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Combining stable insect cell lines with baculovirus-mediated expression for multi-HA influenza VLP production

Daniela P. Sequeira [a,b], Ricardo Correia [a,b], Manuel J.T. Carrondo [a,c], António Roldão [a,b,*], Ana P. Teixeira [a,b,*], Paula M. Alves [a,b]

[a] IBET, Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2780-901 Oeiras, Portugal
[b] ITQB NOVA-Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av. Da República, 2780-157 Oeiras, Portugal
[c] Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Monte da Caparica, Portugal

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ABSTRACT

Safer and broadly protective vaccines are needed to cope with the continuous evolution of circulating influenza virus strains and promising approaches based on the expression of multiple hemagglutinins (HA) in a virus-like particle (VLP) have been proposed. However, expression of multiple genes in the same vector can lead to its instability due to tandem repetition of similar sequences. By combining stable with transient expression systems we can rationally distribute the number of genes to be expressed *per* platform and thus mitigate this risk. In this work, we developed a modular system comprising stable and baculovirus-mediated expression in insect cells for production of multi-HA influenza enveloped VLPs. First, a stable insect High Five cell population expressing two different HA proteins from subtype H3 was established. Infection of this cell population with a baculovirus vector encoding three other HA proteins from H3 subtype proved to be as competitive as traditional co-infection approaches in producing a pentavalent H3 VLP. Aiming at increasing HA expression, the stable insect cell population was infected at increasingly higher cell concentrations (CCI). However, cultures infected at CCI of 3×10^6 cells/mL showed lower HA titers *per* cell in comparison to standard CCI of 2×10^6 cells/mL, a phenomenon named "cell density effect". To lessen the negative impact of this phenomenon, a tailor-made refeed strategy was designed based on the exhaustion of key nutrients during cell growth. Noteworthy, cultures supplemented and infected at a CCI of 4×10^6 cells/mL showed comparable HA titers *per* cell to those of CCI of 2×10^6 cells/mL, thus leading to an increase of up to 4-fold in HA titers *per* mL. Scalability of the modular strategy herein proposed was successfully demonstrated in 2 L stirred tank bioreactors with comparable HA protein levels observed between bioreactor and shake flasks cultures. Overall, this work demonstrates the suitability of combining stable with baculovirus-mediated expression in insect cells as an efficient platform for production of multi-HA influenza VLPs, surpassing the drawbacks of traditional co-infection strategies and/or the use of larger, unstable vectors.

1. Introduction

Influenza viruses are responsible for annual epidemics and, occasionally, pandemics, causing acute febrile respiratory tract disease commonly known as "flu". Vaccination is the primary approach to prevent influenza virus infection. However, because of the rapid evolution of these RNA viruses, the seasonal vaccine needs annual reformulation based on empirical predictions about the circulating virus strains in the coming year. In addition, in case the same host is infected by multiple/different strains, the virus can undergo reassortment and thus generate a novel virus strain for which there is no memory immune response [1]. Therefore, the development of a "universal" influenza vaccine able to provide broad coverage against different strains is a public health priority not only to seasonal epidemic infections but also to pandemic situations.

The major surface glycoprotein hemagglutinin is prone to high mutation rates and is therefore an obvious target for the design of vaccine candidates. The most prominent example is the licensed FluBlok influenza vaccine from Protein Sciences, a recombinant vaccine based on full-length HA protein that spontaneously

* Corresponding authors at: IBET, Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2780-901 Oeiras, Portugal (A. Roldão). ETH Zurich, Department of Biosystems Science and Engineering, Mattenstrasse 264058, Basel, Switzerland (A.P. Teixeira).

E-mail addresses: aroldao@ibet.pt (A. Roldão), anat@ibet.pt (A.P. Teixeira).

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assembles into "rosettes" like structures [2]. More recently, it was shown that a specific region of HA – the stem region – can be recognized by antibodies and consequently able to stimulate cross-reactive immunization leading to protection against several influenza strains subtypes in mice [3–5].

Virus-like particles displaying HA (stem or full-length) and/or neuraminidase (NA) proteins on the surface of a protein scaffold (e.g. influenza matrix M1 protein and ferritin) [3,6] in a packed, orderly fashion, have been increasingly considered as promising vaccine designs. There is a number of preclinical trials [7] showing that these multimeric protein structures can induce strong immune response to virus challenge in mice models [8–11].

Influenza VLPs can accommodate multiple HA and/or NA subtypes. Therefore, they can offer broadened recognition and cross protection against circulating influenza viruses, significantly extending vaccine protection life-span. For example, a multivalent influenza VLP composed of two HA subtypes (H1 and H3) can induce immunity against homologous and closely related virus strains decreasing the viral titers in the lungs [12]. Efforts have also been made to generate vaccines protecting from potentially pandemic strains. In particular, a multivalent VLP comprising three HA subtypes (H5, H7 and H2/H9) protected ferrets against multiple influenza virus challenges, inducing specific antibodies to each HA subtype present in the viruses used [13,14]. More recently, a multivalent VLP generated by co-expressing four HA subtypes derived from avian-origin viruses has shown promising results as a vaccine candidate against virus strains with pandemic potential [15]. All these data suggest that influenza VLPs are already an alternative to seasonal attenuated influenza vaccines and a potential vaccine candidate against infections from pandemic strains.

The insect cell-baculovirus expression vector system (IC-BEVS) has proved to be as efficient as traditional egg- and cell-based strategies in producing influenza vaccines [16] with added benefits such as short production times, high production yields and straightforward scale-up. In addition, the construction of recombinant baculoviruses (rBac) has become simpler, faster and more versatile [17,18] enabling today the combination of genes from multiple influenza virus types and/or subtypes within the same expression vector [13]. However, the widespread and successful implementation of IC-BEVS for production of multivalent VLPs as vaccine candidates can be constrained by the cargo capacity of the expression vector. Indeed, the expression of multiple genes in the same vector usually leads to its instability due to tandem repetition of similar sequences that can lead to recombination events [19]. A commonly used strategy to mitigate this risk is to distribute the number of genes into smaller vectors and infect the host cell with these multiple vectors, strategy named co-infection. The major drawback of co-infection strategies is the uncertainty that each cell is infected with all viral vectors or that the ratio of different viruses entering each cell meets the stoichiometry of the target multi-protein complex [20]. For example, the production of correctly assembled triple layered rotavirus 2/6/7 VLPs is improved in insect *Spodoptera frugiperda Sf*-9 cells infected with tricistronic baculoviruses when compared to co-infection strategies, which are also associated with higher quantities of mono- and double-layered contaminant particles [20]. In another study, Aucoin and co-authors had to fine-tune the ratio of three baculoviruses in order to lessen the generation of empty adeno-associated virus (AAV) capsids [21].

The design of a modular system in which the number of genes to be expressed is rationally distributed between a recombinant baculovirus and a stable insect cell line would therefore be of significant value to surpass the drawbacks of co-infection strategies and/or the use of large, unstable vectors.

In this work, we have successfully developed a robust insect High Five cell-based platform for production of multivalent influenza VLPs by combining stable and baculovirus-mediated expression (Fig. 1 – Modular Strategy). The feasibility of this modular approach was demonstrated by infecting insect High Five cells constitutively expressing two different HA proteins from subtype H3 (named HA1 and HA2) with a baculovirus encoding M1 and three other HA proteins from subtype H3 (named HA3, HA4 and HA5) to produce a pentavalent H3 VLP. Rational optimization of infection conditions was conducted by (i) replenishing culture medium with key nutrients before infection and (ii) manipulating the cell concentration at infection. This combined strategy allowed an improvement of up to 4-fold in HA protein concentration when compared to standard infection conditions. Importantly, the HA production levels attained with this modular strategy were similar to those of a traditional co-infection scenario in which parental insect High Five cells are co-infected with two different baculoviruses (Fig. 1 – Co-infection Strategy). These results demonstrate the potential of the modular approach herein presented for efficient production of multivalent influenza VLPs in insect cells.

2. Materials and methods

2.1. Plasmid construction

Vectors encoding the HA genes from two different virus strains of subtype H3, named HA1 and HA2 were kindly provided by RedBiotech AG (Switzerland). Each HA gene was amplified by PCR and cloned into a KpnI or NotI excised pIZT/V5-His plasmid (Invitrogen), resulting in pIZT/HA1 and pIZT/HA2 vectors, respectively. The OpIE2 promoter and HA2 sequences from pIZT/HA2 vector were amplified by PCR and cloned into pIZT/HA1 vector opened by inverse PCR.

