Flexible insect cell platforms for fast production of pseudotyped virus-like particles

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The incorporation of membrane proteins on the surface of virus-like particles (VLPs) from enveloped viruses, such as Influenza virus and Retrovirus, can be a powerful strategy to providing high concentration of membrane proteins. This study demonstrates the development of insect cell platforms using targeted gene integration based on recombinase mediated cassette exchange (RMCE) technology for production of Gag-VLPs displaying target membrane proteins. Sf9-Gag and Hi5- Gag clones with a red reporter protein (iCherry) previous obtained were thoroughly characterized in order to identify those to then co-express from the same locus the model G-Protein coupled receptor (GPCR), Adrb2. A significant increase in the specific Gag secretion rate was obtained from the populations to the isolated clones, demonstrating the value of the cell line development strategy here implemented. To further improve the stable production of recombinant proteins in insect cells, we tested several bioprocess strategies, such as, the adaptation of cells to hypothermic conditions, addition of sodium butyrate (NaBu) or DMSO, and supplementation with key nutrients. The production of Gag protein using lower culture temperature was successful and we determine that Gag production can be enhanced through adaptation of cells to low culture temperature. In addition, supplementation with DMSO, NaBu or different nutrients also have positive impact in protein production. Ongoing work focuses on the combination of these strategies to assess their synergistic effect and contribute to further increase the production of Gag VLPs in insect cells.

Keywords: virus-like particles, membrane proteins, GPCR, insect cells, RMCE systems, cell line development

Introduction

Membrane proteins represent a large part of the open-reading frames of organism's genome and play crucial roles in basic cell functions. More important, these proteins are the target of about 50% of the small drugs in the market [1,2]. Indeed multispanning membrane proteins (MPs), such as ion channels and G-protein coupled receptors (GPCRs), are extremely important therapeutic targets for treating several diseases [3]. These proteins interact with extracellular ligands to produce multiple biological responses, including cell adhesion, signalling and regulatory events. In particular, GPCRs comprise the largest family of membrane receptors in the mammalian genome with approximately 850 members [4]. The study of membrane proteins is challenging because most often they are present in small quantities in the membrane, and they are difficult to recombinantly over-express and purify at desired amounts [5]. In that way, membrane proteins are considered a highly convenient class of proteins for drug discovery as targets for small drug or antibody discover. Nevertheless these proteins rely on their lipid environment and are often found in very small amounts in native tissues making nearly impossible to purify them in the amounts needed [6,7]. Current immunization approaches using peptides, purified membrane proteins, membrane preparations, or whole cells have had limited success in generating conformational antibodies against many membrane proteins [3]. New technologies providing high concentrations of complex multi-spanning and multimeric membrane proteins with their native structure are essential for generating antibodies against these clinically relevant targets. The incorporation of membrane proteins on the surface of virus-like particles (VLPs) from enveloped viruses, such as Influenza virus and Retrovirus, can be a powerful strategy. Virus-like particles (VLPs) are viral proteins that self-assemble into complex structures mimicking the conformation of the native virus, but devoid of viral genetic material [8]. Enveloped VLPs provide a higher degree of flexibility for integration of more antigens from the same or heterologous pathogens and their production requires co-expression of several structural viral proteins and their assembly into particles (budding) from the cell membrane [9]. Enveloped VLPs when used as scaffolds for membrane proteins enable an enrichment of this specific protein concentration in their native structural conformation. For this the target membrane proteins needs to be co-expressed with capsid proteins from enveloped virus leading to the release of virus like particles displaying the membrane proteins correctly folded on their lipidic surface, the viral core protein will trigger VLP budding and release from lipid raft regions of the plasma membrane taking along the anchored target protein. The main structural protein of the Retroviridae virus family, Gag, has been the mostly used to produce VLPs. In a VLP context, it has been shown that Gag assembles even in the absence of any other viral factor in the lipid raft regions of transduced/transfected cell and leads to the budding of VLPs into the culture supernatant [10]. The use of Gag as a carrier has been mainly explored to produce viral antigen-displaying VLPs that have been proposed as alternatives to conventional vaccines [11–13]. Such pseudo-typed VLPs are safer than live-attenuated viral vaccines and elicit robust and broad immune responses as they present target epitopes at the surface in a clustered fashion, efficiently activating antigen presenting cells [12]. Furthermore, pseudo-typed VLPs could be an ideal

platform for high-throughput screening of large libraries of compounds when searching for inhibitors of highly pathogenic viruses (e.g. H5N1 influenza virus), substituting the use of live viruses for obvious biosafety reasons. The baculovirus/insect cell system has been extensively used for VLP production, both non-enveloped and enveloped VLPs. In clinical development the enveloped VLP vaccines produced in insect cells are among the most advanced [10]. However, baculovirus technology has a number of limitations that primarily affect the production of complex membrane proteins: i) the lytic infection process results in the release of host and viral proteases which may impact the quality of recombinant proteins; ii) certain viral proteins may hamper the secretory pathway; iii) repeated rounds of virus amplification tend to produce defective interfering particles with concomitant reduction in recombinant protein yields [14]. The inefficiencies of Baculovirus-insect cell expression system have motived the development of stably transformed lepidopteran insect cell lines for heterologous proteins expression [15]. To circumvent the laborious traditional cell line development process, our group has been developing insect cell lines using targeted gene integration by recombinase-mediated cassette exchange (RMCE) [8,15,16]. This technology encompasses the replacement of a tagging cassette anchored in a given chromosomal *locus* by a target cassette encoding the gene-of-interest, both flanked by the same pair of recombinase recognition target sites. In this way, RMCE allows repeated use of the same *locus* to produce different proteins, thus circumventing extensive clone screening once a good *locus* is tagged [17–19].The developed RMCE-insect cell lines, based on the yeast-derived Flipase (Flp) recombinase, were used to produce single gene products (DsRed, eGFP, rotavirus VP2 protein) with production levels and development timelines similar to those of the baculovirus expression system, but with the advantage of continuous production [8,15,16] The linker between the fused moieties includes one FRT site enabling a posteriori removal of the GFP gene by cassette exchange. Upon antibiotic selection, the tagged populations were sorted for the top GFP expressing cells. The resulting high expressing populations were then subjected to targeted recombination with a second cassette that includes a red reporter protein (iCherry) to replace the GFP gene in the fusion construct in the cells genome. The main purpose of this cassette exchange at population level was to select the subpopulation of cells tagged in loci supporting cassette exchange.

In this work the best clones earlier obtained were selected and characterized for the co-expression of target membrane proteins with the VLP scaffold. To do that, we have a final cassette exchange process with a target cassette which will take out the fluorescence protein fused to the scaffold protein and will express the target membrane protein from the same locus. In parallel, it was performed a study to improve the protein production based on cell growth arrest by hypothermal growth conditions and chemical supplementation (dimethyl sulfoxide - DMSO and sodium butyrate-NaBu). Also media supplementation is usually implemented to increase cell proliferation, the maximum cell density reached and the culture longevity, that in the end results in an increase in product titer[20] .

