Production of yellow fever VLPs by perfusion cultivation of stable recombinant HEK293 cells

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Abstract

Yellow fever (YF) is a life-threatening viral disease endemic in large areas of Africa and Latin America. Although there is a very efficacious vaccine since the 1930s, YF still causes 29,000-60,000 annual deaths. During recent YF outbreaks there were issues of vaccine shortage due to limited supply of the current egg-derived vaccine; rare but fatal vaccine adverse effects occurred; and cases were imported to Asia, where the mosquito vector circulates and where local transmission could potentially start. In this work, we investigated the production of YF virus-like particles (VLPs) using suspension-adapted stably-transfected HEK293 cells. In order to develop an intensified process, we combined two strategies: the use of sequential FACS rounds to enrich the stable cell pool in terms of high producers, and the use of perfusion processes. At first, shaken tube experiments revealed that FACS enrichment of the cell pool allowed doubling VLP production, and that in pseudoperfusion cultures (with daily medium exchange) lasting 14 days VLP production increased by 8.3 fold as compared to batch cultures lasting 11 days. When true perfusion cultures were carried out in bioreactors, the use of an inclined cell settler as cell retention device showed operational advantages as compared to an ATF system.

1. Introduction

Yellow fever virus (YFV) belongs to the Flavivirus genus and forms a virion of approximately 50 nm that is composed by a capsid, a premembrane/membrane (prM/M) protein and an envelope (E) protein, a single-stranded positive-sense RNA and a lipid envelope (Lindenbach et al., 2007). YFV is an arthropod-borne human pathogen transmitted by mosquitoes that causes a "historically devastating disease" (Paules & Fauci, 2017), for which there is no specific treatment available yet (CDC, 2019).

Before the current live-attenuated egg-derived vaccine became available in the 1930s, yellow fever (YF) was a major health threat, since outbreaks used to kill approximately 10% of the population of affected cities, such as occurred in Philadelphia (USA) in 1793 (Frierson, 2010; Monath & Vasconcelos, 2015; Paules & Fauci, 2017). Despite the availability of a highly protective vaccine, nowadays 29,000-60,000 people die annually from YF worldwide (Garske et al., 2014). Moreover, recent outbreaks in Africa (2016) and Brazil (2017-2018) have shown an inadequate preparedness for YF. In Africa in 2016, depletion of the WHO YF vaccine stockpile along with worldwide vaccine shortage led WHO authorities to start dose fractionation (1/5) as an emergency measure for outbreak control. Despite being the largest world producer of YF vaccine, Brazil also suffered from vaccine shortage in the latest outbreak and started using the 1/5 fractional dose in January 2018 for mass vaccination of the population. Since the African outbreak in 2016, when there were several imported cases to China, representing the first ever documented cases of yellow fever in Asia, concerns have arisen about vaccine availability in case the virus starts to circulate in other world regions where the mosquito vector is present, such as Asia (Paules & Fauci, 2017; WHO, 2016; Wasserman et al., 2016).

Although the existing live-attenuated vaccine confers lifetime immunity in up to 99% of vaccinees (Paules & Fauci, 2017), rare but serious adverse effects have been reported (Seligman, 2014; Porudominsky & Gotuzzo,

2018) and usually become more apparent during mass vaccination campaigns in the setting of outbreaks. Thus, considering the risks of YFV spread to world regions where the population is naïve but the mosquito vector circulates, the recently experienced shortages of the vaccine and the occurrence of rare, but fatal adverse effects demonstrate that there is a need for a new yellow fever vaccine (Shearer et al., 2018; Wong et al., 2019).

In this context, virus-like particles (VLPs) represent a promising alternative, since they are 3D particles formed by structural viral proteins produced in recombinant form, thus mimicking the virus, but lacking its genetic material. Antigens are presented in a repetitive mode, enhancing immunogenicity, so VLPs are considered a vaccine platform that combines good safety and high efficacy (Fuenmayor et al., 2017; Krol et al., 2019; Mohsen et al., 2017; Wang & Roden, 2013).

Vaccines must be affordable and their production needs to be scalable. One way to develop cost-effective production processes is to develop continuous perfusion cultivation processes, using cells stably expressing the product. Perfusion technology usually results in high product quality and high volumetric productivity. However, it is a more complex technology in comparison to batch processes, since cells need to be retained inside the bioreactor while medium is continuously renewed and product is continuously harvested (Castilho, 2015). Finding an appropriate cell retention device may be a challenge, considering that the device needs to work continuously for long periods presenting high efficiency, not causing cell damage and not retaining product in order to allow its continuous recovery in the harvest stream (Bielser et al., 2018; Carvalho & Castilho, 2017).

