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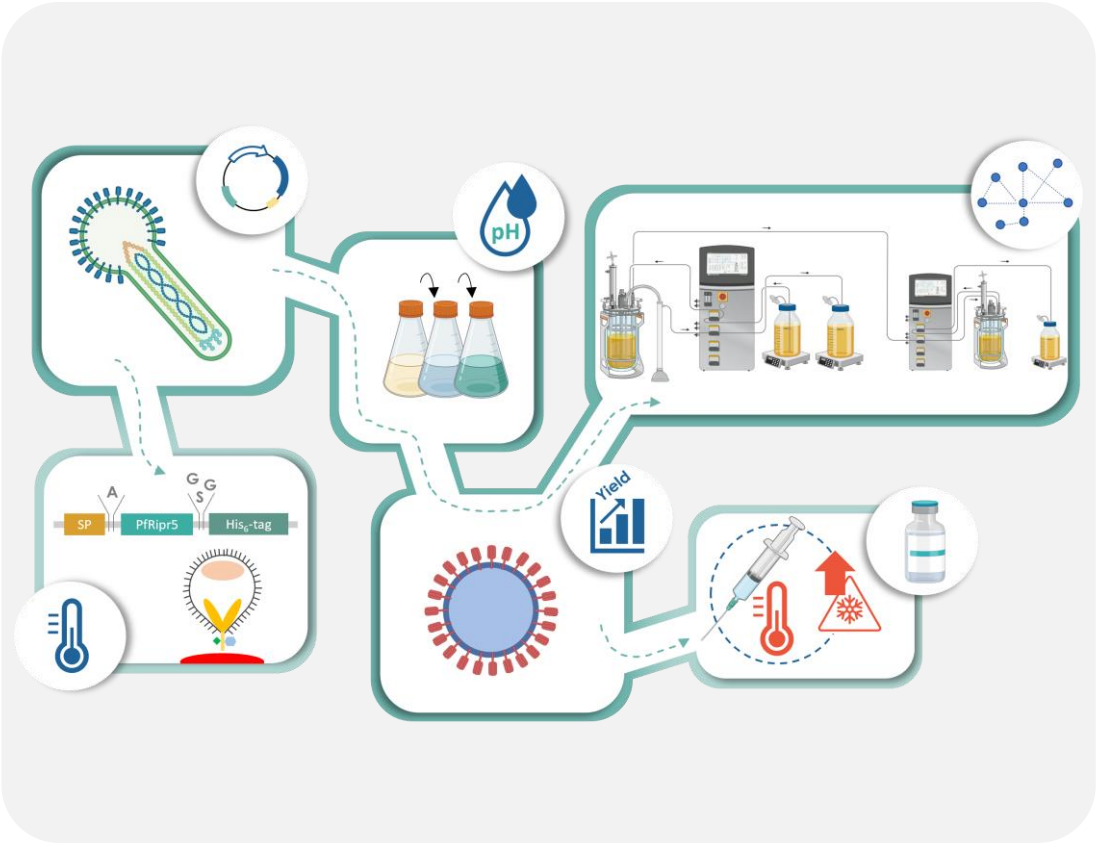
Atypical culture conditions and process intensification to enhance vaccine production using insect cells

Ricardo Correia



Dissertation presented to obtain the **Ph.D degree in Bioengineering**

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Oeiras, April, 2022

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The work developed in this thesis was supervised by:

- **Doctor António Roldão**, Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa (ITQB NOVA) e Instituto de Biologia Experimental e Tecnológica (iBET)
- **Professor Manuel Carrondo**, Instituto de Biologia Experimental e Tecnológica (iBET)

From left to right: Dr. Colin McVey, Dr. Ana Teixeira, Dr. Ricardo Correia, Dr. António Roldão, Dr. Felipe Tapia. Not present in this photo: Dr. Mark Smales, Dr. Dominic Esposito.

Supervisors

- **Doctor António Roldão**, PhD, Head of Cell-based Vaccine Development Lab, Animal Cell Technology Unit (ACT Unit), Instituto de Biologia Experimental e Tecnológica (iBET) and Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa
- **Professor Manuel Carrondo**, PhD, Vice President of iBET and Head of Engineering Cellular Applications & Systems Biology Lab at ACT Unit, iBET

President of the Jury

- **Doctor Colin Edward McVey**, Principal Investigator at Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa

Jury

- **Felipe Tapia**, PhD, Bioprocess Engineering Group at Max Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany, and co-founder at ContiVir Spinoff
- **Mark Smales**, PhD, Professor of Industrial Biotechnology and Director of Industrial Biotechnology Centre, School of Biosciences, University of Kent, Canterbury, Kent, England, UK.
- **Ana Teixeira**, PhD, Senior Scientist at ETH Zurich, Dep. of Biosystems Science and Eng., Basel, Switzerland
- **Dominic Esposito**, PhD, Laboratory Director at Frederick National Laboratory for Cancer Research (FNLCR), Frederick, Maryland, United Stated of America

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Universidade Nova de Lisboa

Do. Or do not. There is no "try"

- *Yoda*

(Star Wars Episode V: The Empire Strikes Back)

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Abstract

Prophylactic vaccination still remains the most efficient measure of counteracting the spread of infectious diseases and attenuating their symptoms. Important advancements have been made over the years to improve the load of vaccines manufactured. Nevertheless, the emergence of novel viruses and virus strains demands the continued development of improved platforms of vaccine production, purification, and storage.

In this thesis, engineering approaches were developed to improve the production and storage of two vaccine candidates, an influenza vaccine candidate based on virus-like particles (VLPs), and a blood-stage malaria vaccine candidate based on the PfRipr5 antigen.