2.2. Cell line and culture media

Insect High Five cells were routinely sub-cultured to 0.3×10^6 cells/mL every 3–4 days when cell density reached $2–3 \times 10^6$ cells/mL in 125 or 500 mL shake flasks (10% working volume – w/v) in an Innova 44R incubator (orbital motion diameter of 2.54 cm - Eppendorf) at 27 °C and 100 rpm using Insect-XPRESS™ medium (Lonza). For the establishment of the stable insect High Five cells population expressing HA1 and HA2 proteins, parental cells were transfected using a lipofection protocol. Cells were transfected at a density of 0.5×10^6 cells/mL in 10 mL culture; for each 1×10^6 cells, 8 μl of Cellfectin® II reagent (Invitrogen), 100 μl of Grace's Insect Medium (Gibco) and 0.3 μg of DNA were used. After 48 h, selection of the transfected cell population started with 0.1 mg/mL of Zeocin selection antibiotic (Invivogen).

2.3. Baculovirus amplification

Recombinant baculoviruses containing (i) no influenza gene (named rBac-Empty), (ii) influenza M1 gene (named rBac-M1), (iii) influenza M1 gene in combination with two HA genes (for expression of HA1 and HA2 proteins) (named rBac-M1|HA1|HA2), or (iv) influenza M1 gene in combination with three HA genes (namely HA3, HA4 and HA5) (named rBac-M1|HA3|HA4|HA5) were kindly provided by RedBiotech AG (Switzerland). Since all H3 genes in rBac-M1|HA1|HA2 and rBac-M1|HA3|HA4|HA5 are expressed from identical promoters in the baculovirus vector (the presence of HA and M1 genes was confirmed by PCR), it is expected that they are present at similar levels in the pentavalent H3 VLPs.

Amplification of baculovirus stocks was performed as described elsewhere [20]. Briefly, *Sf*-9 cells were infected at 1×10^6 cells/mL at a multiplicity of infection (MOI) of 0.01–0.1 infectious particles

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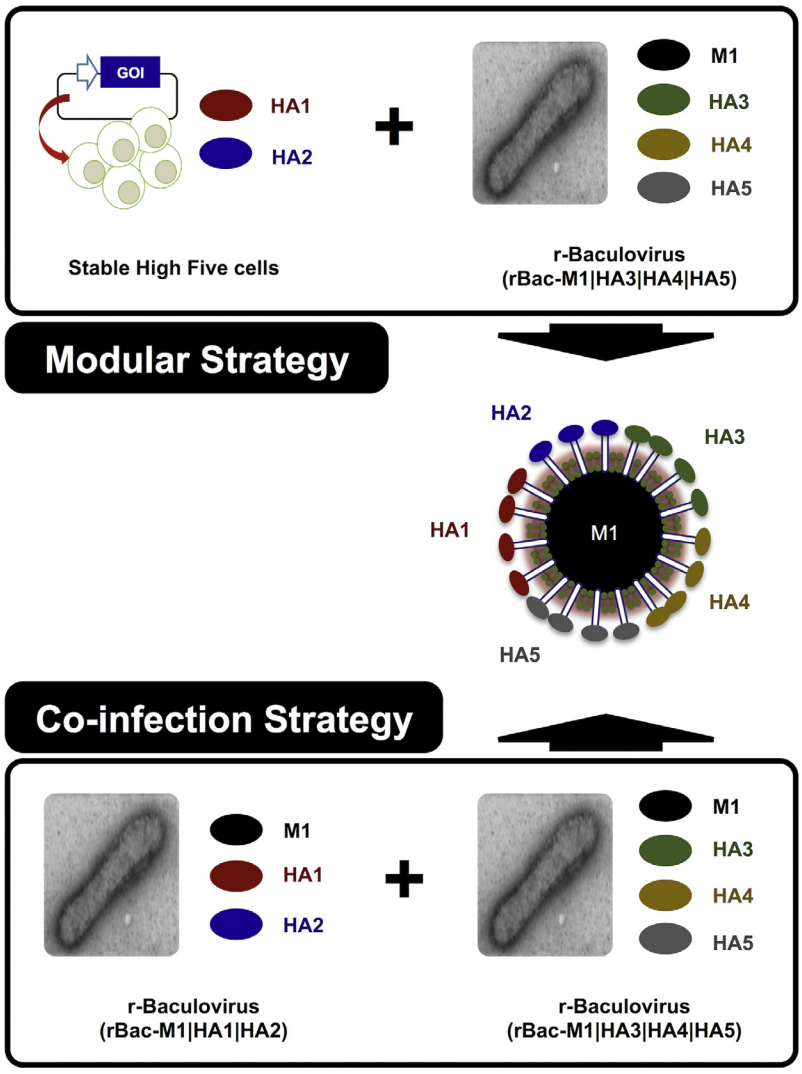

Fig. 1. Combining stable and baculovirus-mediated expression for production of multivalent influenza VLPs. Schematic view of the modular strategy herein implemented for production of pentavalent VLPs composed by influenza M1 protein plus five HA proteins from different H3 virus strains (Modular Strategy). The traditional co-infection scenario is also represented (Co-infection Strategy). **Modular Strategy**: insect High Five cells constitutively expressing two HA proteins (HA1 and HA2) are infected with one baculovirus encoding M1 and three other HA proteins (HA3, HA4 and HA5). **Co-infection Strategy**: parental insect High Five cells are co-infected with two different baculoviruses (rBac-M1|HA1|HA2 and rBac-M1|HA3|HA4|HA5), one encoding for two HA proteins (HA1 and HA2) and the other one encoding for three HA proteins (HA3, HA4 and HA5).

per cell (ip/cell). When cell viability reached 80–85%, cultures were harvested, centrifuged at 200*g* for 10 min at 4 °C. The pellet was discarded and the supernatant was centrifuged at 2000*g* for 20 min at 4 °C. The resulting supernatant was stored at 4 °C until further use.

2.4. Production of influenza VLPs

Influenza VLPs were produced in 250 or 2000 mL shake flasks (10% w/v) and in 2 L glass stirred tank bioreactors. Briefly, shake flask cultures were infected at a CCI of 2, 3 or 4×10^6 cells/mL using a MOI of 1 ip/cell. Specifically, for infections performed at CCI of 4×10^6 cells/mL, when cell concentration reached 2×10^6 cells/mL, the culture medium was supplemented with a mixture containing insect medium supplement 10×, 5 mM glutamine, 10 mM asparagine (Sigma-Aldrich) and 20 mM glucose (Merck Millipore) at a ratio of 10% (v/v) regarding the final culture volume.

Bioreactor culture was performed in a computer-controlled BIOSTAT® B-DCU 2 L vessel (Sartorius) equipped with two Rushton impellers, a sparger for gas supply, a water recirculation jacket for temperature control, and multiple ports for temperature, pH, pO2 (partial pressure of oxygen) probes as well as for additions (e.g. culture medium) and sampling/harvesting of cell culture. The pO_2 was set to 30% of air saturation and was maintained by varying the agitation rate from 70 to 250 rpm and the percentage of O_2 in the gas mixture from 0 to 100%. The gas flow rate was set to 0.01 vvm and temperature was kept at 27 °C. The working volume was 2 L. The culture was inoculated at a cell density of 0.5×10^6 cell/ml, supplemented as described above, and infected at 4×10^6 cells/mL with rBac-M1|HA3|HA4|HA5 (MOI of 1 ip/cell).

2.5. Purification of influenza VLPs

When cells viability reached 60%, cell culture bulk was harvested and processed using two different strategies according to the volume to purify.

For purification of shake flask cultures, cell culture bulk was clarified by centrifugation, first at 200*g*, 4 °C, 10 min for cells removal, and second at 2000*g*, 4 °C, 20 min for removal of cellular

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debris. The supernatant was collected and proteins concentrated by polyethylene glycol (PEG 8000) precipitation method using a solution of 50% (w/v) PEG with 4 M NaCl. After incubation at 4 °C overnight, protein pellets were collected by centrifugation at 4500*g*, 4 °C for 30 min and resuspended in 1.5 mL of HEPES buffer (50 mM) containing NaCl (300 mM) and pH 6.6. Influenza VLPs were purified by size exclusion chromatography with a Superdex200 10/300 column (GE Healthcare). The fractions corresponding to the VLP peak were pooled and sterile filtered using a Whatman cellulose regenerated membrane filter.