In the present work we generated a stable recombinant HEK293 cell line, which constitutively secretes yellow fever VLPs comprised of the prM and E proteins. In order to develop an intensified VLP production process, we combined two strategies: the use of FACS to sort recombinant cells for high producers and the use of the resulting enriched cell pools to develop continuous perfusion processes based on two different cell retention devices (ATF and cell settler). In order to better characterize the VLP product, VLPs were purified by steric exclusion chromatography and then analyzed by conventional biochemical techniques and by transmission electron microscopy.

2. Materials and Methods

2.1. Cell line development, culture conditions and stability evaluation

In order to generate a cell line capable of stably expressing the structural premembrane (prM) and envelope (E) proteins, HEK293SF-3F6 cells (NRC, Canada) were transfected by lipofection in a shaken vented tube (spin tube, TPP AG, Switzerland) using a chemically-defined medium (HEK TF, Xell AG, Germany) and Lipofectamine 3000 reagent (Invitrogen, USA), as described previously (Alvim et al., 2019). Transfection volume was 4 mL.

The gene construct used (named LECC15) was based on an in-house designed signal peptide followed by the sequence encoding prM-E (683 amino acids) of the yellow fever virus (South-America I genotype, strain BeH655417, GenBank # JF912190). The gene construct was codon-optimized, synthetized, subcloned into pCIneo vector (Promega, USA) and supplied ready to transfect by Genscript (USA).

Approximately 48h post-transfection (hpt), cells were transferred to a vented 125 mL-erlenmeyer flask (Corning, USA) and diluted with fresh HEK TF medium in the presence of 100 μg/mL G418 sulfate (Gibco, USA) for selection of stable transfectants. Every 3-4 days, viable cell concentration was adjusted to 1×10^6 cells/mL by diluting cell suspension with fresh HEK TF medium containing 100 μg/mL G418 sulfate. Documented research cell banks of the stable cell pool were cryopreserved at 30 and 60 days post-transfection.

In order to evaluate production profile over time, the cell pool was kept in culture in the presence of the selection antibiotic for up to approximately 120 days post-transfection. During the whole period, cells were kept in a humidified incubator at 37 °C and 5 % CO_2 and shaken at 180 rpm (50 mm orbit). Cell count was performed using an automated cell counter (Vi-Cell®XR, Beckman Coulter, USA) and, when necessary,

glucose and lactate concentrations were monitored using a YSI 2700 Select Biochemistry Analyzer (YSI Inc., USA).

2.2. Enrichment of stable cell pools by FACS

With the aim of selecting high-producer subpopulations, at six weeks post-transfection a total of 200 $\times$ 10^6 cells were stained using 4G2 mAb (biotinylated in house using EZ-Link biotinylation kit #21425, Thermofisher, USA) and Streptavidin-phycoerythrin (PE) (#S866, Thermofisher, USA). Cells were then sorted by FACS (fluorescence-activated cell sorting) in order to collect cells showing higher fluorescent signal, as described previously (Alvim et al., 2019). After these sorted cells (named cell pool 1) recovered viability, a second round of sorting was performed on week 14 post-transfection and high-fluorescence cells were collected and named cell pool 2. Documented cell banks of both cell pools were cryopreserved. Kinetic studies were carried out to evaluate cell growth, metabolism and VLP production.

2.3. Kinetic studies in shaken tubes operated in batch and pseudoperfusion (PP) mode

Cells from sorted pools 1 and 2 were seeded at 0.5 $\times$ 10^6 cells/mL into 50-mL spin tubes containing an initial volume of 15 mL HEK TF medium (Xell AG, Germany). Each condition was evaluated in duplicate, and samples were withdrawn daily to monitor cell density and viability, product formation and glucose and lactate concentrations.

Regarding cells grown in pseudoperfusion mode, from day 4 on cells were centrifuged (300 g , 5 minutes), conditioned medium was collected (partially or completely) and fresh medium was added to achieve a culture volume of 10 mL, mimicking an intermittent perfusion process. The proportion of medium exchanged was defined according to the demand, i.e. based on glucose concentration upon sampling: 0.5 vvd (volume of fresh medium per volume of culture per day) when glucose concentration was higher than 2 g/L; 0.75 vvd if between 1 and 2 g/L; and 1 vvd (i.e. complete medium exchange) when glucose was lower than 1 g/L.