In Chapter 1, the state of the art on existing bioprocess engineering strategies to improve vaccine manufacturing at the different process stages (from the design of the expression vector to the storage of the final purified product) is presented. Whenever possible, this chapter focus on strategies using insect cells (IC) as expression host and the baculovirus expression vector system (BEVS), as this is the expression system employed to produce the vaccine candidates throughout this dissertation.

The use of atypical culture conditions (i.e. non-standard values of culture parameters such as pH, temperature, osmolarity, DO) has been explored to a limited degree to improve productivity of insect cells, with evolutionary engineering being even less investigated. In Chapter 2, insect High Five cells were adapted to grow at neutral pH via adaptive laboratory evolution. These adapted cells allowed to improve influenza hemagglutinin (HA)-VLPs expression by up to 3-fold (specific production rate) and 4-fold (volumetric titer), thus showing potential in improving vaccine production capacity.

Maintaining the vaccine cold chain (i.e. guarantee refrigerated conditions throughout storage and distribution chains) is costly and one of the main reasons for vaccine wastage. In Chapter 3, the storage of influenza HA-VLPs in a trehalose-glycerol natural-deep eutectic solvent (NADES) system was investigated. Formulation of HA-VLPs in this NADES reduced the antigen (HA) degradation rate and improved particle's physical integrity upon storage at 50 °C. At room temperature, HA-VLPs were stable for one month, demonstrating the potential of NADES for storage under non-refrigerated conditions. The storage of vaccines under non-refrigerated conditions provided by the use of water-free formulations such as NADES could significantly alleviate cold-chain constraints, which can be critical in developing countries.

The use of atypical culture conditions to improve productivity in insect cells was further investigated in Chapter 4. A shift in culture temperature from the standard 27 °C to 22 °C was shown efficient in improving production of the malaria's blood-stage antigen protein PfRipr5 by 1.7-fold. The importance of optimizing the expression vector was demonstrated, with the design of new recombinant baculovirus resulting in 2.2-fold improvement. Noteworthy, combination of both strategies improved production yield by over 5-fold, showing that thorough selection of cell line, culture parameters, and expression vector is key to optimize production processes in insect cells.

Traditional setups of vaccine manufacturing are based on batch or fed-batch approaches. However, novel methods of intensifying the process through perfusion at high cell density or continuous operation modes have arisen as alternative to reduce equipment footprint, the need of upscaling, and batch-to-batch variability. The implementation of continuous processes for virus/viral particles production is often difficulted by the lytic nature of virus infection, which may explain why IC-BEVS processes using this operation mode

have been in the shadows of process development for almost two decades. In Chapter 5, the neutral-pH insect High Five adapted cells (from Task 2) were used in a continuous multi-stage bioreactor scheme producing influenza HA-VLPs via IC-BEVS. Virus passage effect was evident and reflected on the decrease of baculovirus infectivity, specific loss of the genes of interest, and inability to maintain productivity of HA-VLPs for prolonged periods. Despite not representing a fully optimized process, this assessment allowed to draw conclusions on some of the main factors playing a role in process success, such as the impact of different residence time in the production bioreactor.

In Chapter 6, the findings of the previous chapters are summarized and discussed. Special emphasis is given to the associated benefits and implications of using non-standard culture conditions, to the challenging task of implementing the continuous production process that is herein attempted, and the expectation of NADES as solution for vaccine storage.

In summary, this thesis demonstrates methods of innovating production and storage of vaccine candidates, evidencing the potential of using non-standard culture conditions as a simple and reliable approach to improve titers in insect cells. It also culminated in the implementation of the first steps to renovate the process of continuous production using IC-BEVS. Altogether, the bioprocess strategies herein devised contribute to advance in the field of vaccines produced in insect cells by targeting different stages of the manufacturing process.

Resumo

Vacinação profilática é ainda a prática mais eficiente para contrariar propagação de doenças infeciosas e atenuar os seus sintomas. Avanços importantes têm sido feitos ao longo dos anos para melhorar a carga de vacinas produzidas. No entanto, o surgimento de novos vírus ou estirpes de vírus exige o desenvolvimento contínuo de melhores plataformas de produção, purificação, e armazenamento de vacinas.

Nesta tese, abordagens de engenharia foram desenvolvidas para melhorar a produção e armazenamento de dois candidatos a vacinas, uma vacina contra influenza baseada em partículas semelhantes a vírus (VLPs), e uma vacina contra malária baseada no antigénio de estágio sanguíneo PfRipr5.

No Capítulo 1, o estado da arte relativo às estratégias de engenharia de bioprocesso existentes para melhorar fabricação de vacinas em diferentes estágios do processo (desde o design do vetor de expressão até ao armazenamento do produto final purificado) é apresentado. Sempre que possível, esta capítulo foca em estratégias usando células de inseto (IC) e o sistema de expressão de vetor de baculovírus (BEVS), sendo este o sistema de expressão implementado para produção dos candidatos a vacina no âmbito desta dissertação.

O uso de condições de cultura atípicas (ou seja, valores não padrões de parâmetros de cultura como pH, temperatura, osmolaridade, oxigénio dissolvido) tem sido explorado de forma limitada para melhorar produtividade em células de inseto, sendo que métodos de engenharia evolucionária têm sido ainda menos investigados. No capítulo 2, células de inseto High Five foram adaptadas a crescer a pH neutro através de evolução laboratorial adaptativa. Estas células adaptadas permitiram aumentar expressão de VLPs de influenza

dispondo hemaglutinina (HA) até 3 vezes (produtividade específica) ou 4 vezes (título volumétrico), demonstrando assim potencial para aumentar a capacidade de produção de vacinas.