Materials and methods

Plasmid construction. pTarget ADRB2 Fusion vector was constructed by In-Fusion® HD Cloning Kit (Clontech). ADRB2 gene was taken by PCR from pCDNA_B2AR-i-pep and built in a house plasmid pTarget.

Cell culture maintenance. For suspension cultures, cells were routinely cultured either in 125mL or 500mL shake flasks (working volume of 10-20mL or 30-50mL, respectively) at 27°C in orbital shakers at 100rpm. Sf-900™ II serum-free medium (Gibco) and Insect-Xpress™ (Lonza) were used for Sf9 and Hi5 cultures, respectively. The cell inoculum was $0{,}5x10^6$cells/mL and $0{,}3x10^6$ cells/mL for Sf9 and Hi5 cells, respectively. Cells were sub-cultured every 3-4 days when cell density reached 2-$3x10^6$ cells/mL. Cell concentration and viability were assessed by haemocytometer counting (Brand) using trypan blue exclusion dye (Merck). For adherent cultures, cells were maintained in T-flasks ($75cm^2$) with the respective medium supplemented with 10% (v/v) of serum (Gibco) and sub-cultured when confluency was reached.

Freezing and Thawing cells. Exponentially growing cells (2-$3x10^6$ cells/mL) were centrifuged at 200g, 4°C for 10 min, and cell pellets were ressuspended in cryopreservation media (CryoStor®, Sigma) to obtain a concentration of 1-$2x10^7$ cells/mL. Aliquots were frozen using a freezing container (Mr. Frosty) (Thermo Fisher Scientific) and stored at -80°C until further use. Thawing was performed by centrifuging cells in 12 mL of medium at 200g for 10 min. Cell pellet was re-suspended in medium, according the volume to achieve the cell density desired.

Transfection and Cassette exchange. For site-specific cassette exchange, cell clones were co-transfected with 0,1 µg of *pTarget* ADRB2 fusion and 0,3 µg iFlp-expressing vector using 40µL of Cellfectin® II reagent (Invitrogen) and 500µL of Grace's Insect medium (Gibco), were used $5x10^6$ cells (5 units of transfection UT). Transfections were conducted in 125 mL shake flasks in 10 mL working volume. Selection was performed with zeocine (0,1 mg/mL; Invivogen). When viabilities dropped to bellow 50% cells were transferred to T-flasks ($75cm^2$). The medium with antibiotic was replaced every four days. Fluorescence intensity and cell colonies growth were evaluated by visual inspection (DMI 6000, Leica). When confluent, cells were transferred back to suspension and cultured with the routinely used medium plus antibiotic.

Culture supplementation schemes. Sodium butyrate (NaBu; VWR) and DMSO (Sigma) were added at different concentrations to shake flask cultures with cell densities of $2x10^6$ cells/mL and $5x10^6$ cells/mL. NaBu was added at 0,75 mM; 1 mM; 5 mM and 10 mM whereas DMSO was added at 0,5%, 1% and 2% (v/v). A mixture containing lipids (Chemically Defined Lipid Concentrate; ref. 11905-031) (Gibco) was added to shake flask cultures at inoculation and 96 hours post-inoculation. A set of different nutrients were also added along culture time, the first being 10 mM Serine (Ser) (Sigma) and 1 mM of Cysteine (Cys) (Fluka) at 96 hours post-inoculation, the second 20 mM Glucose (Glc) (Merk) and 2mM Glutamine (Gln) (Sigma) at 144 hours post-inoculation, and the last 15 mM of Glc and 2mM of Gln at 192 hours post-inoculation. All

nutrients were added to the culture in 1mL of the respective insect growth medium.

Adaptive laboratory evolution. The populations Sf9-Gag and Hi5-Gag were subjected to an adaptation process of three months. The culture temperature was decreased from 28/27ºC to 26ºC, 24ºC and finally to 22ºC. For the adaptation to low temperature, Sf9-Gag and Hi5-Gag cells were cultivated in 125 mL shake flasks (10% working volume) at 24ºC or 22ºC in orbital shakers at 100 rpm. The cell inoculum was $1x10^6$cells/mL and $0,6x10^6$ cells/mL for Sf9 and Hi5 cells, respectively. Cell were sub-cultured when cell density reached $2\text{-}3x10^6$cells/mL. The adaptation period finish when the viability reached more than 90% and the duplication time stabilized along the passages. For the experiments carried out at 24ºC and 22ºC, two different inoculums were used. In the case of Sf9 cells an inoculum of $0,5x10^6$ cells/mL and $1x10^6$ cells/mL were used. Alternatively, for Hi5 cells $0,3x10^6$ cells/mL and $0,6x10^6$ cells/mL for Hi5 cells were used.

Flow Cytometry. CyFlow® space (Partec GmbH) was used to evaluate the recombination efficiency. To characterize the stability of the Gag clones in terms of iCherry fluorescence intensity and percentage, CyFlow® space (Partec GmbH) and BD LSR FortessaTM (BD Biosciences) were used. Samples were collected and diluted in PBS (Gibco). Analysis from 30 000 events per sample was done using FlowJo software. To characterize the Gag-Adrb2 clones was used the wavelength of GFP (509 nm) but the objective was to detect a close wavelength corresponding to Citrine with 527 nm. The fluorescence of the clones is related with the fact of this GPCR (Adrb2) have two fluorescent proteins, Citrine and Cerulean to enable FRET quantification when active by agonist.

MicroBCA quantification. Total protein quantification in cell extracts was performed by the bicinchoninic acid assay (BCA) with Micro BCATM Protein Assay Kit (Pierce Biotechnology). The assay was performed according to manufacturer's instruction.

Quantification of Gag-VLPs. Gag-VLP concentrations were determined by p24 ELISA using the commercially available Lenti-X p24 Rapid Titer Kit (Clontech). The assay was performed according to manufacturer's instruction.

Ultracentrifugation. Upon reaching maximum cell density, cell culture was harvest and centrifuged at 200xg for 10 min. Clarified supernatant was layered over 20% sucrose (VWR) in PBS and then centrifuged for 90 min at 28 000 rpm. Supernatant is removed and the pellet ressuspended in 1 mL PBS overnight. VLPs are then re-pelleted through 20% sucrose in PBS and centrifuged for 45 min at 40 000 rpm. The pellet is ressuspended overnight in PBS.

Polyethylene glycol precipitation. Upon reaching maximum cell density, cell culture was harvest and centrifuged at 200xg for 10 min. Clarified supernatant was collected and PEG (8,5%) and NaCl (0,3 M) were added. This mixture was agitated for 1h30 min at 4ºC, and then centrifuged at 4500xg for 30 min at 4ºC. The pellet obtained was ressuspended in PBS.