For purification of bioreactor culture, cell culture bulk was clarified by centrifugation at 200*g*, 4 °C, 10 min, supernatant collected, mixed with Benzonase (50 U/mL) (Merck Millipore) for digestion of nucleic acids and then passed through a Sartopore® 2 membrane capsule (Sartorius Stedim Biotech). Influenza VLPs were purified by anion-exchange chromatography with a SartoBind Q capsule (Sartorius Stedim Biotech), concentrated by ultrafiltration and diafiltration using 300 kDa cassette regenerated cellulose, and sterile filtered using a Whatman cellulose regenerated membrane filter. The baculovirus titer in the final purified product was 6.68×10^7 pfu/mL.

Influenza VLPs were stored at -80 °C (long-term storage) or at 4 °C (short-term storage) in HEPES buffer (50 mM) containing NaCl (300 mM), trehalose (15% w/v) and pH 6.6.

2.6. Analytics

2.6.1. Cell concentration and viability

Cell concentration and viability were analyzed daily and calculated by trypan blue exclusion method. Cell counting was performed in a Fuchs–Rosenthal hemocytometer chamber (Brand, Wertheim, Germany).

2.6.2. Baculovirus titration

Baculovirus titers were determined using the Virocyt virus counter (Virocyt) (measures total baculovirus particles) or the MTT assay [22,23] (measures infectious baculovirus particles).

2.6.3. Metabolite analysis

Cell culture samples were centrifuged at 200*g* for 10 min and supernatants collected for metabolite analysis. Metabolite quantification was performed by ^{1}H NMR spectroscopy as described elsewhere [24]. Briefly, spectra were recorded in a 500 MHz Avance spectrometer (Bruker) equipped with a 5 mm QXI inversed probe, using a NOESY-based pulse sequence with water presaturation. DSS-d6 (Sigma Aldrich) was used as internal standard for metabolite quantification in all samples. In order to obtain a similar pH between samples, they were mixed with phosphate buffer (pH 7.4) prepared in D_2O at a 2:1 ratio. Each spectrum was phased, baseline corrected and integrated using the Chenomx NMR Suite 8.0 software (Chenomx Inc.).

2.6.4. Western blot analysis

Culture samples were centrifuged at 200*g* for 10 min and supernatants collected and stored at 4 °C until further analysis. Proteins were first denatured by heating the sample to 70 °C for 10 min, then separated under reducing conditions in a NuPAGE 4–12% Bis-Tris protein gel (Thermo Fisher Scientific) and finally transferred to a nitrocellulose membrane using iBlot® gel transfer device (Thermo Fisher Scientific). For HA identification, several antibodies (Abs) were initially tested (polyclonal Abs from National Institute for Biological Standards and Control (NIBSC) and monoclonal Abs from International Reagent Resource (IRR)) in order to find/identify specific Abs to each HA variant included in the pentavalent H3 VLP. The IRR mAbs were unable to recognize any of the HA protein variants included in the VLP. On the contrary, NIBSC pAbs showed cross-reactivity, meaning that they are able to recognize not only the HA variant to which they were raised against but also all other H3 variants included in the pentavalent VLP. Therefore, a sheep polyclonal antibody kindly provided by NIBSC (UK) was used at a dilution of 1:1000. For M1 protein identification a goat polyclonal antibody (Abcam, Cat# ab20910) was used at a dilution of 1:2000. As secondary antibodies, an anti-sheep or anti-goat IgG antibody conjugated with horseradish peroxidase labelling was used at a dilution of 1:5000 (Santa Cruz Biotechnology, Cat# sc-2473) or 1:2000 (Life Technologies, Cat# 81-1620), respectively. Protein band detection was performed with the enhanced chemiluminescence detection system (Amersham Biosciences).

2.6.5. Hemagglutination assay

The hemagglutination assay herein used is a plate-based assay in which the HA titer is determined by comparing the hemagglutination profile of culture samples with that of a standard of known HA concentration. Briefly, cell culture supernatant was 2-fold serially diluted in PBS and incubated for 30 min at 4 °C with 25 µL of 1% chicken red blood cells (RBC) (Lohmann Tierzucht GMBH). Hemagglutination of RBC was identified visually by the formation of a network (lattice structure) of interconnected RBC and HA (positive results). As standard, an influenza vaccine with a known HA concentration was added to each assay experiment. The HA titer value is the inverse of the last dilution of sample that completely inhibited hemagglutination.

2.6.6. Immunofluorescence microscopy

An immunofluorescence protocol was used to visually inspect the presence of HA in insect High Five cell membranes (adapted from [25]). Briefly, culture samples containing 2×10^6 cells were centrifuged at 300*g* for 5 min and the cell pellets collected, washed with PBS twice and then incubated with 50 µl of sheep polyclonal antibody kindly provided by NIBSC (UK) (diluted 1:20 in PBS) at 4 °C in the dark for 1 h. Afterwards, samples were centrifuged and the cell pellets washed twice with PBS and then incubated with 50 µl of Alexa Fluor® 488 donkey anti-sheep antibody (diluted 1:200 in PBS) (Thermo Fisher Scientific, Cat# A11015) for 30 min at 4 °C in the dark. After two washing steps with PBS, samples were resuspended in PBS and fluorescence microscopy analysis was performed to detect GFP (DMI 6000, Leica).

2.6.7. Lysis of insect cells

The insect cells lysis protocol herein used was adapted from [26]. Briefly, insect cell pellets collected after centrifugation at 200*g* for 10 min were resuspended in HEPES buffer (50 mM) containing NaCl (300 mM) and trehalose (15% w/v) at pH 6.6 to a final concentration of 4×10^6 cells/mL and then sonicated in Digital Sonifier 450 (Branson) for 1 min using cycles of 15′ On-Off and an amplitude of 10%. After sonication, samples were centrifuged at 10000*g*, 4 °C for 10–15 min and supernatants collected for further analysis.

2.6.8. Transmission electron microscopy (TEM)

Negative staining TEM was used to assess the conformation and size of purified influenza VLPs. Briefly, 10 µl of purified VLP sample was fixed for 1 min in a copper grid coated with Formvar-carbon (Electron Microscopy Sciences). Grids were washed with H_2O and then stained with 1% (v/v) uranyl acetate for 2 min and left to air dry. Samples were then observed in a Hitachi H-7650 Transmission Electron Microscope (JEOL).

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3. Results

3.1. Establishment of a stable insect High Five cell population expressing two different HA proteins from subtype H3

Parental insect High Five cells were transfected with a vector encoding the HA genes from two different virus strains of subtype H3 (named HA1 and HA2) under control of the OpIE2 promoter (Fig. 2A). Upon two to three weeks under selective pressure (Zeocin selection antibiotic), a robust High Five cell population with viabilities and duplication times averaging 95% and 23 h, respectively, was obtained (Fig. 2B). The presence of both HA1 and HA2 transcripts was confirmed by real-time PCR and total HA protein content in cellular extracts of the stable cell population detected by Western blot (data not shown). Moreover, immunofluorescence microscopy confirmed the localization of HA protein at the cell membrane (Fig. 2C). In addition, intracellular HA protein concentration was determined by hemagglutination assay over 31 passages and no significant differences could be observed, confirming the stability of HA expression along passages (Fig. 2D).