2.4. Perfusion cultivations in stirred-tank bioreactors

2.4.1. XCell^{TM} ATF-2 as cell retention device

A stirred-tank bioreactor (ez-Control, Applikon Biotechnology, The Netherlands) coupled to XCell$^{\text{TM}}$ ATF-2 (Repligen, USA) was set up as described previously (Alvim et al., 2019), with the exception of the hollow fiber cartridge used in the system. In the present work, a polysulfone membrane with 0.4 µm pore size and 0.005 m^2 of surface area (MFSL0005, Asahi Kasei, Japan) was evaluated. Cells from cell pool 1 were inoculated at approximately 0.5 $\times$ 10^6cells/mL in HEK TF medium and working volume was kept at 1 L $\pm$ 0.05 L. The setpoints of pH, temperature and dissolved oxygen were 7.1, 37 °C and 40 % of air saturation, respectively. A pitched-blade impeller was used for agitation, which was kept at 200 rpm during the whole cultivation. Purified air and pure oxygen were injected at a maximum combined flow rate of 0.25 L/min through a drilled hole sparger in order to maintain dissolved oxygen at the setpoint. In this run, permittivity was monitored online using a biomass sensor (FOGALE®Nanotech, France). The software accompanying the biomass sensor used a generic conversion factor for mammalian cells and provided the measured values already converted to million cells/mL.

Perfusion was started on day 4 by feeding fresh HEK TF medium supplemented with 8 mM of L-glutamine at medium exchange rates between 0.17 and 0.67 vvd. ATF recirculation rate was set initially at 0.2 L/min and then gradually increased up to 0.6 L/min, according to variations in viable cell density.

2.4.2. Inclined lamella settler as cell retention device

A stirred-tank bioreactor (RALF, Bioengineering AG, Switzerland) coupled to an inclined cell settler (model CS-10, Biotechnology Solutions, USA) was used. Gases were injected through a ring sparger, and two impellers were used (a Rushton turbine and a pitched blade impeller) to maintain agitation at 150 rpm. The cell inoculum was approximately 0.5 $\times$ 10^6 cells/mL, using an initial volume of 1.1 L. Setpoints of pH, temperature and dissolved oxygen were the same as in 2.4.1. Perfusion was started on day 3 and medium exchange

rates varied between 0.5 and 2 vvd. Once perfusion started, the volume inside the bioreactor decreased to approximately 700 mL due to the dead volume inside the inclined settler and its recirculation loop.

2.5. VLP purification

VLPs were purified in a single step by steric exclusion chromatography (SXC). Chromatography runs were performed at 6 mL/min using an Äkta Purifier system with software Unicorn 5.20 (Cytiva, Sweden). Two buffers were used to obtain the desired PEG concentrations in each step: (i) buffer A: PBS (137 mM NaCl, 2.7 mM KCl, 10 nM Na_2HPO_4, 1.8 mM KH_2PO_4, adjusted to pH 8.0); (ii) buffer B: PBS (pH 8.0) containing 16% (m/v) PEG 6000. For equilibration and wash steps, a 1:1 mixture of buffers A and B was fed to maintain PEG concentration at 8% (m/v). During sample injection, cell culture supernatant and buffer B were in-line mixed (1:1) in order to obtain the desired PEG concentration (8% m/v) while reducing the risk of product precipitation prior to injection (Lee et al., 2012). Elution was accomplished by increasing buffer A proportion in order to decrease PEG concentration and to release VLP.

2.6. Analytical assays

2.6.1. Immunoblot for VLP detection

VLP production was monitored by spot blot: 3 µL of centrifuged cell culture supernatants (300 *g* , 5 minutes) were pipetted onto a nitrocellulose membrane (Vita Scientific, USA) and allowed to dry before proceeding with the immunoblot assay. Primary antibody incubation was performed using either the pan-flavivirus mAb 4G2 (1:8,000) (MAB10216, Merck, USA) or an anti-yellow fever antibody (1:3,000) (Bio-Manguinhos/Fiocruz, Brazil), followed by incubation with anti-mouse IgG HRP-conjugated secondary antibody (1:10,000) (A16011, Invitrogen, USA) and then ECL Prime detection reagent (GE Healthcare, USA). Dengue envelope protein (Prospec, Israel) or in-house purified VLP standards (130-190 µg/mL stock) were used as positive controls and to generate calibration curves for each spot blot. Approximate concentrations of VLPs were determined by densitometry using the image analysis software ImageJ (National Institutes of Health, USA).

2.6.2. ELISA for VLP quantification

A sandwich ELISA previously described (Lima et al., 2019) was used to quantify the VLPs in the chromatography fractions. Briefly, pan-flavivirus 4G2 antibody was used to coat plates, whereas in-house biotinylated 4G2 was used as a secondary antibody followed by avidin-HRP. TMB Single Solution (Invitrogen, USA) was used for detection, reaction was stopped with 1 N HCl, and measured at 450 nm.