Manter a cadeia de frio de vacinas (ou seja, garantir condições refrigeradas durante o armazenamento e distribuição) é dispendioso e uma das principais razões para desperdício de vacinas. No Capíltulo 3, o armazenamento de HA-VLPs de influenza num solvente eutéctico (NADES) composto por trehalose e glicerol foi investigado. A formulação de HA-VLPs neste NADES reduziu a taxa de degradação do antigénio (HA) e melhorar a integridade física das partículas durante armazenamento a 50 °C. à temperatura ambiente, os HA-VLPs mantiveram-se estáveis durante um mês, demonstrando o potencial dos NADES para armazenamento em condições não refrigeradas. O armazenamento de vacinas em condições não refrigeradas, providenciado pelo uso de formulações sem água como NADES, poderia aliviar significativamente os encargos da cadeia de frio, o que pode ser crítico em países em desenvolvimento.

O uso de condições atípicas de cultura para melhorar produtividade em células de inseto foi novamente investigada no Capítulo 4. Uma mudança da temperatura de cultura de 27 °C para 22 °C permitiu melhorar 1.7 vezes a produção de proteína antigénio de malária de estágio sanguíneo PfRipr5. A importância de otimizar o vetor de expressão foi demonstrada, com o desenho de novos baculovírus a resultar numa melhoria de 2.2 vezes. É digno de nota que a combinação de ambas as estratégias permitiu melhorar o rendimento da produção em mais de 5 vezes, demonstrando que a seleção cuidada da linha celular, dos parâmetros de cultura, e do vetor de expressão, é essencial para otimizar processo de produção em células de inseto.

Os esquemas tradicionais de produção de vacinas baseiam-se em abordagens por lotes ou lotes alimentados. No entanto, novos métodos de intensificação de processo através de perfusão a alta densidade celular ou operação contínua têm surgido como alternativa para reduzir a pegada do equipamento, a necessidade de aumentar escala, e a variabilidade de lote para lote. A implementação de processes contínuos para produção de vírus e partículas virais é geralmente dificultada pela natureza lítica da infeção viral e, o que poderá explicar porque processos IC-BEVS usando este modo de operação têm estado na sombra do desenvolvimento de processo durante quase duas décadas. No Capítulo 5, células de inseto adaptadas a pH neutro (do Capítulo 2) foram usadas num processo contínuo de bioreatores multi-estágio para produção de HA-VLPs de influenza usando IC-BEVS. O efeito de passagem de vírus foi evidente e refletiu-se na diminuição de infecciosidade de baculovírus, na perda específica dos genes de interesse, e na incapacidade em manter produtividade de HA-VLPs durante períodos prolongados. Apesar de não representar um processo totalmente otimizado, este ensaio permitiu retirar conclusões sobre alguns dos principais fatores desempenhando um papel no sucesso do processo, como por exemplo o impacto de diferentes tempos de residência no bioreator de produção.

No Capítulo 6, as descobertas dos capítulos anteriores são sumarizadas e discutidas. Uma maior ênfase é dada aos benefícios e implicações do uso de condições de cultura padrão, à tarefa desafiante de implementar o processo contínuo de produção que aqui foi tentado, e à expetativa de usar NADES como solução para armazenamento de vacinas.

Em resumo, esta tese demonstra métodos alternativos de inovar a produção e armazenamento de candidatos a vacinas, evidenciando o potencial de usar condições de cultura não standard como abordagem simples e fiável para

melhorar produção em células de inseto. Também culminou na implementação dos primeiros passos para renovar o processo de produção contínua utilizando IC-BEVS. No seu conjunto, as estratégias de bioprocesso aqui concebidas contribuem para o avanço do ramo de produção de vacinas em células de inseto, ao visarem diferentes fases do processo de fabrico.

Thesis Publications

Correia, R., Fernandes, B., Alves, P.M., Carrondo, M.J.T., Roldão, A., 2020. Improving Influenza HA-Vlps Production in Insect High Five Cells via Adaptive Laboratory Evolution. Vaccines 8, 589.

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Correia, R., Meneses, L., Richheimer, C., Alves, P.M., Carrondo, M.J.T., Duarte, A.R.C., Paiva, A., Roldão, A., 2021. Improved storage of influenza HA-VLPs using a trehalose-glycerol natural deep eutectic solvent system. Vaccine 39, 3279–3286.

https://doi.org/10.1016/j.vaccine.2021.04.033

Correia, R., Fernandes, B., Castro, R., Nagaoka, H., Takashima, E., Tsuboi, T., Fukushima, A., Viebig, N.K., Depraetere, H., Alves, P.M., Roldão, A. (2022). Asexual blood-stage malaria vaccine candidate PfRipr5: enhanced production in insect cells.

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Additional Publications

Sequeira, D.P., **Correia, R.,** Carrondo, M.J.T., Roldão, A., Teixeira, A.P., Alves, P.M., 2018. Combining stable insect cell lines with baculovirus-mediated expression for multi-HA influenza VLP production. Vaccine 36, 3112–3123.

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Fernandes, B., Vidigal, J., **Correia, R.,** Carrondo, M.J.T., Alves, P.M., Teixeira, A.P., Roldão, A., 2020. Adaptive laboratory evolution of stable insect cell lines for improved HIV-Gag VLPs production. Journal of Biotechnology 307, 139–147.