Centrifugation with Vivaspin® centrifugal concentrator. VLPs can be concentrated with appropriate device size and membrane cut-off. We have used Vivaspin 20 300kDa (Sartorius). A passivation procedure by washing the concentrators filled with mili-Q water and spinning the liquid through by centrifugation. The residual water is removed thoroughly by pipetting carefully without damage the membrane with the pipette tip. After the washing procedure, the concentrators are filled with the blocking solution (Triton X-100 5%) (Sigma) and incubated at room temperature for 2 hours. Then the device is washed again with mili-Q water 3-4x very thoroughly, and finally the VLP samples are concentrated.

For evaluation of Gag-VLPs concentration methods, protein recovery (PR) was calculated according to equation 1:

(Equation 1) $$\mathrm{PR}\ (\%) = \frac{\text{final volume} \times \text{final concentration}}{\text{initial volume} \times \text{initial concentration}} \mathrm{x}100$$

Results

Important steps in the development of a re-used and flexible insect cell factory for the production of pseudo typed enveloped VLPs were undertaken in this thesis. Two major tasks were performed during this thesis: 1) Cell line development; 2) Bioprocess engineering strategies in order to enhance VLP production as well as their concentration and purification.

Cell line development

Characterization of Sf9-Gag and Hi5-Gag clones

The first part of this thesis focuses on the generation of a flexible insect cell platform for production of enveloped VLPs displaying membrane proteins of interest using RMCE technology. The work performed can be divided in two tasks: characterization of Sf9-Gag and Hi5-Gag clones, and Co-expression of Gag and target membrane protein from the same locus. The Gag-iCherry expressing Sf9 and Hi5 clones isolated previously were compared based on several characteristics: (1) cell growth performance, (2) iCherry fluorescence intensity, (3) expression of Gag protein, and (4) stability of iCherry expression along passages. In order to compare cell growth performance, isolated clones were cultured in shake flasks and sampled daily to assess cell concentration until viability dropped below 90% (Figure 1 A-B). The Sf9 clones with higher cell growth rate and peak cell density are clones #11, #12 and #13. In contrast to Sf9-Gag clones, Hi5-Gag clones show growth performances similar to the parental cell line. The Hi5 clones with higher cell growth rate and peak cell density are clones #5m and #1. We took advantage of the Gag-iCherry fusion to compare the clones in terms of protein expression. The cell clones were analysed by flow cytometry and fluorescence microscopy. In principle, the clones expressing higher amounts of iCherry will be those producing higher amounts of the Gag protein. The Figure 2C shows the mean iCherry fluorescence intensity of the clones with highest fluorescence intensity from the two cell hosts analysed by flow cytometry. The Sf9-Gag clones with highest fluorescence intensity are clones #14, #10, #13 and #11 and the Hi5-Gag clones with highest fluorescence intensity are clones #4m, #10, #15 and #5m. Noteworthy, Hi5-Gag clones reach higher mean fluorescence intensity as compared to Sf9-Gag clones. Sf9-Gag clones with highest fluorescence intensity have a single population with exception of Sf9-Gag clone 14. Also, in the case of Hi5-Gag clones #4m and #10 don't have a single population as can be observed by flow cytometry analysis (Figure 2E).

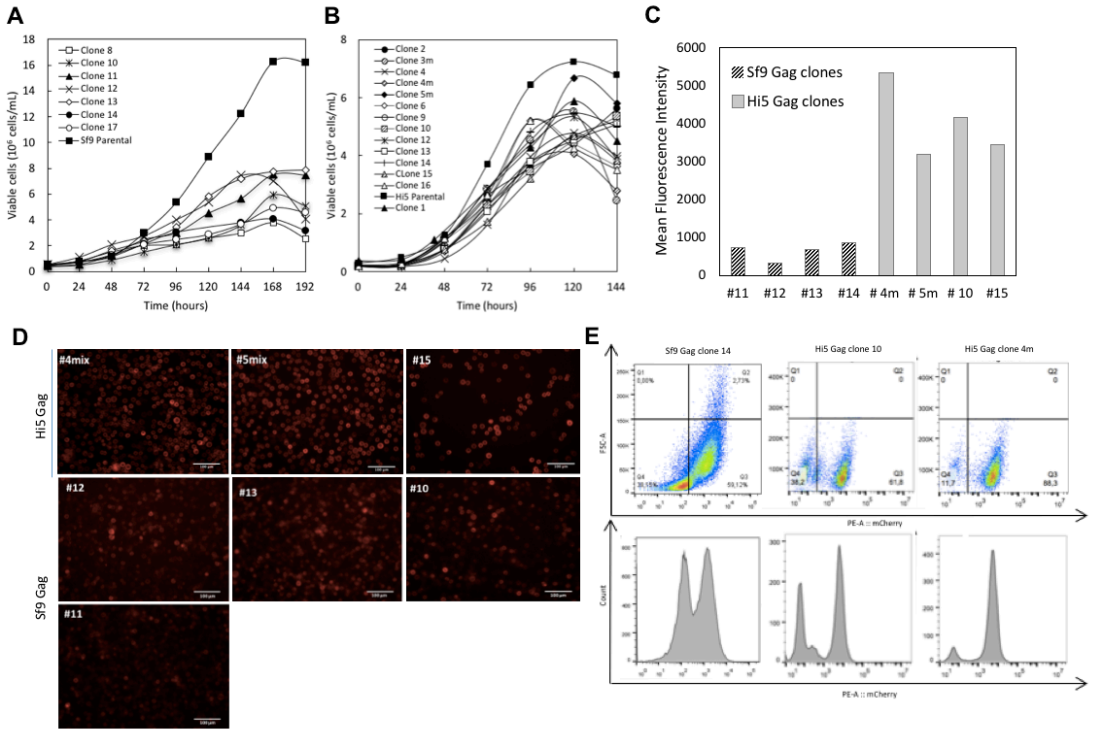

Figure 1 Characterization of Sf9 Gag and Hi5 Gag clones. Growth profiles of (A) parental Sf9 cells and Sf9-Gag clones #8, #10, #11,#12,#13,#14 and #17 along 8 days of culture; (B) parental Hi5 cells and Hi5-Gag clones #1, #2 and #3m, #4, #4m, #5m and #6, #9 ,#10, #12, #13, #14, #15 and #16 along 6 days of culture. (C) Flow cytometry analysis of mean iCherry fluorescence intensity expression 96 h post inoculation (C) Fluorescence microscopy analysis of iCherry expression 96 hours post inoculation (scale bars are in 100µm; all images were obtained with the same parameters (D) Flow cytometry analysis of iCherry expression 96 h post inoculation of Hi5-Gag clones with more mean fluorescence intensity, clones #5m and #10.