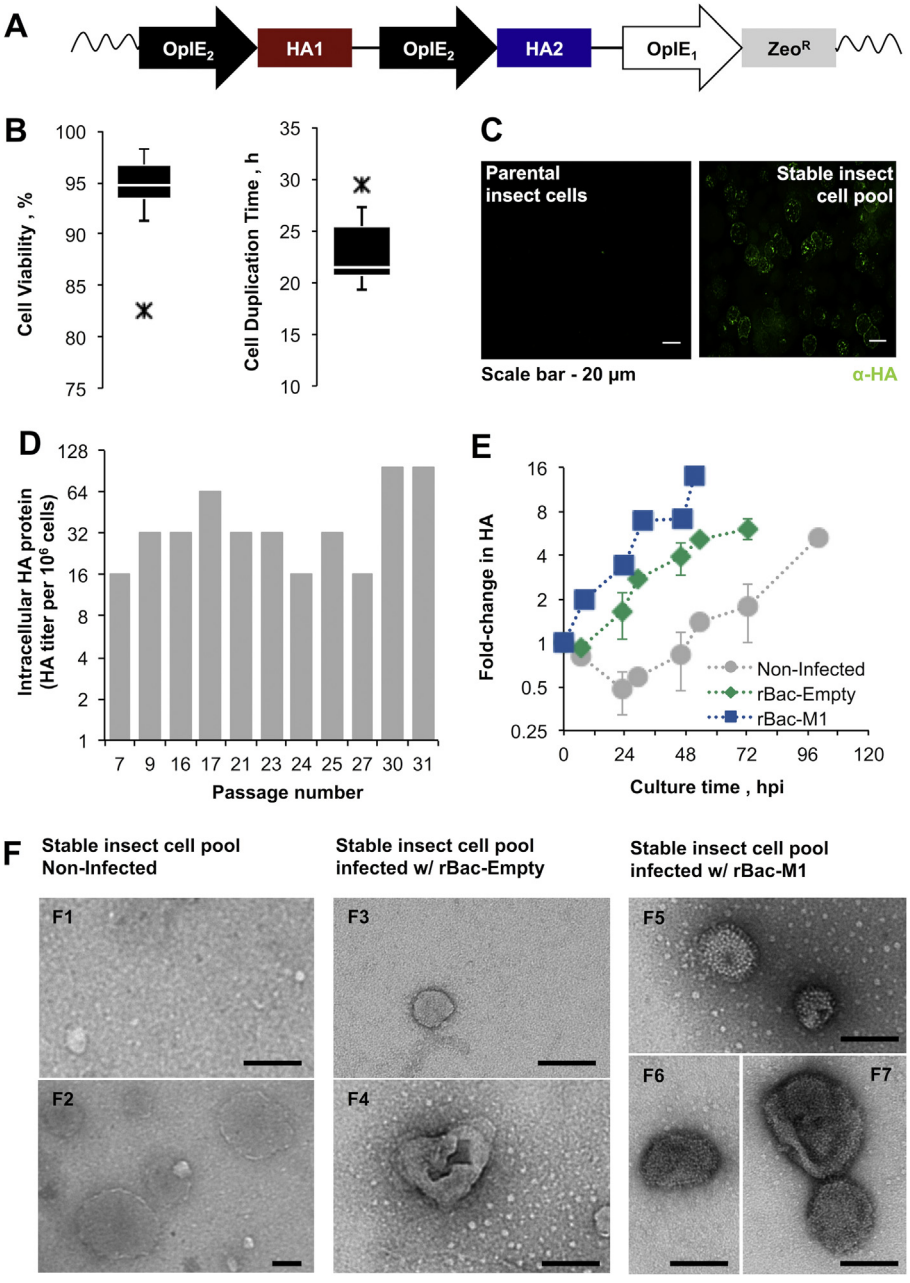

Fig. 2. Establishment of a stable insect High Five cells pool expressing two different HA proteins from subtype H3. (A) Schematic view of the expression vector used for transfection of parental insect High Five cells - OpIE2 and OpIE1 denote the promoters driving the expression of HA proteins (HA1 and HA2) and zeocin resistance marker (Zeo^R), respectively; (B) Box-plot diagrams showing cell viabilities and duplication times of the stable insect High Five cells population over 31 passages - horizontal lines are medians, boxes represent the interquartile range (IQR), error bars show the full range excluding outliers (asterisk) defined using the modified Thompson tau technique (for n = 19); (C) Visualization of HAs anchored to the plasma membrane of stable insect High Five cells by immunofluorescence microscopy - scale bar represents 20 µm; (D) Impact of passage number on intracellular HA protein concentration; (E) Comparing extracellular HA protein concentration of stable insect High Five cells (Non-Infected) with stable insect High Five cells infected either with a baculovirus containing no influenza gene (rBac-Empty) or with a baculovirus containing the influenza M1 protein (rBac-M1) – the HA titer *per* cell (in units) throughout the culture was normalized by the titer obtained at cell concentrations of 2×10^6 cells/ml for each experiment; (F) Negative staining transmission electron microscopy of purified VLP samples from "Non-Infected", "rBac-Empty" and "rBac-M1" cell cultures - scale bar represents 100 nm.

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The HA-expressing cell population was cultured for approximately 120 h and the concentration of HA protein in the culture medium assessed by hemagglutination assay (Fig. 2E - Non-Infected). Noteworthy, HA protein not only could be detected but also seemed to accumulate over time, most likely due to: (i) the decrease in cells viability and subsequent release of HA protein to culture medium, particularly from 60 to 120 hpi when cells viability dropped from 90% to 60% (data not shown) and HA titer *per* cell increased almost 4-fold; and/or (ii) the release of microvesicles containing membrane proteins into the medium (incl. HA protein), similar to what occurs in other insect cell lines (e.g. Drosophila hemocyte-like Kc and S2 cells [27,28]) or in mammalian cell lines [29,30]. In fact, the structures identified by negative staining TEM in Fig. 2F (image F2) closely resemble the exosomes released by Drosophila cells, both in shape and size (200–300 nm) [27,28].

To confirm that accumulation of HA in culture medium is potentiated by baculovirus infection, the stable HA-expressing cell pool was infected with a baculovirus containing no influenza gene (Fig. 2E - rBac-Empty). In parallel, to assess if accumulated/extracellular HA proteins are presented in their native conformation as correctly assembled VLPs, the cells were infected with a baculovirus containing the influenza M1 protein (as scaffold). Extracellular HA protein concentration and VLP formation were assessed and compared to "Non-Infected" cultures (Fig. 2E - rBac-M1). Faster and higher accumulation of HA was observed in the medium of "rBac-Empty" and "rBac-M1" cultures when compared to "Non-Infected" cultures (Fig. 2E). In addition, correctly assembled VLPs (described as HA protein spikes displayed on the surface of the M1 protein scaffold) with the expected size range [27] could be identified in purified samples of "rBac-M1" cultures (Fig. 2F - images F5-F7) but not in purified samples of "Non-Infected" and "rBac-Empty" cultures (Fig. 2F - images F1-F4). This data suggests that (i) accumulation of HA in culture medium of stable insect High Five cells populations is negligible when compared to cell cultures infected with rBac-Empty or rBac-M1 baculoviruses and (ii) the expression of M1 protein (as scaffold) is essential for the generation of correctly assembled VLPs displaying HA proteins.

3.2. Modular strategy for production of multivalent influenza VLPs

3.2.1. Proof-of-concept and comparison with co-infection strategy

The suitability of combining stable and baculovirus-mediated expression to produce a pentavalent H3 VLP was demonstrated by infecting the HA-expressing High Five cell population with a baculovirus encoding M1 and three different HA proteins from H3 subtype (named HA3, HA4 and HA5) (Modular Strategy – Fig. 1). This modular strategy was compared to the standard co-infection scenario in which parental insect High Five cells were co-infected with two baculoviruses, the rBac-M1|HA1|HA2 and rBac-M1|HA3|HA4|HA5 (Co-infection Strategy – Fig. 1). Independently of the approach followed, cells were infected at CCI of 2×10^6 cells/mL using a MOI of 1 ip/cell (standard infection condition). The kinetics of cell growth and HA protein accumulation in culture medium upon baculovirus infection are depicted in Fig. 3. Results show immediate cell growth arrest upon infection in both strategies, as expected when using MOI of 1 ip/cell [31], but a later onset of cell viability decrease for the modular strategy when compared to the co-infection strategy (Fig. 3A). Therefore, in order to