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Fernandes, B., **Correia, R.,** Sousa, M., Carrondo, M.J.T., Alves, P.M., Roldão, A., 2021. Integrating high cell density cultures with adapted laboratory evolution for improved Gag-HA virus-like particles production in stable insect cell lines. Biotechnology and Bioengineering 118, 2536–2547.

https://doi.org/10.1002/bit.27766

Fernandes, B., **Correia, R.,** Carrondo, M.J.T., Alves, P.M., Roldão, A., 2022. Intensified production of Gag-HA virus-like particles in stable insect cells by lowering CSPR in high cell density continuous culture.

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List of Abbreviations

AcMNPV: *Autographa californica* multiple nucleopolyhedrovirus

ALE: adaptive laboratory evolution

ATF: alternating tangential flow

BVM: bee venom melittin

CCI: cell concentration at infection

CDE: cell density effect

DIPs: defective interfering particles

DLS: dynamic light scattering

DO: dissolved oxygen

ELISA: enzyme-linked immunosorbent assay

ER: endoplasmic reticulum

GIA: growth inhibition assay

GOI: Gene of interest

HA: influenza hemagglutinin protein

HCD: high cell density

HF: hollow fibers

IC-BEVS: Insect cell – baculovirus expression vector system

IMAC: immobilized metal affinity chromatography

ITCC: integral of total cell concentration

M1: influenza matrix protein

mAb: monoclonal antibody

MOI: multiplicity of infection

MVS: master virus stock

NADES: natural deep eutectic solvent

NTA: nanoparticle tracking analysis

PEI: polyethylenimine

PfRipr: *Plasmodium falciparum* merozoite Rh5 interacting protein

PTMs: post-translational modifications

rBAC: recombinant baculovirus

RT: residence time

SEC: size exclusion chromatography

SF: shake-flask

SP: signal peptide

SPR: surface plasmon resonance

SRID: single radial immunodiffusion

STB: stirred-tank bioreactor

TEM: transmission electron microscopy

TFF: tangential flow filtration

TGly: trehalose-glycerol NADES system

TOH: Time-of-harvest

TOI: Time-of-infection

VCC: viable cell concentration

VLPs: virus-like particles

WGCFS: wheat-germ cell free system

Chapter 1

Introduction

Author contribution

Ricardo Correia wrote the chapter based on the referred bibliography.

TABLE OF CONTENTS

1. Vaccination

The concept of vaccination dates from centuries ago, when civilizations worldwide began to understand that immunization from previous virus infection would confer protection against re-infection. Edward Jenner's breakthrough immunization study performed in 1796, using cowpox (vaccinia) to generate immune protection against human smallpox infection, paved the way to the use of vaccination as efficient method of disease control and eradication. This would turn out to be not only the first reported case of a modern-day-like consensual and broadly used vaccination method, but also the emergence of the idea that a similar but milder disease-causing virus could be used to circumvent infection with a more deadly pathogen. Since then, scientific and technological advancements have resulted in the development and broad use of vaccines against different infectious diseases, including measles, polio, hepatitis A/B, influenza and, most recently, COVID-19.

In addition to eradicating smallpox and almost eliminating polio, national vaccination programs allow to spare over 2 million lives every year and have almost extinguished reports of the major childhood death-causing diseases in developed countries (Delany et al., 2014; Pollard and Bijker, 2021). Besides conferring protection to those inoculated, intensive vaccination programs can induce indirect protection even to those unvaccinated through herd immunity (Anderson, 1992), although this may often be challenging and require a large percentage of the population to be vaccinated (Anderson et al., 2020), and through vaccination during pregnancy (Swamy and Heine, 2015).

Given the long (over 10 years) traditional process of vaccine development (Pronker et al., 2013), from conceptualization to commercialization, novel vaccine technologies, expression systems, and production processes allowing rapid reaction and adaptation to respond to market and healthcare demands

are utterly needed. Such technological flexibility has recently allowed to respond to global urgency with a record-time development of vaccines against COVID-19 (Jeyanathan et al., 2020; Tregoning et al., 2021). Nevertheless, continued scientific research is needed to develop novel expression systems, production setups and vaccine technologies with capacity to guarantee high vaccine quality and production load in response to unpredictable infectious diseases outbreaks.

1.1. Vaccine modalities

Vaccines vary greatly on e.g. their mode of action, biological material contained, and valency. The ideal vaccine type depends on different factors including the level of protection desired, the type of disease being targeted, and the characteristics of the subject (Vetter et al., 2018). A combinatorial treatment with different vaccine modalities may allow to develop stronger immune responses, including against difficult-to-target infectious diseases such as HIV (Hallengärd et al., 2012). Overall, vaccines can be divided in whole pathogen (live attenuated, inactivated), subunit (antigenic proteins, toxoids, virus-like-particles, conjugate), nucleic acid (DNA, RNA) and viral vectored vaccines.

1.1.1. Whole pathogen vaccines

Whole pathogen-containing vaccines are the most well-known modality dating back to Jenner's study, but these have not always been the safest option for posing risk of actual infection. This raises the importance of Pasteur's discovery of live attenuated vaccines (1881), which comprise a weakened version of the pathogen. By performing consecutive passages in non-human cells, a strain with lower to no capacity to infect human cells but allowing the same degree

of antigen display is obtained (Hajj Hussein et al., 2015). Despite being highly immunogenic, attenuated pathogens still pose risk of infection and are contraindicated for immunocompromised individuals. To tackle this, pathogens can be inactivated via heat or chemical treatment. Despite being safer, inactivated vaccines are usually less immunogenic, requiring multi-dosing to boost immune protection (Cid and Bolívar, 2021). Live-attenuated and inactivated technologies are still the most widely used in human vaccination (Francis, 2018).