Consequently, they are not a good option to use for the final cassette exchange. Hi5 and Sf9 clones were also analysed by fluorescence microscopy (Figures 2 D). The Sf9-Gag clones showing highest fluorescence are clones #12, #13, #10 and #11. Although the Sf9-Gag clone 12 was not within those with higher mean fluorescence by flow cytometry, it seems to be one of strongest by fluorescence microscopy. In agreement with the flow cytometry analysis, the Hi5-Gag clones 4m, 5m and 15 seem to have higher iCherry expression. It is important that the expression of the recombinant protein is stable along passages even after the selective pressure was removed. The clones were kept under G418 selection to eliminate the cells which did not exchange cassettes, i.e. cells that did not turn into red-expressing cells. Once the selection is finished we can test the effect of removing the antibiotic. The stability of iCherry expression along twelve passages (with and without antibiotic) was assessed by flow cytometry for Sf9-Gag clones #11, #12, #13 and Hi5-Gag #1, #5m, #10, #15 clones. After twelve passages without antibiotic the Sf9-Gag clones and Hi5-Gag clones the fluorescence intensity doesn't change along passages with exception of Hi5-Gag clone 10. Hi5-Gag clone 10 had already shown instability of iCherry, but along passages get worse. As a result, Hi5-Gag clone 10 was not considered a candidate for the final cassette exchange. In addition, was observed that there is no impact of antibiotic in stability of the clones. The expression of Gag protein in Sf9-Gag and Hi5-Gag clones was assessed by ELISA (p24 protein quantification) (Table 1).

Table 1 Quantification of p24 protein in the culture supernatant of Sf9 (day 9) and Hi5 (day 5) cell clones.

ID	[p24] (pg/mL)	Specific productivity (pg p24/(10^6cells.h))
Sf9 Gag Clone 11	6680 ± 964	7.3
Sf9 Gag Clone 12	3000 ± 500	3.6
Sf9 Gag Clone 13	3360 ±136	3.0
Hi5 Gag Clone 1	160 ±3	0.2
Hi5 Gag Clone 5m	3950 ± 154	4.7
Hi5 Gag Clone 15	7080 ± 727	15.9

Results show that p24 concentration varies significantly within Sf9-Gag and Hi5-Gag clones. The Sf9-Gag clone 11 secretes over 2-fold higher Gag protein than clones 12 and 13. These results do not correlate directly with the flow cytometry analysis, from which the strongest of the three clones was the clone 13. Regarding the Hi5 clones, the p24 quantification correlates better with the flow cytometry analysis: the clones 5m and 15 are secreting much more Gag protein than clone 1, so this one was not used in the further studies. In terms of specific productivities, the Hi5-Gag clones surpass the Sf9-Gag clones by up to 2,2-fold, since the former reach lower maximum cell densities and have shorter culture times.

Co-expression of Gag and target membrane protein from the same locus

The last step in the cell line development process is to perform RMCE in the Gag-iCherry expressing clones using a cassette encoding the target membrane protein (the GPCR Adrb2), generating cells that produce Gag VLPs decorated with Adrb2 proteins. In this step, the fluorescent protein fusion (iCherry) is removed from the VLP and the Adrb2 is expressed from the same locus. To assess the Adrb2 expression and secretion, we first performed a transient expression assay in which parental Sf9 cells and the Sf9-Gag population were transfected with a plasmid encoding the adrb2 gene controlled by the OpIE2 promoter. Five days after transfection, we analysed the supernatant from both cultures by western blot and observed and increased amount of the receptor in the Sf9-Gag population (Figure 2C), suggesting that Gag increases the secretion of the receptor. We then co-transfected three clones from each cell host - the Sf9 clones 11, 12 and 13 and the Hi5 clones 5m and 15 - with the target cassette encoding the receptor and a plasmid encoding iFlp. Two days post-transfection, zeocin was added in order to initiate the selection process of the cells that had exchanged cassettes. In order to evaluate the cassette exchange and selection process, the clones were analysed by flow cytometry and fluorescence microscopy along time. The fluorescence of the clones is related to the fact that this GPCR is fused to two fluorescent proteins, Citrine (mCir) and Cerulean (mCer). Twelve weeks after targeting, it is possible to see that the cells lost the red fluorescence and are all mCir positive (Figure 2A).

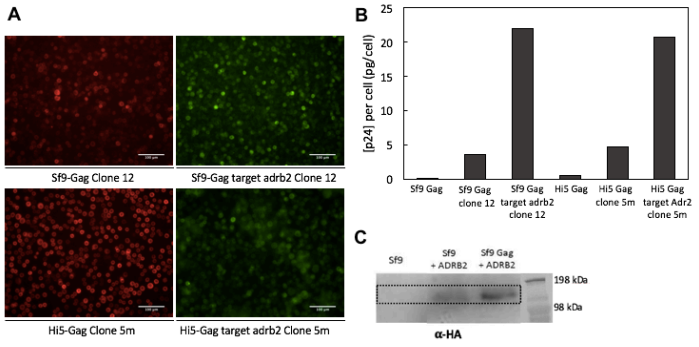

Figure 2 Final target cassette exchange – (A) Fluorescence microscopy images of Sf9 Gag clone 12 and Sf9 Gag target adrb2 clone 12; Hi5 Gag clone 5m and Hi5 Gag target adrb2 clone 5m 12 weeks after targeting; (B) Quantification of p24 protein of the different stages of cassette exchange in order to have the insect cell line specialized in the production of VLPs displaying membrane proteins of interest with Sf9 and Hi5 cell lines by elisa assay (C) Western blot analysis of Sf9-Gag target adrb2 clone #12 comparing with Sf9-Adrb2 and Sf9.

The concentration of p24 protein accumulated in the supernatant at the end of the cultures was also assessed and compared with the amount produced by the Gag-expressing cell pools and by the respective Gag-expressing clones from which they were derived (Figure 2B). In both Sf9 and Hi5 cell hosts, there was a significant increase in the specific and volumetric productivities from the population to the clone stage, confirming the powerfulness of the RMCE-FACS based screening and selection process we propose. In particular, the specific productivity of the Sf9 clone 12 is 36-fold higher than that of the population it was derived, and the specific productivity of the Hi5 clone 5m is 9-fold higher in relation to the Hi5 Gag cell pool. Surprisingly, the subsequent iFlp-mediated cassette exchange at the clone stage, which were transformed into producers of Gag and Adrb2 proteins, further increased the secretion of p24, by 6-fold and 4-fold for Sf9 clone 12 and Hi5 clone 5m, respectively. Similar fold increases were observed for all clones selected for the final target cassette exchange, confirming the platform efficiency.

Bioprocess and product optimization

The second part of this work focuses on the design of bioprocess engineering strategies for optimization of VLPs production.