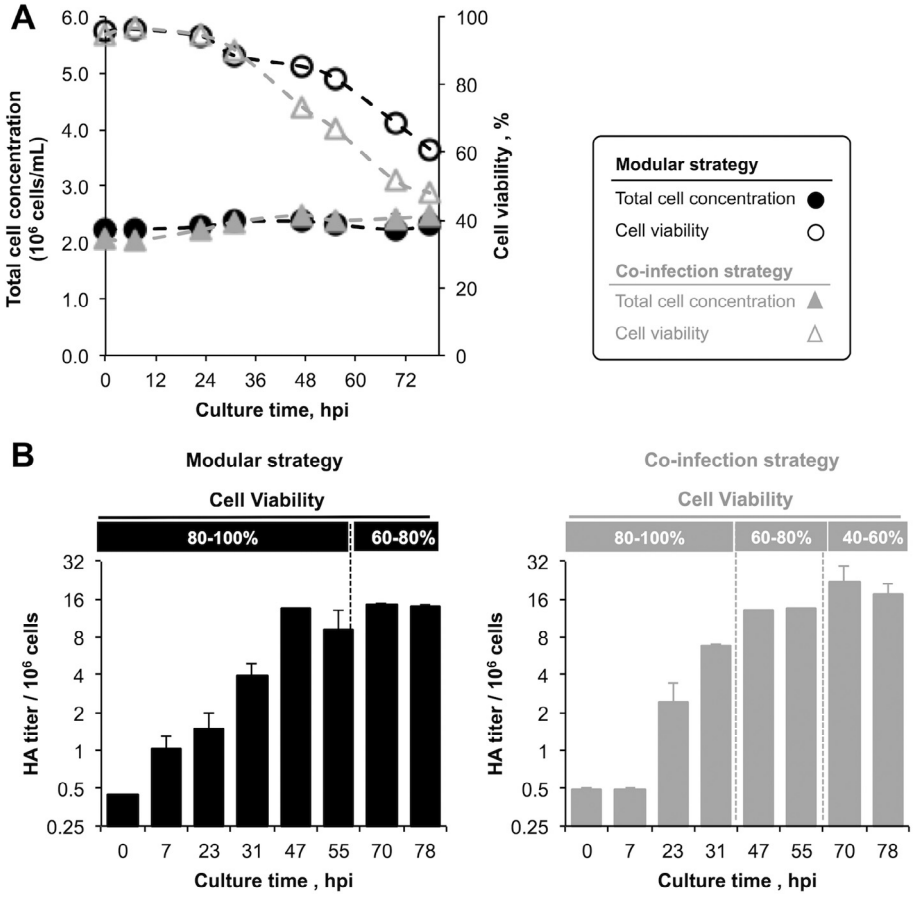

Fig. 3. Modular strategy vs co-infection strategy for production of pentavalent H3 Influenza VLPs. (A) Total cell concentration and cell viability profiles upon baculovirus infection for both infection strategies. (B) Accumulation of HA protein in the culture medium upon baculovirus infection for the modular strategy and for the co-infection strategy. Error bars denote standard deviations (n = 3). hpi denotes hours-post infection.

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evaluate performances, the HA production levels attained in each strategy were compared at similar cell viabilities. Data presented in Fig. 3B show that, at comparable cell viabilities, the HA production levels attained with the modular strategy were similar to those of the co-infection strategy, thus demonstrating the potential and competitiveness of the modular approach herein presented for efficient production of multivalent influenza VLPs in insect cells.

3.2.2. Impact of CCI on HA protein production

Aiming at increasing HA protein production, stable insect High Five cells were infected at CCI of 3×10^6 cells/mL with the rBac-M1|HA3|HA4|HA5 baculovirus (Modular Strategy – Fig. 1) using a MOI of 1 ip/cell. The kinetics of cell growth and HA protein accumulation in culture medium upon baculovirus infection are depicted in Fig. 4 and compared to the standard infection condition. Cell growth is arrested immediately upon infection regardless of the CCI used (Fig. 4A). Importantly, the onset of cell viability decrease due to infection as well as its profile is similar in both conditions evaluated (Fig. 4A), thus allowing a direct comparison between the HA titers attained in each CCI tested. Data presented in Fig. 4B shows a clear reduction in the HA titer *per* cell when stable insect High Five cells are infected at CCI of 3×10^6 cells/mL in comparison to standard CCI of 2×10^6 cells/mL, a phenomenon known as "cell density effect", i.e. drop in the specific productivity in the insect cells-baculovirus expression system when cells are infected at high cell densities [32]. Consequently, the HA titers *per* mL obtained at CCI of 3×10^6 cells/mL at most matched those achieved at CCI of 2×10^6 cells/mL (Fig. 4C). The "cell density effect" was also observed in stable insect High Five cell cultures infected with the rBac-M1 (data not shown) thus demonstrating that this phenomenon is independent on the baculovirus used.

3.3. Identification of nutrients depleted over culture time

The concentration of stable HA-expressing High Five cell population peaks around 72–96 h post-inoculation, reaching values close to 3.5×10^6 cells/mL, after which a steep decrease in cell viability onsets (Fig. 5A). In order to identify key metabolic drivers contributing for the onset of the cell death phase and concomitantly the "cell-density effect" observed above (see Section 3.2.2 for details), exometabolome analysis by ^{1}H NMR was performed (Fig. 5B). Results show that cells avidly consume asparagine (Asn), exhausting the 8.5 mM available at inoculation in less than 48 h. Nonetheless, Asn seems to be a non-essential metabolite for cell growth as cells continue to grow even after its exhaustion. In addition, lactate (Lac) accumulates up to 4.8 mM during the first 72 h, after which it starts decreasing as glucose (Glc) concentration becomes limiting. Glutamine (Gln) is the second most consumed amino acid, reaching values close to zero around 120 h post-inoculation.

Based on these results, a refeed strategy was designed that consisted in supplementing the culture 72 h after inoculation with the key nutrients identified above, Asn, Gln and Glc, plus a mixture of lipids, proteins and vitamins for insect cells (Insect Medium Supplement 10×) previously identified as potential enhancer for VLPs production [33]. The kinetics of cell growth obtained in supplemented cultures is shown in Fig. 5A. ^{1}H NMR data shows that (i) Gln and Glc concentrations were restored to values near those found at the inoculation time and (ii) lactate concentration

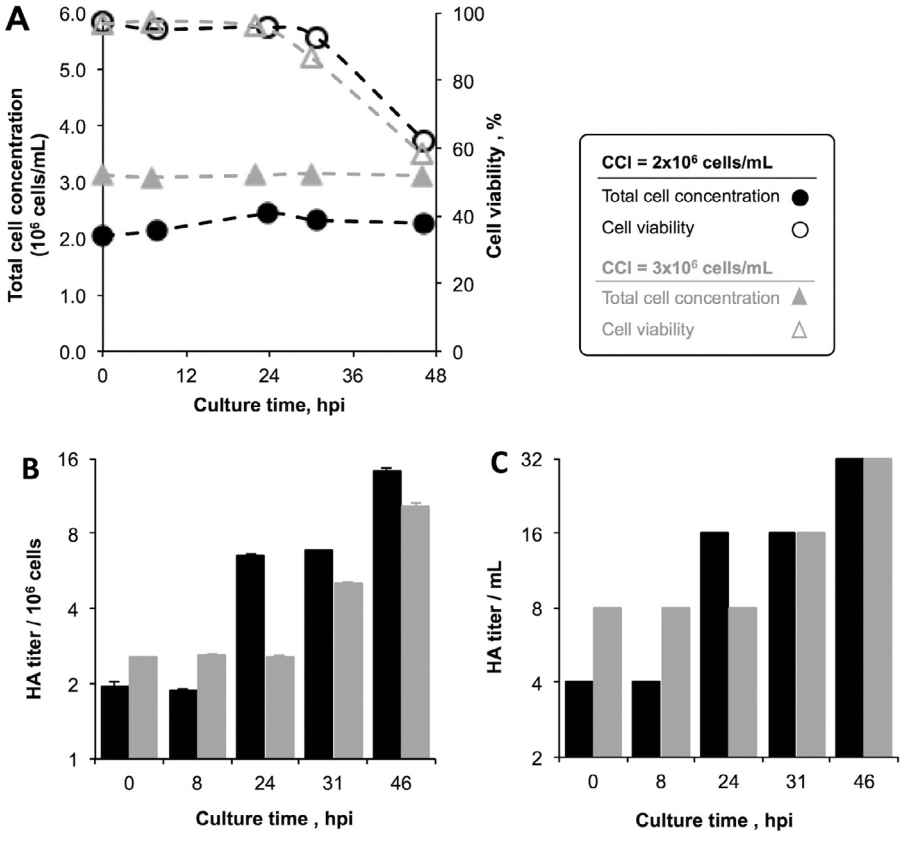

Fig. 4. Modular strategy for production of pentavalent H3 Influenza VLPs: impact of CCI on HA protein production. (A) Total cell concentration and cell viability profiles upon baculovirus infection for CCI of 2×10^6 cells/mL and 3×10^6 cells/mL. HA protein accumulation in the culture medium upon baculovirus infection for CCI = 2×10^6 cells/mL (black bars) and CCI = 3×10^6 cells/mL (grey bars): HA titer *per* 10^6 cells (B) and HA titer *per* mL (C). MOI = 1 ip/cell. Error bars denote standard deviations (n = 3). hpi denotes hours-post infection.