1.1.2. Nucleic acid vaccines

Rather than relying on antigen presentation, nucleic acid-based vaccines deliver the genetic information coding for the target antigen to host cells. As a result, the foreign antigen is produced by the cell's machinery which consequently triggers an immune response. This type of technology focusing on the delivery of genetic material allows to build immunity specifically against the most immunogenic antigens commonly presented by the wild type pathogen. Nucleic acid vaccines can be composed of RNA (as messenger RNA) or DNA (as plasmids) (Qin et al., 2021) and were the basis for the development of the first approved COVID-19 vaccines (Pfizer–BioNTech and Moderna) (Tregoning et al., 2021).

1.1.3. Viral vectored vaccines

Viral vectored vaccines work by delivering genetic material, coding for the target antigen, incorporated into a harmless carrier virus (vector). Adenoviruses are the most widely used as they offer high genetic stability and

efficiency of infection and transgene expression (Robert-Guroff, 2007). The delivered genetic information will serve as backbone to produce the target antigens by the host cells, consequently triggering immune response. Together with nucleic acid vaccines, this is one of the newer vaccine technologies to be marketed, being employed in two Ebola vaccines (Matz et al., 2019) and four COVID-19 vaccines (Tregoning et al., 2021).

1.1.4. Subunit vaccines

Subunit vaccines are composed of parts of the pathogen rather than the whole organism, therefore being safer than whole pathogen vaccines, and smartly engineered to contain only the essential biological material to build up a strong immune response upon vaccination. These include antigen proteins (natural or recombinant) (e.g. hepatitis B vaccine which comprises the hepatitis B surface antigen (Zhao et al., 2020)), toxoids (detoxified toxins) (Vetter et al., 2018) (e.g. tetanus vaccine), conjugate (Rappuoli et al., 2019) (e.g. haemophilus b vaccine), or viral proteins that self-assembly into virus-like particles (VLPs) (Roldão et al., 2010) (e.g. human papilloma virus vaccine). VLPs mimic the structure of the native viruses allowing for a similar degree of antigen display in organized arrays, while lacking viral genomic material thus not being able to infect host cells (Nooraei et al., 2021). As subunit vaccines usually generate lower immunogenicity than traditional vaccines, VLPs are especially promising due to their high level of antigen display (Tan and Jiang, 2017), potentially conferring broader protection than vaccination with whole viruses (Bright et al., 2007). Structural proteins from a model virus can serve as scaffold to generate chimeric VLPs displaying antigens from a variety of other pathogens on their surface (Liu et al., 2011). A good example is the malaria vaccine RTS,S/AS01 Mosquirix® (GSK), which consists of hepatitis B surface antigen VLPs

displaying the *Plasmodium falciparum* circumsporozoite protein (Casares et al., 2010). Additionally, antigens from different strains can be combined in the surface of the same particles yielding multivalent VLPs to confer broader protection (Kang et al., 2021).

2. Cell culture-based vaccine production

With re-emergence of new virus strains and appearance of unprecedented organisms, vaccine demand tends to keep on increasing. Nonetheless, old techniques of vaccine production such as the egg-based are still amongst the most widely used to produce hundreds of millions of vaccine shots every year (Zahoor et al., 2016). This platform relies on the supply of high-quality eggs which limits significantly vaccine production load, reducing capacity of response to epidemics and pandemics. Importantly, egg-produced vaccines may have reduced efficacy due to antigen alterations (Yang et al., 2019) from adaptation of strains to replicate in eggs.

Contrastingly, cell culture-based vaccine production enables to significantly increase the production capacity through theoretically unlimited *in vitro* cell propagation, while allowing for superior process control and reproducibility (less batch-to-batch variability). Despite offering higher flexibility, there is no ideal cell line to produce all types of vaccines (Zahoor et al., 2016).

2.1. Different hosts to produce vaccines

Continuous cell lines from different origins have been used to produce licensed vaccines and other candidates that are currently at different stages of development.

The use of mammalian cells for the production of recombinant protein vaccines can be costly and yield low expression levels (Cid and Bolívar, 2021). Nevertheless, a herpes zoster vaccine based on varicella zoster virus glycoprotein E and produced in the CHO cell line has been approved for human use (Shingrix®, GSK) (James et al., 2018). The capacity of mammalian cells to promote efficient protein folding and other post-translational modifications (PTMs) of higher complexity (Dumont et al., 2016) ensures a proper biological function to a higher variety of complex biologics being produced. Mammalian cells are natural hosts of many viruses infecting humans and therefore are suited to produce virus-based vaccines for human use. Different cells lines have been employed to produce commercially available virus-containing vaccines targeting many diseases, including vaccinia-smallpox (ACAM2000®, Sanofi Pasteur, produced in Vero cells), rotavirus (e.g. RotaTeq®, Merck & Co., produced in Vero cells) and influenza (e.g. Flucelvax®, Seqirus, produced in MDCK cells).

Mammalian cells may not be the preferred choice for production of all cell culture-based vaccines. For instance, avian cell lines may be the ideal choice to produce avian strains of influenza virus (Babar et al., 2013). Also, yeast and insect cells are the most extensively used platforms for production of recombinant protein-based vaccines approved for human use, being also the preferred systems for VLPs manufacturing (Cid and Bolívar, 2021). Examples of VLP-based vaccines commercialized for human use and produced in such systems include two vaccines against human papillomavirus (Gardasil®/Gardasil-9®, Merck & Co., produced in yeast, and Cervarix®, GSK, produced in insect cells) and the abovementioned Mosquirix® malaria vaccine (GSK, yeast). The bacteria (mostly *E. coli*) was the first expression system to be established for production of recombinant protein, however it lacks the

capacity to promote eukaryotic protein PTMs often resulting in misfolded or nonfunctional proteins (and therefore not immunogenic). Such disadvantage impaired the use of this system to produce the first recombinant vaccine approved for human use (the hepatitis B vaccine using hepatitis B surface antigen proteins self-assembling into VLPs) which was later developed using yeast cells (Recombivax HB®, Merck & Co.) (Cid and Bolívar, 2021). Cell lines from hosts such as insects or plants are also able to promote the mammalian-like protein PTMs and ensure the expected biological activity of a certain antigen (Fischer et al., 2004; Fung and Liu, 2018).