Evaluating Gag-VLPs concentration methods

Different methods were evaluated regarding their potential to concentrate Gag-VLPs, namely (1) ultracentrifugation, (2) centrifugation with or without using a Vivaspin® centrifugal concentrator and (3) PEG precipitation. The Gag-VLPs used as model were collected at day 5 and day 6 from cultures using the populations Sf9-Gag and Hi5-Gag, respectively. The methods were compared in terms of p24 ELISA assay (Table 2).

Table 2 Quantification of p24 protein of Sf9 Gag and Hi5 Gag with different methods of protein concentration by elisa assay

	ID	p24 (pg/mL)	Total protein (ug/mL)	Protein recovery (%)	Purity (10^{-5} %)
Sf9 Gag	Without concentration	223 ± 12	3383 ± 146	-	1
	PEG precipitation	3063 ± 182	2872 ±122	9%	11
	Centrifugation with Vivaspin® centrifugal concentrator	8127 ± 671	2604 ±635	24%	31
	Ultracentrifugation	2442 ± 273	823 ± 78	7%	30
Hi5 Gag	Without concentration	493 ± 8	1889 ± 279	-	3
	PEG precipitation	3118 ± 487	2689 ± 492	4%	12
	Centrifugation with Vivaspin® centrifugal concentrator	21289 ± 1168	6398 ± 265	29%	33
	Ultracentrifugation	2860 ± 62	1456 ± 72	4%	20

The clarified supernatants were concentrated by 150-fold using the different methods before p24 analysis. The results obtained suggest that the best concentration method is the centrifugation with Vivaspin® centrifugal concentrator, with p24 concentrations much higher than PEG precipitation and ultracentrifugation methods for both cell hosts. Furthermore, comparing the Gag protein to total protein ratios obtained with the different methods, the ultracentrifugation and centrifugation with Vivaspin® centrifugal concentrator methods allowed higher ratios. This means that these methods are more specific to purify the protein of interest.

Adaptive evolution of cells to non-optimal growth temperatures

Aiming at increasing recombinant Gag production, we tested the effect of growing the cells at hypothermic conditions. Culturing cells under non-physiological temperatures has been widely used in different mammalian expression systems with the objective of increasing the protein production [20–23].

To determine whether the beneficial effect of low culture temperature on Gag production can be enhanced through adaptation, we adapted the Hi5-Gag and Sf9-Gag cell populations to low temperature (24ºC and 22ºC).

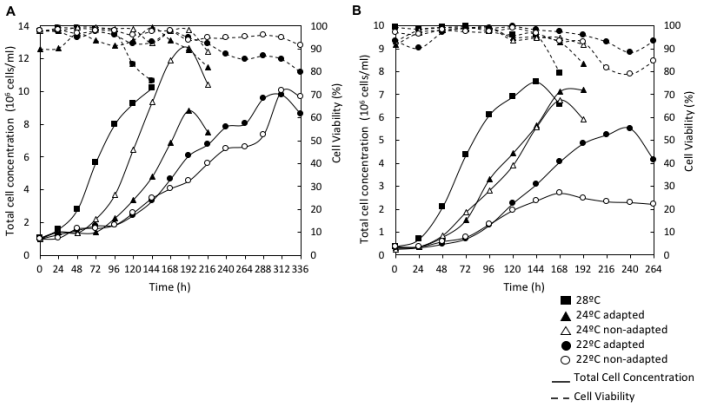

Figure 3 Growth profiles of cells adapted and non-adapted to 24ºC and 22ºC and control at 27ºC (A) Sf9 Gag population with inoculum of $1x10^6$ cells/mL (B) Hi5 Gag population with inoculum of $0,3x10^6$ cells/mL

Shake flask cultures of both populations were sampled daily to assess cell concentration until viability dropped (Figure 3). The adapted cells at 24ºC and 22ºC were compared with non-adapted cells at this temperature and with cells at normal culture temperature (27ºC). Furthermore, we compared two cell inoculums for each cell host in order to identify which one allows higher protein production when the cell lines grow at lower temperatures. The behaviour of the two cell hosts (adapted and non-adapted) was different at lower culture temperature. In atypical culture conditions, Sf9-Gag cells exhibit more susceptibility than Hi5-Gag cells. When using a low inoculum ($0.5x10^6$ cells/mL), Sf9-Gag cells do not growth and the cell viability was lower than 90%. Despite this, Sf9-Gag cells were able to produce more Gag protein than cells non-adapted or cells growing at 27ºC using the same inoculum, meaning that the specific productivity was significantly higher in this condition (Figure 4). However, with a higher inoculum ($1x10^6$ cells/mL), it was possible to have a better growth performance, and the p24 protein titers reached were much higher than that of cells non-adapted or growing at 27ºC. In the case of Hi5-Gag cells, the growth performance was less affected by the lower culture temperature; at a lower inoculum ($0.3x10^6$cells/mL) the cells growth but slower than at a higher inoculum ($0.6x10^6$cells/mL). As this cell growth arrest increases the protein production, there is no need to increase the culture inoculum in Hi5-Gag cells adapted to 22 ºC.

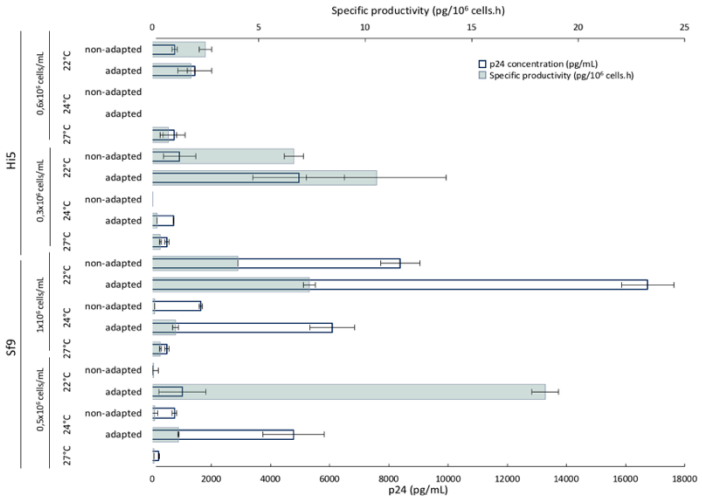

Figure 4 Comparison of Gag protein production in Sf9 Gag and Hi5 Gag at different growth temperatures (27ºC, 24ºC, 22ºC); cell inoculums ($0,5x10^6$ cells/mL and $1x10^6$ cells/mL for Sf9-Gag cells; $0,3x10^6$ cells/mL and $0,6x10^6$ cells/mL for Hi5-Gag cells); and adapted and non-adapted cells.