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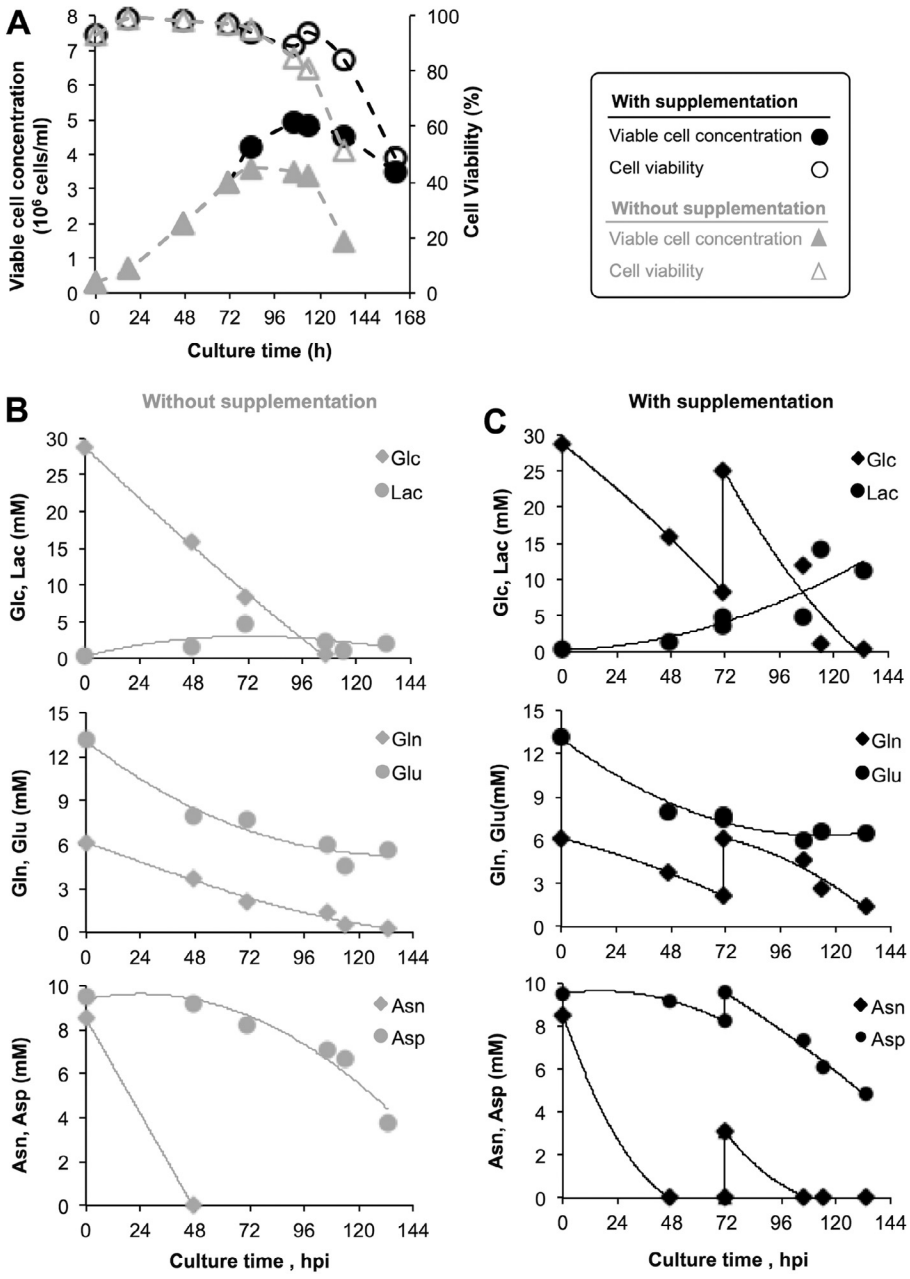

Fig. 5. Identification of key metabolic drivers during growth of stable insect High Five cells. (A) Viable cell concentration and cell viability profiles of stable insect High Five cell cultures with (black symbols) and without supplementation (grey symbols) at 72 h post-inoculation. The tailor-made refeed strategy consisted in a mixture of glucose, glutamine, asparagine, lipids, proteins and vitamins for insect cells (see text for further details). Time profile of metabolite concentration in the medium of non-supplemented (B) and supplemented (C) insect High Five cell cultures. Lac - lactate; Glc – glucose; Gln – glutamine; Glu – glutamate; Asn – asparagine; Asp – aspartate. Metabolite analysis was performed using 1H NMR. hpi denotes hours-post inoculation.

increases significantly after glucose addition reaching 14 mM at 114 h post-inoculation (Fig. 5C). Noteworthy, the refeed strategy herein adopted was able to sustain cell cultures at concentrations higher than those previously achieved, peaking at 5×10^6 cells/mL around 96–120 h post-inoculation.

3.4. Improved production of pentavalent H3 VLPs through a tailor-made refeed strategy

3.4.1. Impact of a tailor-made refeed strategy on HA protein production

To assess the impact of the tailor-made refeed strategy on HA protein production, the HA-expressing cells were supplemented 72 h post-inoculation and subsequently infected at CCI of 4×10^6 -cells/mL with the rBac-M1|HA3|HA4|HA5 vector (Modular Strategy – Fig. 1) using a MOI of 1 ip/cell. The kinetics of cell growth and HA protein accumulation in culture medium upon baculovirus infection are depicted in Fig. 6 and compared to the standard infection condition without supplementation. Cell growth is arrested immediately upon infection independently of the CCI used (Fig. 6A). Noteworthy, the onset of cell viability decrease is delayed for approximately 24 h in cultures infected at CCI of 4×10^6 cells/mL when compared to CCI of 2×10^6 cells/mL (Fig. 6A) suggesting that the tailor-made refeed strategy impacts positively on cells viability during infection phase. Therefore, in order to evaluate productivity performances, the HA production levels attained in each condition