2.6.3. SDS-PAGE and Western blot

SDS-PAGE was performed using 10% polyacrylamide handcast gels in the Mini-Protean Tetra Cell system (BioRad, USA) operating at 150V and 3.0 A for 90 minutes. A 4-fold concentrated sample buffer (0.25 M Tris-HCl, 40 g/L SDS, 20% v/v glycerol, 0.5 g/L bromophenol blue) was added (1:4) to the samples, which were then heated at 99 °C for five minutes before applying 40 ?L of each sample to the gel. The tank was filled with running buffer (3.04 g/L Trizma$^{(r)}$-base, 14.6 g/L glycine, 1 g/L SDS, pH 8.4). The gel was silver stained and photographed.

For the Western blot, the gel and a nitrocellulose membrane were wetted with transfer buffer (5.82 g/L Trizma-base, 2.93 g/L glycine, 20% v/v methanol) and proteins were transferred to the membrane using a Trans-Blot$^{(r)}$ SD Semi-Dry Transfer Cell (BioRad, USA) set at 15 V and 3.0 A for 90 minutes. After the transfer, the membrane was blocked using a 5% (m/v) skimmed milk solution followed by incubation with 4G2 antibody (MAB10216, Merck, USA) diluted 1:8,000 and anti-mouse IgG HRP-conjugate (A16011, Invitrogen, USA) diluted 1:10,000. Then the membrane was incubated with ECL Prime detection reagent (GE Healthcare, USA) and image was obtained using a FluorChem E system (ProteinSimple, USA).

2.6.4. DNA and total protein assays

PicoGreen dsDNA Assay Kit (Invitrogen, USA) and Pierce BCA Protein Assay Kit (Thermofisher, USA) were used according to manufacturer's instructions to quantify DNA and total protein, respectively.

2.6.5. SEC-HPLC

Cell culture supernatant and a SXC-purified sample, previously filtered using 0.45-μm syringe filters, were analyzed in a LC-20 Prominence HPLC system (Shimadzu, Japan) fitted with a TSKgel G3000SWXL column (7.8 mm ID x 30 cm, stainless steel). 30-μL samples were applied to the column and the analyses were carried out at 1 mL/min using a 12.5 mM phosphate buffer with 250 mM NaCl at pH 7.0.

2.6.6. Analytical size exclusion chromatography (A-SEC)

In order to further evaluate purity and size distribution of SXC eluates, samples (250 μL at 158 μg/mL) were directly injected into a Superose 6 Increase 10/300 GL column (29-0915-96, Cytiva, Sweden). All runs were performed in PBS at a flow rate of 0.7 mL/min, and the absorbance was monitored either at 280 or 620 nm using an ultra fast liquid chromatography equipment (Shimadzu, Japan). The column was calibrated using thyroglobulin (670 kD), γ-globulin (158 kD), ovalbumin (44 kD), myoglobin (17 kD), and vitamin B12 (1.35 kD) (151-1901, Bio-Rad, USA). Blue dextran with molecular mass of 2,000 kD (D5751, Sigma-Aldrich, USA) was used to determine the void volume of the column.

2.6.7. Transmission electron microscopy (TEM)

Samples were stored at 4°C up to grid preparation. Briefly, 4 μL of a sample at 134 μg/mL was applied to a previously discharged carbon film on 300 mesh copper grids (CF300-CU-TH, Electron Microscopy Sciences, USA) for 1-2 min, gently dried with filter paper and stained for 2-5 s with 15 mg/mL uranyl formate (22450, Electron Microscopy Sciences, USA). Negatively stained samples were imaged on a Hitachi HT-7800 microscope operated at 80 kV.

3. Results and Discussion

3.1. Cell line generation and FACS-aided enrichment of stable cell pools

We used suspension-adapted HEK293 cells originating from an animal component-free GMP cell bank (from NRC, Canada) to express VLPs comprising the structural proteins prM and E of the yellow fever virus. The HEK293 cell line was chosen due to its ability to produce VLPs of different viruses and to grow well in continuous suspension cultivation processes (Fontana et al., 2015; Fuenmayor et al., 2018; Alvim et al., 2019; Venereo-Sanchez et al., 2017).

The prM-E gene construct was inserted into a plasmid containing a selection marker cassette based on the neomycin phosphotransferase gene in order to allow selection of stable transfectants by including G418 antibiotics in the medium. Selection pressure by G418 addition was started on day 2 post-transfection, and cell culture showed low viability and enhanced cell death for the following 2 weeks, due to death of cells where the heterologous DNA had not been integrated into the cell genome. After that period, cell viability recovered, indicating that stable producer cells had been successfully selected. A research cell bank of the original cell pool was cryopreserved 4 weeks after transfection, and then FACS-aided cell sorts were carried out to select subpopulations of high-producer cells.