Comparing adapted cells to non-adapted cells the protein titers and the specific productivities are in general higher in adapted cells, evidencing the importance of the adaptation process of cells to low temperature. In agreement with the different susceptibilities of the two cell hosts to atypical culture conditions, the differences between specific productivities in cells adapted and non-adapted are smaller in Hi5 cells. In addition, Hi5-Gag cells with a higher inoculum ($0.6x10^6$cells/mL) demonstrated lower production comparing to a lower inoculum ($0.3x10^6$cells/mL); cultures starting at higher cell densities are much shorter (96h versus 168h), decreasing the Gag-VLPs accumulated at time of harvest. For both cell hosts, the protein production is increased at 22ºC, comparing with 24ºC and 27ºC. In this atypical culture condition, despite the drop in the specific growth rate, the metabolic activity is reduced, allowing high cell viabilities during longer periods, contributing to increase the accumulation of Gag protein in the supernatant. Still to determine is whether the quality of the Gag-VLPs is affected or improved in these new culture conditions, as the ELISA assay quantifies all p24 protein (free and assembled).

Effect of productivity enhancers on insect cells growth and Gag expression

There are several chemical compounds (such as NaBu and DMSO) that are known to promote the expression of recombinant proteins in mammalian cells. Their adoption for stable expression in Sf9 and Hi5 cells was hardly tested. To evaluate their effect on growth performance and Gag production, shake flask cultures of Hi5-Gag cells were independently supplemented with different concentrations of DMSO and NaBu at different cell concentrations. The cell concentration was followed daily until the viability started to

decrease below 90% and was compared with cultures without supplementation. For DMSO, three concentrations (0.5%, 1%, 2%) were added when a cell concentration of $2x10^6$ cells/mL was reached (Figure 5C). There was a small decrease in the cell growth for 0.5% and 1% of DMSO comparing to control conditions, and the viability was maintained above 90%. However, when 2% of DMSO was used, a significant decrease in the growth rate was observed and the viability started to decrease below 90% sooner and much faster. Because of its cytotoxicity, this DMSO quantity was not used in further studies. We also tested the effect of adding DMSO (0.5% and 1%) at higher cell densities ($5x10^6$cells/mL), which impacted less the integral of viable cells. To study the impact of NaBu on growth and Gag production, we used 4 different concentrations (0.75, 1, 5 and 10 mM), also at two different growth stages ($2x10^6$ cells/ml and $5x10^6$cells/mL) (Figure 5 A-B).

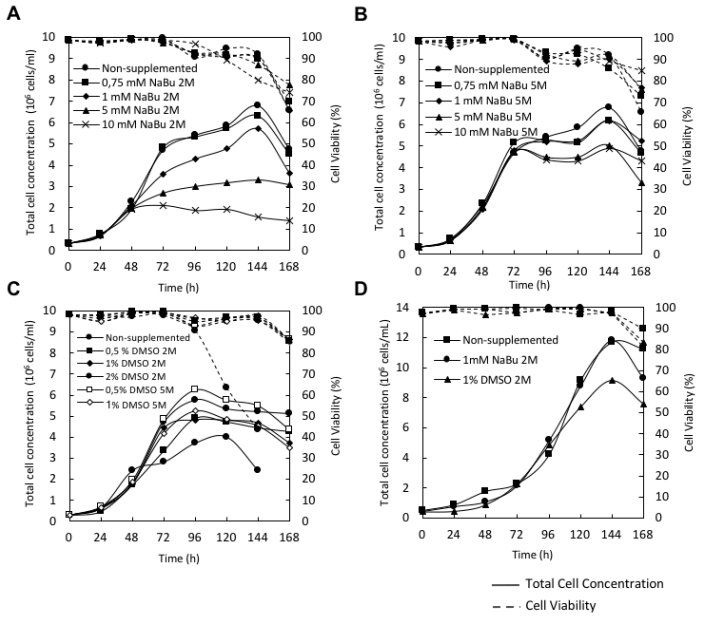

Figure 5 Growth profiles of supplemented and non-supplemented (control) (A) Hi5 Gag with NaBu supplementation of 0,75 mM; 1mM; 5mM and 10 mM when the cells reached the cell densities of $2x10^6$ cells/mL (2M) (B) Hi5 Gag with NaBu supplementation of 0,75 mM; 1mM; 5mM and 10 mM when the cells reached the cell densities of $5x10^6$ cells/mL (5M) (C) Hi5 Gag with DMSO supplementation of 0,5%, 1% and 2% of cell culture volume when the cells reached the cell densities of $2x10^6$ cells/mL (2M) and $5x10^6$ cells/mL (5M) (D) Sf9 Gag supplemented 1% DMSO and 1mM NaBu at cell density of $2x10^6$ cells/mL

When NaBu was added at 5mM and 10mM concentrations the cells could not divide anymore, causing a cell growth arrest. The lowest concentration tested (0.75mM) did not affect the growth performance, while 1mM had a small negative impact when added in the mid-exponential growth phase. The impact of the different concentrations of the compounds on Gag production was assessed at 144 hours post inoculation. All conditions tested allowed higher protein titers and higher specific productivities than the control cultures (Figure 6). DMSO allowed up to 3.5-fold increase in protein titer, while the lowest concentrations of NaBu allowed up to 7.0-fold increase in protein titer. In terms of specific productivity, the highest increase was observed for the culture in which 10 mM NaBu was used (25.4-fold increase in relation to untreated cultures). The DMSO and NaBu conditions allowing higher Gag concentrations in Hi5-Gag cells were applied to Sf9-Gag cell cultures. While the NaBu treatment did not affect the Sf9 growth performance, with the DMSO treatment the maximum cell density reached was lower than non-treated cultures (Figure 5D). Concerning Gag production, both treatments increased significantly the amount of p24 protein in the supernatant (Figure 6), with a slightly higher fold increase achieved with DMSO (7.5-fold versus 6.2-fold).

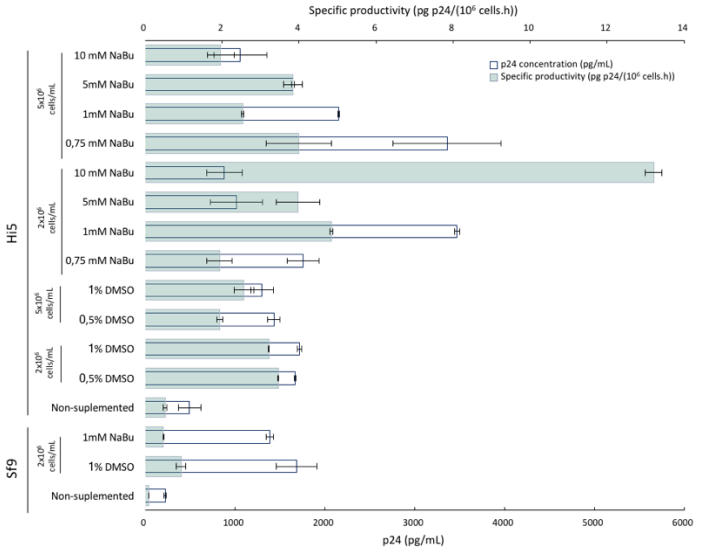

Figure 6 Comparison of Gag protein production in Sf9 Gag and Hi5 Gag supplemented (NaBu, DMSO, nutrients, lipids) and non-supplemented. Supplementation of DMSO (0.5% and 1%), NaBu (0.75 mM; 1mM; 5mM and 10 mM) at different cell densities ($2x10^6$ cells/mL and $5x10^6$ cells/mL). Gag production was evaluated by a p24 ELISA assay.