2.2. Production of vaccines using insect cells and BEVS

Insect cells are a powerful host for recombinant protein expression including to produce complex VLPs (Fernandes et al., 2013). The insect *Spodoptera frugiperda Sf*9 cell line (a clone of insect *Spodoptera frugiperda Sf*21 cell line) and the High Five™ cell line (BTI-TN-5B1-4, a clone of the insect *Trichoplusia ni* cell line) are the most widely used for production of recombinant proteins with therapeutic purposes. Both these insect cell lines can promote mammalian-like protein PTMs, such as glycosylation, phosphorylation, disulfide bond formation and further protein folding and processing (Davis and Wood, 1995). Despite not being able to synthesize mammalian-specific complex glycan structures such as processing of N-glycans to terminally sialylated complex-type structures by default, modified cells lines have been established for this end (Okada et al., 2010).

Stable insect cell lines have also been established for production of such biologics (Dias et al., 2021; Vidigal et al., 2018), however these often offer low productivities. The transient insect cell-baculovirus expression vector system

(IC-BEVS) allows to achieve considerably higher production yields (Kost et al., 2005; López-Vidal et al., 2015). This system is based on the baculovirus propensity to infect insect cells, using this virus as vector for foreign gene expression (Figure 1); the most common is the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) (Ayres et al., 1994). This vector can be genetically modified to include the gene(s) coding for the protein(s) of interest in replacement of genes that are non-essential for virus' *in vitro* infection and replication. Most common recombinant baculovirus (rBAC) constructs involve replacement of the polyhedrin or p10 genes whose promoters allow very high gene expression levels (Contreras-Gómez et al., 2014; Min and Bishop, 1991); however other less conventional promoters can be explored (Grose et al., 2021; Martínez-Solís et al., 2016). While *Sf*9 cells are most suited for virus replication thus favoring rBAC amplification, High Five cells are more appropriate to produce higher titers of heterologous recombinant protein (Wilde et al., 2014).

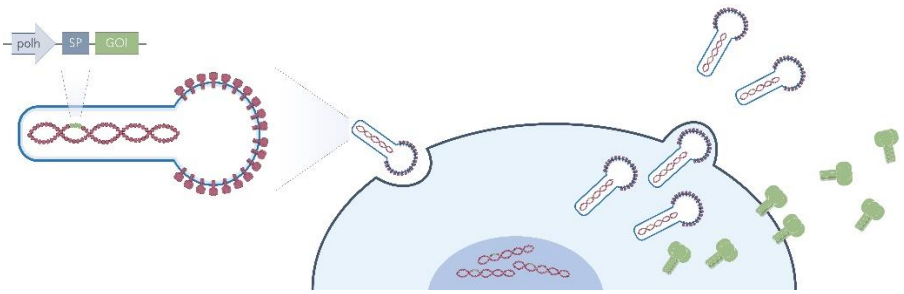

Figure 1 Schematic representation of the use of a generic recombinant baculovirus genetically modified to include the sequence coding for a gene of interest (GOI), used to infect insect cells (represented in blue) to produce the recombinant protein of interest (represented in green). SP denotes signal peptide, polh denotes the polyhedrin promotor.

A single rBAC vector can be used for heterologous production of one or multiple proteins of interest, enabling the generation of multi-component

particle structures such as VLPs (Mi et al., 2021). Different antigens of interest (targeting different diseases or different strains of the same pathogen) can be displayed on the surface of the same particle resulting in multivalent VLPs (Shima et al., 2016), either by infecting insect cells with a rBAC comprising multiple genes of interest (GOI) or by co-infecting with as many rBAC, each coding for one of the GOI. Stable insect cell lines and IC-BEVS can be combined to produce VLPs with higher valency, overcoming the instability of rBAC resulting of including a high number of foreign GOI (Sequeira et al., 2018). Production of multivalent vaccine formulations is especially important to confer broader protection (Schwartzman et al., 2015), with this strategy being employed by some conventional vaccines (e.g. current influenza virus vaccines contain antigens targeting three – trivalent – or four – quadrivalent – strains of the virus); it can also be the key to develop "universal" vaccines. The IC-BEVS has been employed to produce VLPs as vaccine candidates against infection by influenza (Pushko et al., 2005), Ebola (Ye et al., 2006), Chikungunya (Metz et al., 2013), rabies (Bernardino et al., 2021), SARS-CoV-2 (Naskalska et al., 2021), MERS (Wang et al., 2017), human papillomavirus (Touzé et al., 1996), amongst many others.

To date, four drug products approved for human use and produced by rBAC-infected insect cells are/were commercially available: two vaccines (Cervarix®, GSK, against human papillomavirus; Flublok®, Protein Sciences, against influenza) and two therapeutics (Provenge®, Dendreon, for prostate cancer; Glybera®, uniQure, world's first gene therapy). This expression system has also been used to produce seven veterinary vaccines (Cid and Bolívar, 2021; Felberbaum, 2015).

