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Use of hollow fiber tangential flow filtration for the recovery and concentration of HIV virus-like particles produced in insect cells

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A B S T R A C T

Attenuated viruses, inactivated viruses and virus like particles (VLPs) are known to be efficient vaccines partially due to their particulate structure. A potential HIV vaccine candidate engineered as a VLP (HIV gag-VLP) and produced in insect cells is currently under preclinical trials demanding large amounts. Due to their extreme fragility and sensitivity to shear forces the recovery and concentration of these extracellular enveloped particles of approximately 120 nm in size is challenging. The current bench scale gradient ultracentrifugation and precipitation methods have been found unsuitable for larger scale processes. In this study a two-step tangential flow filtration (TFF) process using hollow fibers was developed for the clarification and concentration of HIV gag-VLPs. The first step is microfiltration for cell removal and the second step is ultrafiltration for concentrating the HIV gag-VLPs. The chosen parameters for the microfiltration step were hollow fiber membranes of 0.45 μm cut off 5000 s^{-1} shear force and a flux of 10 LMH. The chosen parameters for the ultrafiltration step were a 500 kDa cut off membrane, 6000 s^{-1} shear force and a trans-membrane pressure (TMP) of 1.25 bar. The utilization of these parameters provided with concentrated HIV-gag VLPs from 2 L of starting cell suspension within 6 h of processing time. These downstream processing conditions are extremely valuable for the further large-scale purification process development for HIV gag-VLPs and other particulate bioproducts.

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1. Introduction

The baculovirus-insect cell system is a well established method for the production of multimeric protein complexes including ~120 nm in size, virus-like particles (VLPs). The advantages of this system are the short specific growth rates in serum-free or protein-free media, and high levels of protein expression (Drugmand et al., 2012; Mena and Kamen, 2011). The VLPs have similar capsid conformation to the native virus but without the viral genetic material. VLPs are considered good vaccine candidates as a result of the high immunogenicity conferred by their particulate structure and their non-replicating nature which minimizes safety concerns (Bolhassani et al., 2011; Roy and Noad, 2008). Some examples of VLP-based vaccines include HEV, influenza, BTV, Ebola, HIV, HPV, rotavirus, PPV and SARS (Roldão et al., 2010). The subject of this work is a promising HIV candidate vaccine produced as VLP (HIV gag-VLP) in insect cells which is currently undergoing preclinical testing. This HIV gag-VLP is composed of the HIV-1 p55 viral core protein and surface spikes. It presents neutralizing determinants in membrane-proximal external region of the HIV-1 gp41 envelope glycoprotein. The recombinant baculovirus used for its production expresses the p55 and gp41 genes under the P10 and Pph promoters, respectively.

One of the weaknesses of the VLPs production is the lack of consistency in the downstream processing. The VLPs have to be purified from many contaminants including cell debris, organelles, incomplete VLPs, baculoviruses, and media components. The number of steps required depends on different properties of the VLPs such as size, density, presence or absence of envelope, biochemical characteristics of the surface, and on the complexity of the media composition. The recovery and concentration of VLPs with intact membrane, which is required for maintaining immunogenicity is challenging because the membrane is very fragile and susceptible to damage by shear forces (Chalmers, 1996). Fragility is more evident in extracellularly-produced enveloped VLPs in comparison to non-enveloped VLPs. The preferred bench scale methods for the recovery and purification of VLPs include sucrose or cesium chloride gradient ultracentrifugation, poly (ethylene glycol) precipitation, tangential flow filtration (TFF) in flat sheet membranes, ion exchange, affinity and size exclusion chromatography (Peixoto et al., 2008; Morenweiser, 2005; Bernard et al., 1996). However,

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not all these methods are suitable for a large-scale production, since in addition to robustness, efficiency, and reproducibility short processing time is required (Vicente et al., 2011).

Despite of the cellular localization of the produced VLPs, the downstream processing starts with the clarification step, or separating the cells from the cell culture media. Several centrifugation and filtration based technologies have been developed as clarification steps (Bernard et al., 1996). Centrifugation either in batch or continuous mode, is commonly used but becomes limiting due to the scale-up adjustments and capital investment needed. On the other hand, the use of membranes for TFF is advantageous because the scale-up process is relatively straightforward, and relatively low shear forces are applied (Saha et al., 1994; Maiorella et al., 1991).

The principle of TFF is based on pumping the feed in a tangential direction to the membrane to avoid build up and clogging of the membrane pores. Simultaneously, pressure is applied in order to pass through the membrane "permeate". Fluid that did not cross the membrane "retentate" contains particles larger than the pore size and is re-circulated for re-processing. The success of the TFF is based on several important factors such as operating pressure, flux, selectivity, scalability and low capital cost. The microfiltration and ultrafiltration of viruses using flat sheets operated in TFF mode has been reported (Wickramasinghe et al., 2005), and at the same time the use of hollow fibers has been reported for the removal of bacteria, viruses, and parasites from water (Liu et al., 2012; Smith and Hill, 2009). However, the use of hollow fibers for the recovery and concentration of VLPs has not been extensively exploited (Morenweiser, 2005).