Effect of productivity enhancers on insect cells growth and Gag expression

The supply of limiting nutrients (e.g., sugars, amino acids, lipids, vitamins) can improve the growth rate of cells, increase the culture longevity and consequently increase the protein production[20,24]. We performed cultures of Sf9-Gag and Hi5-Gag cells in which we replenished essential nutrients that are known to be exhausted during typical batch cultures. Furthermore, as Gag-VLPs bud through the plasma membrane taking along lipidic envelope, we tested if supplementing the cultures with extra lipids could improve culture performance (see in Materials and Methods). Regarding the growth performance, both cell hosts supplemented with nutrients achieved higher cell densities comparing to control cultures ($15x10^6$cells/mL in Sf9-Gag cells and $9x10^6$cells/mL in Hi5-Gag cells) (Figures 7). On the other hand, the supplementation with lipids (1× and 5×) didn't increase the total cell concentration, comparing to non-supplemented cells in both cell hosts.

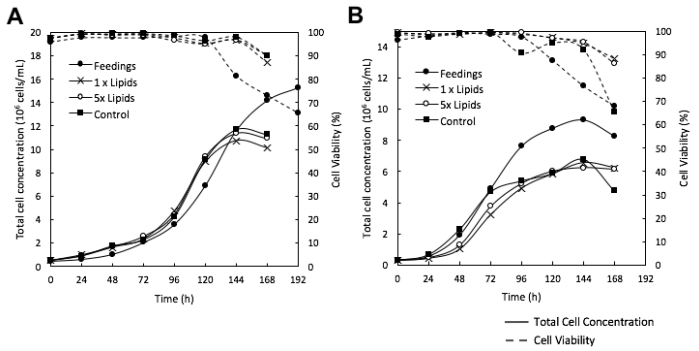

Figure 7 Growth profiles of supplemented and non-supplemented (control) (A) Sf9 Gag supplemented at 96h (10mM Ser and 1mM Cys) and 144h (20mM Glc and 2mM Gln), 1xLipids and 5xLipids (B) Hi5 Gag supplemented at 72h (5mM Gln, 10 mM Asp and 20 mM Glc)., 1xLipids and 5xLipids.

From the ELISA results (Figure 8), the supplementation of essential nutrients allowed higher Gag concentrations than supplementation with lipids for both cell populations. In the case of Hi5-Gag cells, extra lipids (1× or 5×) have almost no increase in Gag production.

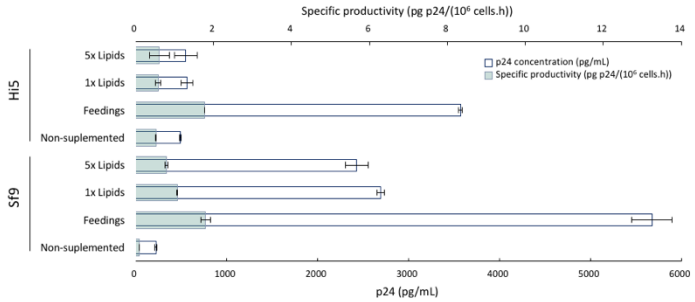

Figure 8 Comparison of Gag protein production in Sf9 Gag and Hi5 Gag supplemented with glucose and specific amino acids, or lipids (see Materials and Methods). Gag production was evaluated by a p24 ELISA assay when the maximum cell concentration was reached in each condition.

Comparing all the tailor-made nutrients and/or chemical supplements, with 10mM NaBu was obtained higher specific productivity but there was a cell growth arrest and the growth performance of the cells was affected. Regarding the Gag concentration obtained in each conditions the feeding with nutrients was the greatest in the both cell hosts.

Discussion

Cell line development

RMCE technology has been widely adopted for production of recombinant proteins in several mammalian cell lines[19,25] and more recently also in insect cells, including contributions from our lab [8,15,16]. The focus of this work was to establish re-usable high producer insect cell lines, relying on the flipase site-specific recombination system, bypassing the need to perform extensive clone screening for every new target membrane protein to be produced at the surface of Gag-VLPs. Indeed, the incorporation of membrane proteins on the surface of VLPs from enveloped viruses, such Retrovirus, can be a powerful strategy for applications such as drug screening or manufacture of vaccines. Our new platform can combine the production of Gag-VLPs with any membrane protein of interest by means of RMCE. The Gag core protein will trigger VLP budding and release from lipid raft regions of the plasma membrane, taking along the anchored target protein, thus providing a native conformation for downstream assays. The use of RMCE with a Gag fused to fluorescent reporter proteins enabled the use of FACS to screen higher producer clones. It has been reported that such fusion proteins result in VLPs which are morphologically indistinguishable from VLPs formed by Gag only [26]. The linker between the fused moieties includes one FRT site enabling the removal of the reporter gene by cassette exchange. Cells tagged in loci supporting high transcription rates and high Flp recombination efficiencies can be pre-selected at population level, a remarkable advantage of this tagging/targeting strategy. In this way, all clones tested supported cassette exchange, which did not happen when using the conventional RMCE cell line development process [15.] Furthermore, the fact that we have an FRT site composing the linker fusing the two genes does not seem to impact the recombination efficiency, as the percentage of cells which exchanged cassettes is similar to those achieved with standard FRT cassettes (unpublished results). After the first cassette exchange at population level, several clones from Gag-iCherry expressing Sf9 and Hi5 cell populations were isolated by cell sorting, which were then subjected to a comprehensive characterization. In both cells hosts, the clones with best growth performance were not necessarily those with high fluorescence intensity or high Gag protein production. Furthermore, some clones show high intracellular fluorescence intensity but low Gag production; this could be because Gag-iCherry may be staying within the cell instead of coming out to the supernatant. In addition, it is important that the clones have stable expression along passages (fluorescence stability), such that the antibiotic can be removed after the selection process. Improved growth performance is normally associated to higher amounts of recombinant protein which accumulate into the supernatant. However, the production of Gag VLPs could have influence in the growth of the cells because the stress related with the disruption of the membrane when they are released [10]. Clones with different morphogenetic characteristic found during the selection and cloning process were found to release VLPs inefficiently [27]. This phenomenon indicates that maintaining similar morphology and growth characteristics with the parental cells may be important in the engineering cell lines with high efficiency and stable exogenous gene expression. To produce Gag-VLPs displaying the Adrb2 receptor, several clones were submitted to RMCE and selected with zeocin to isolate the cells which exchanged cassettes. All clones lost red fluorescence and gained the fluorescence associated to the receptor, demonstrating successful cassette exchange, but the selection process was very long. Consequently, we are still optimizing the