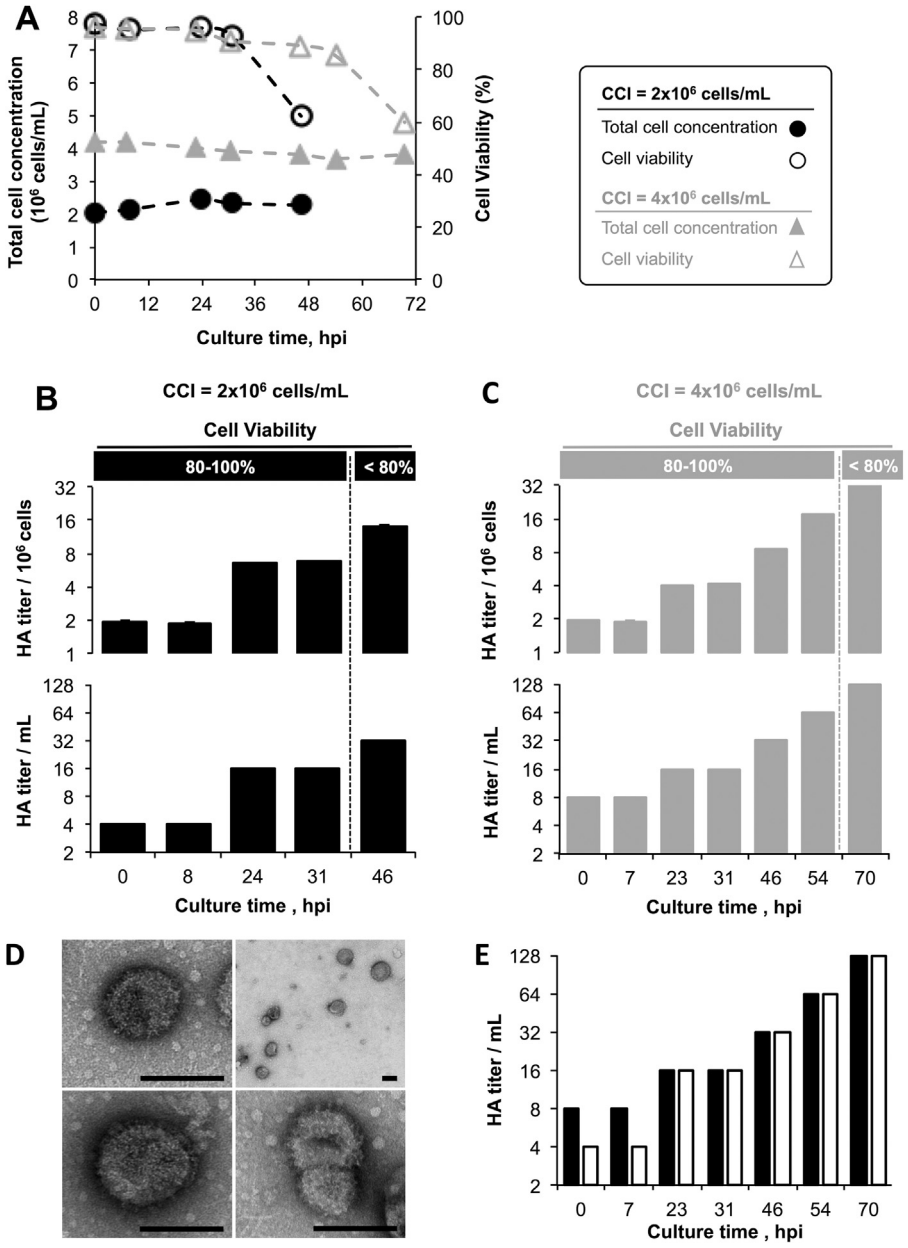

Fig. 6. Modular strategy for production of pentavalent H3 Influenza VLPs: impact of a tailor-made refeed strategy on HA protein production at high CCIs. (A) Total cell concentration and cell viability profiles upon baculovirus infection for CCI = 2×10^6 cells/mL and CCI = 4×10^6 cells/mL. HA protein accumulation in the culture medium upon baculovirus infection for CCI = 2×10^6 cells/mL (B) and CCI = 4×10^6 cells/mL (C). Negative staining TEM of purified pentavalent H3 Influenza VLPs sample - scale bar represents 100 nm (D). HA protein accumulation in the culture medium of stable insect High Five cells infected at CCI = 4×10^6 cells/mL (w/ supplementation) with the rBac-M1|HA3|HA4|HA5 baculovirus (filled bars) or with the rBac-M1 baculovirus (empty bars) (E). MOI = 1 ip/cell. Error bars denote standard deviations (n = 3). hpi denotes hours-post infection.

were compared at similar cell viabilities. Fig. 6B and 6C shows that, at comparable cell viabilities, the HA titers *per* cell obtained in cultures infected at CCI of 4×10^6 cells/mL were higher than those of CCI of 2×10^6 cells/mL, leading to an increase of up to 4-fold in HA titer *per* mL. Negative staining TEM images of purified samples from cultures infected at CCI of 4×10^6 cells/mL (Fig. 6D) show VLPs that closely resemble influenza viruses both in size and morphology [9].

In order to validate the potential of the tailor-made refeed strategy for HA protein production, stable insect High Five cells were infected with a different baculovirus construct (rBac-M1) using the same infection scheme as described above (i.e. culture supplementation at 72 h post-inoculation and CCI of 4×10^6 cells/mL). The kinetics of cell growth and HA protein accumulation in culture medium upon infection were assessed and compared to standard infection condition without supplementation (data not shown). The profiles obtained were similar to those achieved above for cultures infected with the rBac-M1|HA3|HA4|HA5 baculovirus (example is shown for HA titer *per* mL - Fig. 6E) thus confirming the potential of the tailor-made refeed strategy herein developed for attenuating the "cell density effect" and improving HA protein production.

3.4.2. Proof-of-concept in 2 L stirred-tank bioreactors

The feasibility of the modular strategy herein proposed for production of multivalent influenza VLPs was demonstrated in a

computer-controlled 2 L stirred-tank bioreactor. HA-expressing High Five cells were grown up to 4×10^6 cells/mL using the tailor-made refeed strategy aforementioned and subsequently infected with the rBac-M1|HA3|HA4|HA5 baculovirus (Modular Strategy – Fig. 1) using a MOI of 1 ip/cell. The kinetics of cell growth and HA protein accumulation in culture medium upon baculovirus infection are depicted in Fig. 7 and compared to those achieved in small-scale shake flasks. Total cell concentration and cell viability profiles obtained in the 2 L bioreactor were very similar to those of shake flask cultures, with cell growth being arrested immediately upon infection and the onset of cell viability decrease occurring around 48 hpi (Fig. 7A). In addition, the HA titers *per* cell obtained in both culture systems were fairly comparable (Fig. 7B). Western blot analysis of in-process samples collected from the bioreactor culture allowed the identification of HA and M1 proteins with the expected molecular weight (64 kDa for HA and 28 kDa for M1) as well as their accumulation in culture medium with the course of infection (Fig. 7C). Fig. 7D shows negative staining TEM images of purified samples from the bioreactor culture and the presence of VLPs resembling influenza viruses, both in size and morphology, could be confirmed. The results obtained confirm the scalability of the modular strategy herein proposed (incl. the tailor-made refeed supplementation scheme) for production of pentavalent H3 VLPs.

4. Discussion

A modular insect cell-based platform for efficient production of multivalent influenza VLPs is herein proposed to surpass the drawbacks of traditional co-infection strategies and/or the use of large, unstable viral vectors. To achieve such goal, three major tasks have been performed: (i) generation of a stable insect High Five cell population expressing two different HA proteins from subtype H3, (ii) identification of nutrients depleted over culture time to design a tailor-made refeed strategy for improved HA protein production and (iii) proof-of-concept in 2 L stirred-tank bioreactor.

To our knowledge this is the first time stable expression of influenza HA genes was successfully undertaken in insect High Five cells. This work demonstrates that it is possible to obtain a population of cells expressing two different HA proteins from a single subtype with similar growth performances along several passages (Fig. 2B). In addition, the concentration of HA protein being expressed in this cell line is relatively constant across more than 31 passages (Fig. 2D), hence corroborating the concept of insect cells being a competent platform for stable protein production [34]. The observation that HA protein accumulates in culture medium over time without baculovirus infection or the assistance of a scaffold (in this case influenza M1 protein) may be related to (i) the decrease in cells viability and subsequent release of HA protein to