The objective of this study was to develop a process for the recovery and concentration of HIV gag-VLPs produced in insect cells. The use of hollow fibers in TFF mode was selected for both recovery and concentration steps because of the relatively low processing shear forces required. Successful and scalable use of hollow fibers for the recovery and purification of VLPs has not been reported and it is especially important for HIV gag-VLPs because these particles are enveloped and sensitive to shear force. An optimized process providing high yield of HIV gag-VLPs while maintaining their integrity will supply sufficient VLPs needed to complete the pre-clinical trials for the potential HIV candidate vaccine. Additionally, it will also contribute with valuable information for the large-scale process development for similarly designed VLP-based vaccines.

2. Materials and methods

2.1. Cell culture

All the chemicals and cell culture media were obtained from Life Technologies, Grand Island, NY unless otherwise stated. The insect cells *Spodoptera frugiperda* (Sf9) (Life Technologies) were grown in Sf-900™ II SFM (Serum Free Medium). Cells were inoculated at 5.0×10^5 cells/mL and viability greater than 98%. The cell culture was incubated at 27 °C and 150 rpm. For the non-infected cells experiments, cells were harvested at a concentration of around 2.0×10^6 cells/mL. For the infected cells experiments, cells growing at this concentration were infected with recombinant baculovirus containing the gag protein. The recombinant HIV gag baculovirus were kindly donated by Dr. Prasad Vennakalanti and Dr. Carol Weiss from FDA. A multiplicity of infection (MOI) of 0.01 was used and cells were harvested at 48 h post infection (hpi). Cell count was performed in triplicates by trypan blue method using an automatic cell counter BioRad TC10 (BioRad, Hercules, CA) and by PI (propidium iodide) method using the Guava™ flow cytometric automatic cell counter (Millipore, Billerica, MA). The parameters measured included viability, total cell count, viable cell count, and cell debris index. Cell debris is excluded from cell count results based on negative staining with the nuclear dye and it is expressed as percentage. High cell debris index values correspond to high breakage of cell membrane.

2.2. Tangential flow filtration

An automated TFF system ÄKTACrossflow™ (GE Healthcare, Pittsburg, PA) was used for both clarification and concentration experiments. A hollow fiber cartridge containing 6 fibers of 0.45 μm pore size with a total membrane area of 50 cm^2 (GE Healthcare) was used for clarification of cell culture. For concentration of VLPs a hollow fiber cartridge with 50 fibers of 500 kDa pore size and 50 cm^2 total membrane area (GE Healthcare) was used. The method used in this study was created with a pre-product cycle to rinse and condition the hollow fiber with PBS buffer. This was followed by a product cycle where the actual filtration took place and finally the post-product cycle where the membrane was cleaned in place using 0.2 N NaOH. Distilled water was used to remove any residual NaOH. For all samples, 200 mL of feedstock was used and the flux was maintained at 25 LMH. In all cases, feedstock, retentate, and permeate were maintained on ice during the bioprocessing. For the clarification of non-infected cells, flux was maintained constant at 25 LMH and the TMP was measured at different shear forces including 3000 s^{-1}, 5000 s^{-1}, 7000 s^{-1}, and 15000 s^{-1}. The parameters used for clarification of infected cells included constant shear force of 5000 s^{-1} and flux was measured at different values of TMP including 0.005 bar, 0.1 bar, 0.2 bar, 0.3 bar, 0.4 bar, and 0.5 bar. The optimization of concentration of VLPs was performed at 4000 s^{-1}, 6000 s^{-1}, 8000 s^{-1}, and 10,000 s^{-1} shear forces. Each shear force was evaluated at TMP of 0.5 bar, 0.75 bar, 1.0 bar, 1.25 bar, and 1.5 bar. Concentration factor was set to 6 for all samples. The UNICORN™ software (GE Healthcare) was used to control and monitor the filtration parameter including flux, TMP, shear force, pNFF, and concentration factor obtained were used for data analysis. For the 2 L scale experiment, two hollow fibers with a surface area of 420 cm^2 (A/G Technology Corporation, Needham, MA) with same pore size that those from 200 mL scale were used. These hollow fibers had 50 fibers of 1 mm internal diameter. The pressure from feed and permeate was measured with pressure gauges connected to those ports. The feed was supplied using a peristaltic pump set at specific flow rate. Exactly the same maintenance and conditioning processes were used for all hollow fibers.

2.3. Sample preparation for protein analysis

Collected samples from feed and retentate were centrifuged at 600 g for 5 min. The supernatant samples were precipitated using trichloroacetic acid (TCA, Sigma–Aldrich, St. Louis, MO) as described elsewhere. Briefly, TCA was added to final concentration of 5% in a total volume of 1.5 mL. After 30 min incubation on ice, the fraction was clarified by centrifugation at 15,000 × *g* for 15 min at 4 °C. Proteins were then concentrated by making up the final concentration of TCA in the supernatant to 25%. Samples were centrifuged under same conditions and the pellet obtained was washed with 500 μl acetone (Sigma–Aldrich). Pellets were resuspended in PBS and total protein was quantified.

2.4. Protein quantitation

For total protein concentration, the micro BCA protein assay kit™ (Thermo Fisher Scientific, Rockford, IL) was used as described elsewhere. Briefly, serial dilutions of triplicates were placed on a microplate. After incubating sample with mix of reagents (containing bicinchoninic acid and uric sulfate), the absorbance was