Our focus is on VLPs that are secreted to the extracellular medium, but since secreted VLPs can be transiently found on the cell membrane on their way to the extracellular medium, it is possible to stain the cells and sort for high producers. The first round of cell sorting was carried out 40 days after transfection by collecting 0.3% of cells presenting the highest levels of 4G2-PE fluorescence. These cells were named cell pool 1 and a cell bank was cryopreserved. The second round of cell enrichment was carried out starting from cell pool 1 by again collecting the cells with highest fluorescent intensity and then expanding them. This double sorted cell pool was named cell pool 2 and a cell bank was cryopreserved. Passage history of the original stable cell pool and of cell pools 1 and 2 is shown in Figure 1A.

With the purpose of evaluating VLP production after each round of cell sorting, an immunoblot assay was

performed (Figure 1B). The results show that the concentration of VLPs in the supernatant increased after each round of cell sorting, confirming that cell enrichment was successful and that FACS is a powerful high-throughput technique also for selection of cells secreting a recombinant product (Sutermaster & Darling, 2019).

3.2. Evaluation of sorted cell pools under batch and pseudoperfusion modes

In order to compare cell growth and VLP production of cell pools 1 and 2, cells were cultivated in shaken spin tubes under batch (Figure 2A) and pseudoperfusion modes (Figure 2B). Spin tube cultures carried out under pseudoperfusion mode have been shown to serve as a good scale-down model for perfusion processes, reaching high viable cell concentrations comparable to those achieved in a continuous perfusion bioreactor process, with the added advantages of much lower media expenditure and possibility of simultaneously screening many different conditions before moving to bioreactor scale (Nikolay et al., 2020a).

Regarding cell growth in batch mode, cell pools 1 and 2 reached maximum viable cell concentrations of 7.6 and 9.9 million cells/mL, respectively (Figure 2A). Cells of pool 2 grew quicker, which led to earlier glucose depletion (Figure 2C) and a consequent earlier drop in cell viability when compared to cell pool 1. When both cell pools were grown in pseudoperfusion mode (PP) with daily medium exchange from day 4 on, much higher cell concentrations were obtained for both cell pools reaching maxima of approximately 40 and 50 million viable cells per mL for cell pools 1 and 2, respectively (Figure 2B). Enhanced cell growth in pseudoperfusion was enabled not only by the daily feeding of fresh medium (Figure 2D), but also by daily harvesting of the spent medium, promoting removal of any inhibitory metabolites. Since a fresh stock of nutrients was provided daily, the duration of the pseudoperfusion cultures was longer and could have been even longer if more than one medium exchange per day had been performed, but in these exploratory experiments we limited the medium exchange rate to 1 vvd (1 complete medium exchange per day).

Concerning product formation, maximum VLP concentrations in batch cultures were 98 and 163 mg/L for cell pools 1 and 2, respectively (Figure 2E). In pseudoperfusion, in spite of daily product harvesting, the high cell densities achieved allowed that high amounts of product were produced daily, so that maxima of 118 and 270 mg/L were achieved in PP for cell pools 1 and 2, respectively. Based on the volumes harvested at the end (10 mL) of batch cultures, or on day 4 (12 mL) and on all the following days (9 mL/day) for PP, cell pool 1 in batch mode resulted in a total harvest of 1.0 mg of VLPs on day 11, whereas in PP 9.0 mg were harvested altogether from day 4 to day 17. In the case of cell pool 2, batch culture resulted in 2.2 mg of VLPs, and PP culture allowed an accumulated harvest of 16.2 mg of VLPs. These data show that by doing a second FACS round it was possible to approximately double VLP production, both in batch and in PP. On the other hand, by moving from batch to PP allowed an average increase of 8.3 fold in total VLP produced for both cell pools. By combining both strategies – FACS enrichment of cell pools and pseudoperfusion operation – an increase of approximately 16.5-fold in total VLP production was achieved, for a process just somewhat longer (11 days for cell pool 1 in batch, as compared to 14 days for cell pool 2 in PP). Thus, the present data demonstrate that both strategies exploited herein contribute to process intensification and, if combined, can significantly increase VLP production and decrease production costs.

3.3. Perfusion runs using XCell^{TM} ATF-2 and inclined cell settler as cell retention devices

Based on the promising results of pseudoperfusion cultures obtained at spin tube scale, perfusion bioreactor cultivations were carried out. At first, a XCell^{TM} ATF-2 was chosen as cell retention device, since it has been widely used not only for production of recombinant biopharmaceutical proteins, such as monoclonal antibodies (Clincke et al., 2013), but has also been reported in the recent years for virus (Coronel et al., 2019; Nikolay et al., 2018) and VLP production processes (Lavado-García et al., 2020; Alvim et al., 2019).