protocol in order to have a faster and efficient recombination and selection process. Of note, a significant increase in Gag production was achieved from both Sf9- and Hi5-Gag tagging populations to the isolated Gag-iCherry clones, demonstrating the efficiency of the RMCE strategy here proposed. When these clones were further submitted to cassette exchange to co-express Gag (without iCherry fusion) and the receptor, an additional increase in Gag production was observed for all clones tested. Although an increase was expected due to the removal of the reporter protein fused to Gag, we were surprised by the observed improvements (up to 6-fold depending on the clone), These results deserve further investigation; for instance, assessing by real time PCR the amount of Gag transcripts before and after the co-expression with adrb2.

Bioprocess Engineering Strategies

In the biotechnological field is important to have highly productive bioprocesses [28]. In the second part of this work, we explored different bioprocess strategies to further improve the recombinant protein titers produced by the developed RMCE insect cell lines, and make them strong alternative platforms to the baculovirus-insect cell system. In addition to protein production improvement, it is also important to establish the optimal concentration method for Gag-VLPs, bearing in mind scalability, costs, protein recovery and purity. Indeed, centrifugation with Vivaspin® centrifugal concentrator yields more protein and enables higher purification levels, although being dependent on costly material. Despite the lower protein recovery obtained with ultracentrifugation as compared to what has been reported [29,30], it enabled reasonable VLP purification yields. This method is dependent on the ressuspension of VLPs, which can be problematic given that those are particularly sensitive particles [31]. However, both methods described above are challenging when higher volumes of culture are processed. Consequently, protein concentration with PEG precipitation would be more suitable for large scale protein production, although the low efficiency.

Hypothermal growth conditions (i.e. culturing cells at temperatures lower than the optimal for growth) or the addition of small chemical compounds have been applied to different expression systems to enhance recombinant protein production [20]. Furthermore, the supplementation of essential nutrients is normally employed to improve cell proliferation and culture longevity as a means to also increase final product concentration [20,32] . For both cell hosts used in this thesis, the hypothermal growth increased the production titers of the Gag protein, with the minimum temperature tested, 22ºC, yielding the largest improvements. In addition, cells adapted to this culture temperature showed a higher fold increase in Gag production than cells non-adapted. The culture temperature at 22ºC allowed up to 34-fold increase in specific productivity in Sf9-Gag cells, while in Hi5-Gag cells allowed up to 10-fold increase in specific productivity, demonstrating that the adaptation process is important to have optimal yields. Culturing *Drosophila melanogaster* derived S2 cells at 22ºC and reducing the temperature of suspension cultures of CHO cells from 37ºC to 31ºC during late exponential growth phase was also described to enhance production of recombinant proteins[33]. Indeed, the growth capabilities of the cells decrease at low temperatures. Also the metabolites are consumed are lower rates, contributing to increase the culture longevity, and consequently, allowing higher protein titers to be accumulated. Nevertheless, in this work we were assessing the total p24 protein accumulated in the supernatant. As future work, it is important to evaluate the Gag-VLPs stability at 22ºC and confirm that hypothermic growth conditions do not have influence in the assembling of the VLPs. This can be performed by using a Vivaspin® centrifugal concentrator to concentrate only Gag-VLPs based on molecular weight, and then analyse p24 protein by ELISA. Also still to be performed is the observation of the produced Gag-VLPs in the different conditions by electron microscopy.

The treatment with the chemicals DMSO and NaBu allowed to obtain higher protein titers when compared to non-treated cultures. It has been shown that DMSO act as a stabilizing agent during protein folding and also have an extensive impact on the transcription rate [20]. For example, the production of rRVGP in S2 cells increased 3.6-fold[33] in the presence of DMSO in relation to non-treated cultures. It has also been demonstrated that DMSO induces an efficient and reversible G1 phase arrest in CHO cells [20]. In our study, the concentrations of DMSO used did not cause cell growth arrest but allowed a 10 and 6-fold increase in specific productivity of p24 in Sf9 and Hi5 Gag cells, respectively. However, the higher concentrations of NaBu used caused growth arrest in Hi5 cells, condition which also allowed the highest increase in the specific productivity (25-fold increase). NaBu is known to cause cell blockage at the G1-phase of the cell cycle, inhibit histone deacetylases, induce cell differentiation and apoptosis[20]. Hyperacetylation of histones through the inhibition of histone deacetylases has been found to up-regulate transcription by opening up nucleosome structures. These modifications would make a recombinant gene more accessible to the transcription machinery, thus increasing its transcription rate [23].

Fed-batch is a common strategy used for industrial manufacture of recombinant therapeutics in animal cell cultures [32]. The objective of fed-batch strategy is to increase final product concentration which is possible by extending culture duration and/or by increasing peak viable cell concentration by using feeding strategies based on the consumption rates of nutrients [20]. Supplementing Sf9-Gag and Hi5-Gag cell culture with glucose and amino acids enabled to reach higher cell concentrations and extend culture duration, which positively impacted the

production titer of p24 leading to 20 and 3-fold increase in specific productivity.

Conclusion and future work

In this study, we developed insect cell platforms for production of enveloped VLPs displaying membrane proteins of interest. The baculovirus-insect cell expression system is commonly used for recombinant protein production, especially for difficult-to-express proteins such as receptors and other membrane proteins. Nevertheless, stable cell lines are advantageous over transient expression, due to potential to scale-up and improvement of the production by process optimization. Also, stable insect cell lines don't have the negative effect on protein processing pathways caused by the lytic baculovirus infection cycle, sometimes critical for the protein quality. Our system is particularly beneficial for proteins expressed in lower quantities as membrane proteins, because of the ability to secrete them in their own native environment without the need to used chemical or physical methodologies for extraction that in the end could interfere with their own conformation and functionality. Combining RMCE and FACS we could identify cell clones which produce much more Gag than the populations from which they were derived, confirming the potential of our approach. In parallel, by using three independent strategies we could improve the Gag titers produced by the populations. The next step would be to combine these strategies to evaluate the synergistic effect of supplementing cells adapted to low culture temperature with key nutrients, and in the presence of DMSO/NaBu. Furthermore, it is important to assess the impact of such strategies on the protein titers produced by the final clones. As proof-of-concept, it is important to perform lab scale bioreactors with the purpose of assess the scalability of the strategy.

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