).

Downstream processes (DSP) account for 70% of the overall manufacturing cost in vaccine production (Morenweiser, 2005). The elevated costs in the DSP are mainly due to chromatography and nano- or ultrafiltration, each of which are regularly applied to vaccine manufacturing (Ray, 2011). Even though chromatography is the main unit operation for virus purification, viruses are large biomolecules that have limited diffusion in conventional resins and therefore have difficulties accessing the high internal surface area of the resins (Trilisky and Lenhoff, 2007). Another common method employed in virus purification is nano- or ultrafiltration. However, the efficiency of membranes can be affected by fouling (Bolton et al., 2006), leading to longer filtration time, high transmembrane pressure, and low flux through the membrane (Bolton et al., 2006; Morenweiser, 2005). Due to the limitations of chromatography and nano- or ultrafiltration, we propose to purify virus particles by flocculation in osmolytes, followed by microfiltration. Microfiltration, not typically used to retain viruses, would increase the flux and decrease the fouling as compared to nano- or ultrafiltration.

Here, we demonstrate that a potential platform approach to vaccine purification is flocculation with osmolytes. Osmolytes are natural compounds found in the cells of many organisms. Their main function is to stabilize intracellular proteins and maintain cell volume when exposed to environmental stresses. There are two types of osmolytes, protecting and denaturing. Protecting osmolytes have the ability to stabilize proteins through the preferential exclusion of osmolytes from the protein surface, whereas denaturing osmolytes directly interact with the protein backbone (Street, 2007). Osmolytes are commonly used as excipients in protein formulations (Roberts et al., 2013), making it likely that the osmolytes would not have to be removed to extremely low concentrations for formulation. In our previous work, we have shown that protecting osmolytes preferentially flocculate a model non-enveloped virus, porcine parvovirus (PPV). Our greatest removal were found with glycine, which demonstrated a 96% removal with a 0.2 μm microfiltration filter while leaving model proteins, which

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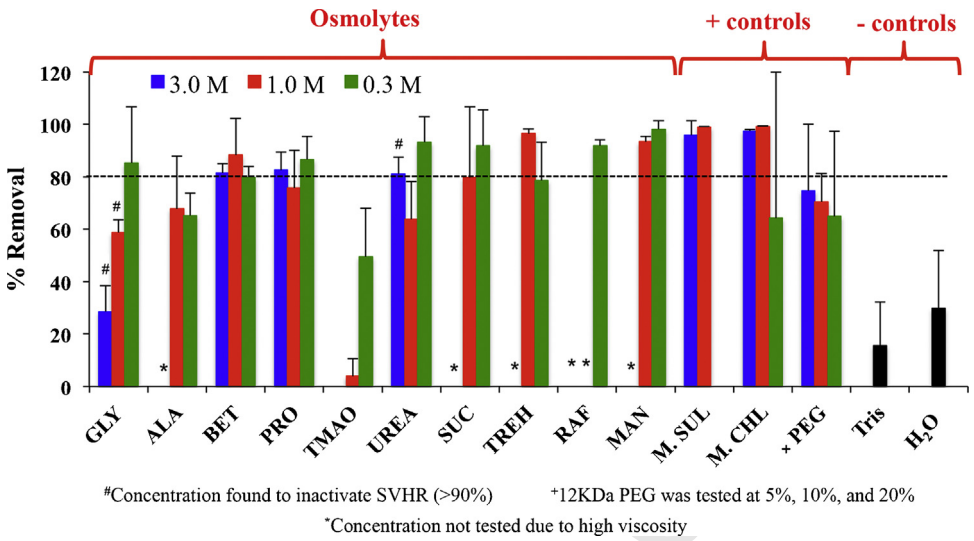

Fig. 1. High-throughput screening of osmolytes as virus flocculants. A variety of osmolytes were compared to the positive control salts and PEG and the negative controls, Tris, and water. Flocculation and removal of SVHR was carried out in a 96-well filtration plate containing a 0.2 μm micropore filter and centrifuged as described earlier (Gencoglu et al., 2014). 80% removal was used as the cut-off to pursue testing. % removal is defined in Eq. (1). All data points are the average of three separate experiments and the error bars represent the standard deviation.

are likely less hydrophobic, in solution (Gencoglu et al., 2014). In this work we demonstrate that protecting osmolytes also flocculate an enveloped virus and allow removal with a microfiltration membrane. This further validates our hypothesis that osmolyte flocculation could create a platform purification process for viral products.