In our previous work on zika VLP production (Alvim et al., 2019), although a perfusion run could be stably operated for 30 days at approximately 30 million cells/mL, product retention inside the bioreactor due to membrane fouling was observed. However, considering a recent report (Nikolay et al., 2020b) that showed a polysulfone hollow fiber cartridge to be highly permeable for the yellow fever virus, we decided to use a cartridge of hollow fibers having the same characteristics as reported by these authors, with the aim of

avoiding fouling.

Cell pool 1 was inoculated at 0.5 × 10^6 cells/mL and maximum viable cell density (VCD) was 28.8 × 10^6cells/mL on day 13, as measured offline by trypan blue exclusion in a cell counter. We also monitored VCD online using a capacitance-based biomass sensor. As shown in Figure 3A, the profiles of viable cell density determined using both methods were very similar, with the online biomass sensor showing a trend to be somewhat more sensitive. The use of this sensor can be very important in processes where the cell-specific perfusion rate is controlled, since the online biomass data can be used to automatically adjust the fresh medium feed rate (Dowd et al., 2003).

Cell viability remained above 90% until day 16 (Figure 3A), but then decreased because of nutrient depletion (Figure 3B). Until day 10, perfusion rate had been gradually increased along time in order to meet nutrient demands by the growing cell concentration. However, by the time viable cell density was approximately 25 million cells/mL, no increases in permeate withdrawal rate beyond 0.45 vvd were possible anymore (D_harvest, Figure 3B) due to progressive fouling of the membrane cartridge. Because the rate of permeate withdrawal was limiting, fresh culture medium addition rate (D_feed, Figure 3B) for 3 consecutive days exceeded the permeate and affected bioreactor working volume. Even so, from day 15 on glucose exhaustion in the bioreactor occurred, causing cell death. Another evidence that membrane fouling was progressively occurring was the fact that product retention inside the bioreactor started being observed from day 12 on, causing an increase in VLP concentration inside the bioreactor and a decrease in VLP recovery in the harvest (Figure 3C).

As discussed above, product retention had already been observed for virus and VLP production when typical polyethersulfone ATF cartridges with 0.2 μm pore size and 0.13 m^2 filtration area were used (Alvim et al., 2019; Coronel et al., 2019; Lavado-García et al., 2020). In spite of using in the present work a different membrane cartridge, having the same characteristics (polysulfone membranes with 0.4 μm pore size and 0.005 m^2 filtration area) as the cartridge that Nikolay et al. (2020b) found to promote an efficient yellow fever virus recovery, product retention did occur again.

In their work, Nikolay et al. (2020b) tested the cartridge in a tangential-flow filtration experiment lasting just a few hours, whereby a bioreactor with YFV-infected BHK-21 cells set in a closed recirculation loop was just used to provide cell/virus suspension to challenge the membrane cartridge – no perfusion cultivation was carried out. Thus, considering the results obtained in the present work, it seems probable that in order to effectively avoid fouling in a long-term perfusion process, a membrane cartridge with the characteristics selected by Nikolay et al. (2020b) would need to have a much larger filtration area, such as for example the 0.13 m^2 of the traditional cartridge sold by the ATF manufacturer for the ATF-2 system.

Thus, to overcome product retention and membrane fouling issues observed in ATF-based perfusion herein, large-area membrane cartridges of specific materials and porosities could possibly be a solution and should be tested. However, in view of the low production cost that is desirable for a vaccine to be accessible also for the population of low-income countries, we considered it more adequate to proceed investigations using an inclined lamella settler, which is a low-cost cell retention device that does not use membranes or any other consumables and is intellectual-property free. Although no previous reports of HEK293 perfusion processes using a settler was found in literature, this cell retention device has been shown to provide very high separation efficiencies with no product retention in previous works with other mammalian cell lines (Coronel et al., 2020a; Coronel et al., 2020b).