3. Bioprocess engineering to improve vaccine manufacturing using insect cells

Different factors can play a role on the performance of the insect cell expression system. Productivity and/or quality of the product can be enhanced either through a biological approach (e.g. engineering of cells and expression vectors) or a bioprocess approach (e.g. fine-tune expression conditions, tailor-made supplementation and feeding regimens, manipulation of cell culture conditions, and optimization of the bioreactor setups and designs towards intensified and integrated processes). The ideal manufacturing process can also be highly dependent on the type and/or complexity of the biological product being produced (e.g. monomeric protein vs VLPs, growth-associated product vs rBAC-driven, intracellular vs extracellular).

3.1. Engineering rBAC and cell lines

rBAC constructs can be modified towards improved recombinant protein production by rearranging rBAC-derived genetic regulatory elements (Gómez-Sebastián et al., 2014) or deleting genes that are non-essential for cell culture (Hitchman et al., 2010). New rBAC vectors enabling to improve protein's glycosylation profile in insect cells have been suggested to mitigate these cells' lower PTMs complexity versus mammalian cells (Palmberger et al., 2012). Also, the inclusion of the *vankyrin* anti-apoptotic gene in the rBAC vector allows to extend the timespan of infection by delaying cell lysis due to rBAC infection (Steele et al., 2017). This strategy allows for a prolonged and therefore more productive infection process and promotes harvesting at higher cell viability, thus facilitating further purification processes and reducing product degradation by release of proteases.

Signaling sequences are essential to be included in the rBAC construct if the product is to be re-directed to a specific cellular compartment. Secreted proteins require a signal peptide (SP) sequence in order to be harvested in the extracellular culture compartment (Owji et al., 2018). The selection of the most suitable SP sequence is still largely an empirical process as there is no optimal candidate to secrete every protein (Possee et al., 2020); the choice of the SP highly impacts the efficiency of protein secretion (Jarvis et al., 1993; Olczak and Olczak, 2006). The use of a SP of insect or rBAC origin can be the key for heterologous protein expression using IC-BEVS (Murphy et al., 1993; Tessier et al., 1991).

In addition to modifying the vector driving recombinant protein production, engineering the host cell might prove beneficial for recombinant protein expression. The *vankyrin*-expression strategy described above has also been employed via constitutive expression in insect cells (Fath-Goodin et al., 2009; Steele et al., 2017). Likewise, suppression of cell apoptosis by suppressing caspase-1 in stable insect cells has also resulted in improved recombinant protein production (Lai et al., 2012). Such finding has been correlated with higher expression of molecular chaperones, whose overexpression may improve recombinant protein production, secretion, and/or solubility in insect cells (Hsu and Betenbaugh, 1997; Snead et al., 2022; Zhang et al., 2003). Insect cell lines free of latent virus infection (nodavirus in High Five, rhabdovirus in *Sf*9) (Geisler and Jarvis, 2018) have also been established. Elimination of such adventitious agents facilitates thorough product characterization and further approval (Possee et al., 2020) and shows promising improvements in virus and recombinant protein production (Maghodia et al., 2016). Insect cell lines have also been engineered to promote protein mammalian-like N-glycosylation (Mabashi-Asazuma et al., 2013).

3.2. Fine-tuning process parameters

Optimization of infection parameters such as cell concentration at infection (CCI), multiplicity of infection (MOI), time of infection (TOI) and time of harvest (TOH) is usually performed in small-scale experiments prior to being applied in large scale productions (Weber et al., 2002). Other process parameters that may influence cell growth and recombinant protein production include aeration rate, stirring speed, and dissolved oxygen (DO) concentration (Cruz et al., 1998; Gotoh et al., 2002). Novel attractive solutions for high-throughput screening of production conditions, such as micro-bioreactors, can be used to accelerate early development and give insight on upscaling outcome (Strobl et al., 2020).

CCI and TOI are critical parameters influencing productivity. The IC-BEVS is usually more efficient in producing rBAC and recombinant proteins when infection is performed at a lower-exponential phase of cell growth (Pillay et al., 2009). Infections performed at a CCI higher than the ideal early-exponential phase often result in lower cell-specific productivity, a phenomenon known as cell-density effect (CDE) (Bernal et al., 2009). Alternative strategies such as perfusion or supplementation allow to efficiently grow and/or infect insect cells at higher CCI and cope with CDE (discussed below).

There is less consensus on the ideal MOI to be used (Maranga et al., 2003). High MOIs result in synchronous infection of all cells, while in low MOIs only a proportion of cells is infected followed by a cycle of virus replication and further infection of previously non-infected cells. Therefore, the use of low MOI allows for an initial stage of cell proliferation resulting in higher cell density at the time at which all cells are infected. This increase in the number of producing cells can promote higher volumetric titers but also result in lower cell-specific productivity. Some studies report that higher productivity is

achieved at high MOI (Targovnik et al., 2019; Yamaji et al., 1999) whereas others report that lower MOI is more beneficial (Nguyen et al., 1993). The selection of the MOI has significant impact on the volume of virus required for each process, and therefore lower MOI is preferred at an industrial scale as it increases the lifespan of the (costly) certified master virus stocks (Zhou et al., 2011) and reduces significantly the volumes being handled.

The selection of the TOH depends on the infection kinetics as these differ with the CCI and MOI being used. In a batch process, the ideal TOH is usually 72-120 hours post-infection, before cell viability decreases below a detrimental level at which proteolytic activity (Cruz et al., 1999) becomes a concern. Harvesting earlier, at higher cell viability, facilitates further downstream processing by reducing the amount of impurities and cell debris to be removed, but also reduces the amount of product of interest being processed.