Sindbis virus heat resistant strain (SVHR), our model enveloped virus, is a single-stranded, RNA, icosahedral virus, with a diameter between 60 and 70 nm (Norkin, 2010). A variety of osmolytes, such as dihydrate, glycine (GLY), D-alanine (ALA), betaine (BET), L-proline (PRO), trimethylamine N-oxide (TMAO), urea, sucrose (SUC), D-(+)-trehalose dehydrate (TREH), D-(+)-raffinose pentahydrate (RAF), and D-mannitol (MAN) were screened to demonstrate their ability to flocculate SVHR and the subsequent removal of the virus with a 0.2 μm pore-sized filter (Fig. 1). Polyethylene glycol (PEG) and the salts magnesium sulfate (M. SUL), and magnesium chloride (M. CHL) were selected as positive controls since salts and PEG have been used to precipitate filamentous bacteriophages (Branston et al., 2012). Tris(hydroxymethyl)aminomethane hydrochloride (TRIS) and water (H_2O) were used as negative controls. Osmolytes, such as proline, betaine, sucrose, trehalose, raffinose, and mannitol were able to flocculate the enveloped virus SVHR, and showed a high removal (<80%) at all concentrations tested. Percent removal is defined in Eq. (1),

$$\% \text{ Removal} = \left[1 - \left(\frac{C_f}{C_i}\right)\right] \times 100 \quad (1)$$

where c_f is the concentration of infectious virus or protein after filtration, and c_i is the concentration of infectious virus or protein before filtration. Infectious titer was determined by the MTT assay, as described previously (Mi et al., 2014).

In our previous work we found that the protecting osmolytes, including glycine and mannitol, have the ability to flocculate the non-enveloped virus PPV (Gencoglu et al., 2014). Due to the fact that a variety of protecting osmolytes flocculate viruses, we propose that this could be used as a novel process for vaccine purification. The osmolytes urea and glycine showed >80% virus removal at low concentrations, but at high concentrations, they inactivated SVHR (>1 LRV) (see Table S1). Virus inactivation was confirmed by testing the virus titer of each flocculating solution prior to filtration and comparing to the virus titer of the water control solution. Osmolytes, such as D-arginine (ARG) and L-serine (SER), and the salt ammonium sulfate (A. SUL) were also found to inactivate SVHR (>1 LRV) (see Table S1). Urea at high concentrations has been shown to inactivate enveloped viruses, such as Sindbis, herpes simplex-1 and vaccinia (Roberts and Lloyd, 2007), likely due to the compound destabilizing viral proteins. Arginine has been shown to inactivate other enveloped viruses, such as the influenza virus and the herpes simplex virus (Yamasaki et al., 2008). It has been suggested that arginine binds to proteins without denaturing them (Yamasaki et al., 2008) and likely interacts with the lipid membrane (McCue et al., 2014). In this study, we focused on flocculants that demonstrated high removal without inactivating SVHR.

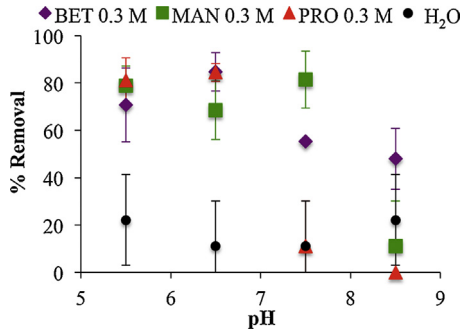

Fig. 2. pH effect on SVHR flocculation with a micropore filter. The pH of the osmolyte solutions was adjusted by the addition of HCl or NaOH and filtered as described in Fig. 1. % Removal is defined in Eq. (1). All data points are the average of three separate experiments and the error bars represent the standard deviation.

The osmolytes betaine, mannitol and proline were used to study the effect of pH on SVHR flocculation (Fig. 2). These osmolytes were selected because they represented three different catagories of osmolytes, *n*-oxide, sugar/polyol and amino acid, respectively. Osmolyte solution pH was adjusted to change the overall charge of the virus. SVHR has been shown to be stable in the pH range of 5–8 (Roberts, 2008), so a pH range of 5.5–8.5 was selected in this study. It was confirmed that virus inactivation did not occur, as stated earlier. The highest removal occurred as the pH neared the isoelectric point (p*I*) of SVHR, which has been found to be ~4.2