As shown in Figure 4, a perfusion run with a CS-10 inclined settler using cell pool 2 resulted in a maximum VCD of 41.2 × 10^6 cells/mL on day 12, thus reaching the same viable cell concentration as obtained in pseudoperfusion on the same cultivation day (Figure 2B). Medium exchange rate was gradually increased during the run, with the aim of maintaining glucose levels at approximately 1 g/L (Figure 4B). In order to sustain VCDs above 25 million cells/mL, perfusion rates above 1.5 vvd had to be set, confirming how importantly membrane fouling in the ATF-based perfusion affected the necessary nutrient supply. Regarding the VLP product, as expected no product retention was observed, and VLP concentrations inside the bioreactor and

in the perfusate harvest were of approximately 200 mg/L from day 10 on (Figure 4C) at dilution rates of up to 2 vvd (Figure 4B), thus reaching volumetric productivities of approximately 400 mg/L/d, which are higher than those obtained in the pseudoperfusion run (270 mg/L at 1 vvd, thus volumetric productivity of 270 mg/L/d). Based on these results, the use of the inclined cell settler combined to the double-sorted cell pool 2 holds a promising potential to establish an intensified perfusion process for yellow fever VLP production.

3.4. Purification and characterization of the yellow fever VLPs

In parallel to the investigation of perfusion processes discussed above, the VLPs produced by stable cell pool 2 were purified and characterized in order to allow evaluation of product attributes and to establish hallmarks for future integration of upstream and downstream processing.

Steric exclusion chromatography (SXC) was selected for single-step purification of YF VLPs due to the good results reported for this technique for concentration and purification of large biomolecules, such as nucleic acids, large proteins and viruses (Lee et al., 2012; Marichal-Gallardo et al., 2017; Levanova & Poranen, 2018). SXC works by capturing the large biomolecules at a non-reactive hydrophilic surface by their mutual steric exclusion of polyethylene glycol (PEG) (Lee et al., 2012). Since the contaminants in the cell culture supernatant are much smaller than the VLPs, they are eliminated during feed in the flowthrough, whereas VLPs are subsequently eluted by reducing PEG concentration.

Figure 5A shows a typical chromatogram obtained for a single-step purification from cell culture supernatant using SXC with 8% (m/v) of PEG 6,000. The chemiluminescent immunoblot of the fractions collected during chromatographic steps shows that most VLPs were recovered as a concentrated eluate fraction and that no product was lost either in the feed flowthrough or in the wash step. Some product was detected in the fraction of the regeneration with 1M NaCl, indicating that there was some degree of adsorption of VLPs to the membrane used as stationary phase. Although adsorption is not the intended mechanism of SXC, the low intensity of the signal in the regeneration shows that it is a minor part of the VLPs that is lost in this fraction.

Panels C, D and E (Figure 5) show SDS-PAGE, Western blot and SEC-HPLC analyses performed to evaluate purity, identity and multimeric state of the VLPs, respectively. As shown in Figure 5C, sample complexity was significantly decreased when the cell culture supernatant is compared to the SXC eluate. The Western blot in Figure 5D shows that two protein bands in the supernatant, in the SXC eluate (containing 8% PEG) and in a concentrated/diafiltered sample of the eluate are recognized by the pan-flavivirus 4G2 antibody. The presence of these two bands having molecular mass (MM) around that of the envelope protein (50 kDa) correspond to two different glycoforms of the E protein, as we have shown in a previous work (Lima et al., 2019). In the SXC eluate the presence of PEG changed protein migration, but upon diafiltration the pattern is similar to the one observed in the supernatant sample. SEC-HPLC analysis of SXC eluate (Fig. 5D) further shows that contaminants, such as other proteins or eventually monomeric E protein not assembled in VLPs, were successfully removed in the purification step.

Concentrations of VLP, total proteins and DNA in the cell culture supernatant and in the SXC eluate were quantified in order to have an overview of SXC performance as a purification step for YF VLPs (Table 1). VLP recovery and DNA removal were 72.3% and 60.3% respectively, which are lower levels than usually reported for SXC, but in a range comparable to other types of chromatography (Pato et al., 2019). On the other hand, there was 99.9% total protein removal, which is comparable to values reported by other authors for SXC (Lee et al., 2012; Marichal-Gallardo et al., 2017; Levanova & Poranen, 2018).

The SXC-purified VLPs were further analyzed by transmission electron microscopy (TEM) and analytical size exclusion chromatography (A-SEC) to further investigate VLP structure and size. Negative-stained TEM confirmed the 3D structure of VLPs (Fig. 6A), whereas A-SEC runs revealed a non-symmetric and broader peak when compared to the protein standards used (Fig. 6B), which goes in line with the variability in sizes observed in the panel of micrographs (Fig. 6A).