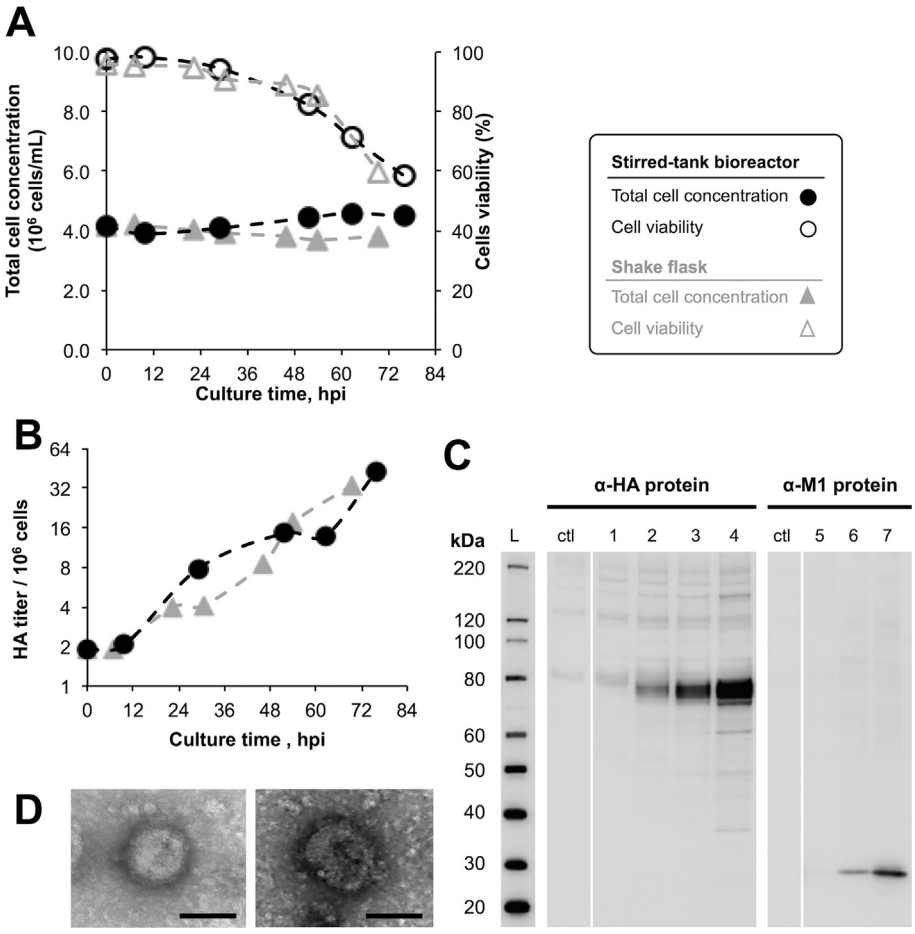

Fig. 7. Production of pentavalent H3 Influenza VLPs in 2 L stirred-tank bioreactor. Stable insect High Five cells were supplemented 72 h post-inoculation and infected at CCI of 4×10^6 cells/mL with the rBac-M1|HA3|HA4|HA5 baculovirus (Modular Strategy – Fig. 1) using a MOI of 1 ip/cell. Kinetics of cell growth (A) and HA protein accumulation in culture medium (B) upon baculovirus infection for shake flask and bioreactor cultures. (C) Analysis of in-process (culture medium) samples from bioreactor experiment by Western blot using a sheep polyclonal antibody (α-HA protein) or a goat polyclonal antibody (α-M1 protein) as primary Ab. The numbers 1–7 denote the culture time at which samples were collected: t = 0 hpi (lane 1), t = 29 hpi (lane 2 and 5), t = 52 hpi (lane 3 and 6), t = 76 hpi (lane 4 and 7). The letters "L" and "ctl" denote the MagicMark™ XP Western protein standard and the negative control sample (culture medium of parental insect High Five cells) used, respectively. The volume of samples 1–7 and "ctl" loaded into the gel was the same (15 μL). The expected MW of HA and M1 proteins are 64 kDa and 28 kDa, respectively. (D) Negative staining TEM of purified pentavalent H3 Influenza VLPs sample - scale bar represents 100 nm.

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culture medium and/or (ii) the release of microvesicles containing membrane proteins into the medium (incl. HA protein), similar to what occurs in other insect cell lines (e.g. Drosophila hemocyte-like Kc and S2 cells [27,28]) or in mammalian cell lines [29,30].

In order to produce a multi-subunit product, co-infection strategies are commonly used [35]. However, they enclose a major drawback which is the uncertainty that each cell is infected with all viral vectors and, most importantly, by the same number of viruses from each construct thus hampering the development of a robust and rapid platform [20]. In this work, we show that constitutive gene expression in combination with baculovirus-mediated expression under single infection conditions can be as competitive as traditional co-infection approaches in producing a multi-subunit product, namely a pentavalent H3 VLP.

In the last couple of decades substantial effort has been committed to optimizing the IC-BEVS in order to enhance specific and volumetric productivities, including the choice of the cell line [36], oxygen supply [37,38], bioreactor design [39] or the infection strategy (e.g. best combination of CCI and MOI) [31]. Regarding the latter, the production of recombinant proteins is usually impaired at CCIs above 2×10^6 cells/mL - the so called "cell density effect" [40,32]. This phenomenon is not well understood, but key factors implicated are the lack of nutrients and/or oxygen and accumulation of toxic by-products [41]. In this work, a similar outcome was observed when stable insect High Five cell cultures infected at CCI of 3×10^6 cells/mL showed lower HA titers *per* cell in comparison to standard CCI of 2×10^6 cells/mL (Fig. 4B).

To lessen the "cell density effect", a number of strategies ranging from nutrient supplementation schemes [42], total or partial replenishing of medium at infection [40], improving medium composition, fed-batch [43] and perfusion [44] processes have been proposed/implemented. In this work, we replenished key nutrients which were depleted during cell growth (namely glucose, asparagine and glutamine), together with a cocktail of proteins, vitamins and lipids, allowing to increase the peak of cell density (Fig. 5A) and, more importantly, the HA titers (Fig. 6B-C) by performing infections at a CCI of 4×10^6 cells/mL. The results suggest that the metabolic boost induced a greater fitness to infected cells allowing the infection to be extended giving cells more time for protein production before the onset of cell death.

As proof-of-concept, the production of pentavalent H3 VLPs was scaled-up from shake-flasks to 2 L stirred-tank bioreactor. The target CCI (4×10^6 cells/mL) and the consequent increase in oxygen consumption upon infection require high oxygen supply [45]. Although a rapid consumption of glucose was observed during infection this was not accompanied by an increase in lactate concentration (data not shown). These results suggest that careful monitoring and control of oxygen supply in the bioreactor is essential to avoid build-up of toxic compounds. At the end, cell growth and viability profiles obtained in the 2 L bioreactor were very similar to those of shake flask cultures (Fig. 7A). Noteworthy, the HA titers *per* cell obtained in both systems were fairly comparable (Fig. 7B), thus confirming the scalability of the modular strategy herein proposed (incl. the tailor-made refeed supplementation scheme) for production of pentavalent H3 VLPs.

The pentavalent H3 VLPs herein produced and purified were used in a mouse vaccination study to evaluate their performance in inducing an immunological response when compared to respective monovalent VLPs, i.e. VLPs encompassing each of the five H3 HA variants included in the pentavalent VLP (data not shown). Results show that (1) immunization with monovalent VLPs mainly induce strain-specific IgG while immunization with pentavalent VLPs show increased breadth, and (2) ELISA IgG titers obtained with pentavalent VLPs are similar to those obtained with monovalent VLPs. Although these results suggest that VLPs generated contain all HA variants, precise quantitation of VLP components will require additional studies (e.g. isotope dilution mass spectrometry using peptide sequences "unique" to each HA variant included in the VLP).

The application of the modular strategy herein proposed is not limited to multi-HA VLPs. For example, it may play a critical role in alleviating the gene cargo and vector size needed in a single expression system for production of multi-protein complexes such as AAV and triple-layered rotavirus-like particles (processes commonly relying on co-infection schemes). It might be useful when targeting the generation of multi-serotype vaccine-conjugates (e.g. combining antigen targets for viral and bacterial infections), allowing the distribution of antigens (and their number) by each expression system (stable cell line and baculovirus vector). Another potential application is to combine the modular strategy with an inducible system for expression of toxic products (e.g. AAV Rep protein). In fact, it has been shown that induction of a toxic product inserted in the cell genome can be achieved by infection with baculoviruses [46]. In addition, the extra degree of freedom provided by the modular strategy may allow to fine-tune transgene expression, for example, by using different promoters in stable cell lines and in baculovirus vectors. It is widely described that stoichiometric ratios between the different components of the final product (e.g. multi-layered VLPs) are critical to ensure the right morphology as well as its immunological response. Therefore, by being able to combine the strength of different promoters, differential gene expression can be achieved for each gene. Stable cell lines combined with baculovirus infection also give the opportunity to optimize several infection parameters such as the CCI. For instance, a perfusion system could be applied in order to maximize intracellular protein content before infection, theoretically enriching the VLP content in target protein. The examples given demonstrate the high potential of the modular strategy herein proposed for expression of complex biologics.

5. Conclusions

This work demonstrates the suitability of combining stable and baculovirus-mediated expression to produce rapidly and efficiently multi-HA influenza VLPs, thus being a promising strategy to overcome specific downsides of the IC-BEVS platform and an alternative way to deliver complex products to industrial purposes.

As future work, a comprehensive study of stable insect High Five cells metabolism before and upon baculovirus infection will be essential to design refeed strategies capable of further extending cell growth and CCI, potentially inducing higher HA expression. A significant effort will also be dedicated to the development of analytical methods capable of identifying and quantifying each HA protein within the multivalent VLP in order to better characterize the product generated.

Conflicts of interest

None.

Acknowledgements

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