4. Conclusions

Considering that yellow fever virus is endemic in many countries in Africa and Latin America and that it could spread in the near future to the other continents where the mosquito vector is already present, a new vaccine is needed to overcome the limited production capacity of the current vaccine manufactured in embryonated eggs. Recombinant virus-like particles (VLPs) are an alternative in this context. In the present work we have shown that HEK293 cells can stably express YF VLPs, and that production process intensification can be achieved by combining two different strategies: (i) sequentially enriching cell pools by FACS to get more homogeneous populations of stable high producers; (ii) developing continuous perfusion processes that can provide higher volumetric productivities, increasing production capacity and decreasing production costs. To perform perfusion cultivations for VLP production, the membrane-based ATF system presented problems of fouling causing underfeeding of the culture and resulting in undesirable product retention. On the other hand, an inclined lamella settler showed promising results and should be deeper explored as a cell retention device for the production of VLPs. Purification by a very simple chromatography technique (steric exclusion chromatography) allowed achieving high purities in a single step. The purified particles were analyzed by different techniques, and their identity and 3D-structure was confirmed.

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Conflict of interest

The authors declare no conflict of interests.

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TABLE

Table 1: Steric exclusion chromatography performance, according to VLP concentration (measured by ELISA), total protein concentration (measured by the BCA assay) and host-cell DNA (measured by the Picogreen assay).

	Volume (mL)	[VLP] (mg/L)	Yield (%)	[Total protein] (mg/mL)	Protein removal (%)	[DNA]
Supernatant	230	3.6	-	4.720	-	465.5
SXC purified VLP	12	49.9	72.3	0.069	99.9	3541.6

LEGENDS OF FIGURES

Figure 1: Generation of cell pools named 1 and 2, enriched by means of 1 or 2 rounds of FACS for YF-VLP high-producers, respectively. (A) Passage history showing viable cell density (VCD) and viability of mock transfection control, of original stable cell pool HEK293-YF and of enriched cell pools 1 and 2. The first round of cell sorting was performed 40 days after transfection (vertical red dashed line) and the second round 98 days after transfection (vertical blue dashed line). (B) Comparison of VLP production by cell pools 1 and 2 generated by FACS-enrichment. Dengue envelope protein (Prospec, Israel) was used as positive control and pan-flavivirus monoclonal antibody 4G2 was used as primary antibody.

Figure 2: Cell growth and glucose and lactate concentrations along time for cell pools 1 and 2 cultivated in shaken vented tubes in batch and pseudoperfusion (PP) modes. (A) Viable cell density (VCD) and viability of cell pools 1 and 2 grown in batch mode. (B) VCD and viability for cell pools 1 and 2 cultivated in pseudoperfusion mode. (C) Glucose (Glc) and lactate (Lac) concentrations along batch cultures of cell pools 1 and 2. (D) Glucose concentration and daily medium exchange rate applied during PP experiment with cell pools 1 and 2. Feeding strategy started on day 4 and samples were taken before and after feed addition. (E) Yellow fever VLP production for cell pools 1 and 2 when cultivated in batch and pseudoperfusion modes.

Figure 3: Production of YF VLPs by cell pool 1 cultivated in perfusion mode using ATF-2 as cell retention device. (A) Viable cell density (VCD), online biomass measurements and viability during perfusion cultivation. (B) Glucose and lactate concentrations and rates of medium addition (D_feed) and harvest (D_harvest) during perfusion cultivation. Due to membrane fouling, D_feed was higher than D_harvest from day 10 on. (C) VLP concentration inside the bioreactor and in the harvested perfusate.

Figure 4: Production of YF VLPs by cell pool 2 cultivated in perfusion mode using the inclined lamella settler CS-10 as cell retention device. (A) Viable cell density (VCD) and viability during perfusion cultivation. (B) Glucose and lactate concentrations and medium exchange rate during perfusion cultivation, given as volume of fresh medium per culture volume per day. (C) VLP concentration inside the bioreactor and in the harvested perfusate.

Figure 5: VLP purification from cell culture supernatant. (A) Typical chromatogram for steric exclusion chromatography (SXC): the solid line represents the absorbance at 280 nm and the dashed line the PEG 6000 concentration. (B) Immunoblot of the fractions collected during the chromatography using the pan-flavivirus monoclonal antibody 4G2 as primary antibody. (C) Silver-stained SDS-PAGE. Lane 1: molecular mass marker. Lane 2: clarified supernatant. Lane 3: purified VLP (SXC eluate). (D) Western blot analysis using 4G2 as primary antibody. Lane 1: molecular mass marker. Lane 2: clarified supernatant. Lane 3: purified VLP (SXC eluate). Lane 4: purified VLP after ultra and diafiltration for concentration for PEG removal. (E) SE-HPLC chromatograms using the TSKgel G3000SWXL column for cell culture supernatant and SXC-purified VLP samples.

Figure 6: Characterization of SXC-purified yellow fever virus-like particles. (A) Zoomed panel of images acquired by negative-staining transmission electron microscopy (scale bar: 30 nm). (B) Line plots showing size exclusion chromatograms using a Superose 6 10/300 column.

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