The set of ideal production conditions should be assessed for each process individually. Depending on their origin, different rBAC stocks might even be prone to different degrees of passage effect (Krell, 1996; Pijlman et al., 2003, 2002), which by itself is a probabilistic event, hence why it becomes difficult to normalize one set of parameters for all processes.

3.3. Bioprocess intensification

The increasing vaccine demand has boosted the interest in intensifying cell culture-based vaccine production processes through top edge technologies. Significant efforts have allowed to change from less versatile linearly-scalable batch processes of vaccine manufacturing to novel methodologies such as high cell density, perfusion, continuous, and integrated processes. Together, these have allowed to increase significantly titers and reduce process time and/or

eliminate turnovers, while enabling to reduce footprint and processing through an almost completely closed environment from seed train to final product.

3.3.1. Using atypical culture conditions to improve productivity

In vitro cultivation is characterized by a set of standard culture parameters (e.g. temperature, pH, DO, nutrient concentration) that are ideal for each cell line. However, the use of atypical (non-standard) values of such cell culture parameters has been shown to modulate cell performance towards improved cell growth, nutrient utilization and recombinant protein production (Lee and Palsson, 2010; Sunley et al., 2008). Two main approaches in using atypical culture condition may be pursued (Figure 2): single/multiple time-point shift(s) (i.e. change one or more culture parameters at specific time-points) or adaptive laboratory evolution (ALE) (i.e. adaptation of cell populations to grow efficiently at atypical culture conditions by changing culture routine through consecutive cell sub-culturing under the influence of that selective pressure). The use of ALE takes inspiration from microbial culture in which it is used to establish enhanced or resistant strains with industrial potential (Mundhada et al., 2017).

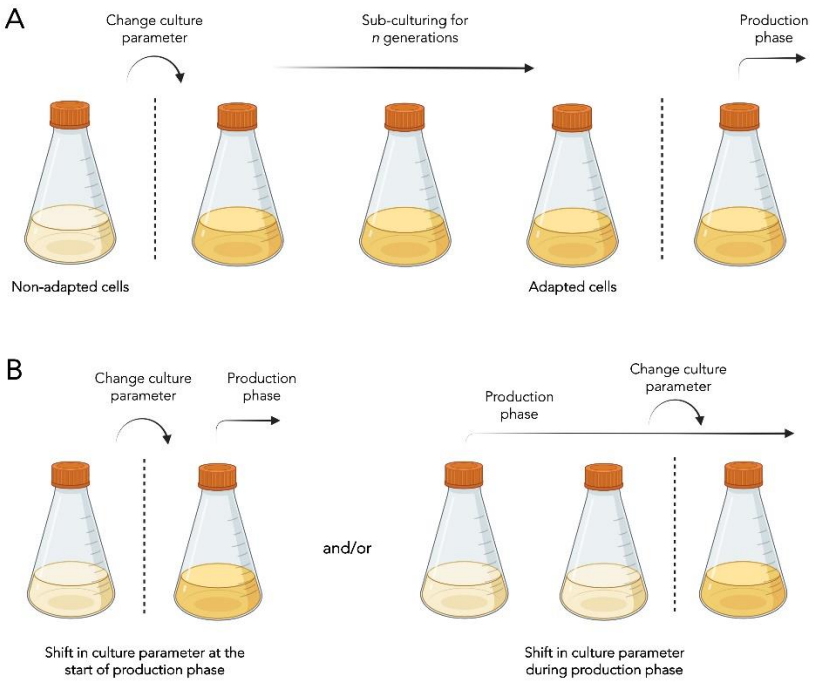

Figure 2 Two main approaches employed for using atypical cell culture condition. (A) Adaptive laboratory evolution (ALE). (B) Examples of strategies for (single or multiple) time-point shift in culture parameters.

There is no universal strategy to improve the production of proteins or vaccine candidates when manipulating culture parameters. The performance of the expression system can be highly dependent on the type and characteristics of the product (e.g. monomeric proteins *vs* VLPs), the cell line being used, the culture parameters being changed, the production platform (e.g. stable cells *vs* rBAC infection), the strategy chosen (shift *vs* adaptation), among others. While in some cases a shift in culture parameters is sufficient to improve productivity (Ikehara et al., 2016), in others ALE is required to improve titers (Correia et al., 2020). Even though ALE (if successful) generally allows to maximize production over a shift-based strategy, both strategies can individually result in higher performance of a specific process (Fernandes et al., 2020) or need to be

combined in other to improve production (Wagner et al., 2014). When comparing with other cell hosts, the use of atypical culture conditions especially through ALE has been fairly limited in insect cells.

Insect cells are normally cultured at 27 °C and changing culture temperature impacts cell growth, rBAC infection and recombinant protein production (Lynn, 2001; Reuveny et al., 1993; Shao-Hua et al., 1998). For instance, lowering culture temperature has been shown to improve recombinant protein production in both stable insect cells (Fernandes et al., 2020; Rossi et al., 2012) and IC-BEVS (Somasundaram et al., 2016), and allow more complete protein glycosylation (Donaldson et al., 1999) or products with higher biological activity (Ikehara et al., 2016). In general, lowering culture temperature allows for a prolonged protein processing through the endoplasmic reticulum (Donaldson et al., 1999), contributing for more efficient protein folding and consequently secretion. When employed in non-lytic systems such as stable insect cells, lower culture temperature allows to prolong significantly the timespan of cell culture, benefiting recombinant protein production. Manipulation of culture temperature has been widely explored to improve performance of other cell hosts (Lin et al., 2015; Yang et al., 2018; Yoon et al., 2003).

Changes in insect cell culture pH have resulted in improved productivity of e.g. Zika VLPs (Wagner et al., 2014) and influenza VLPs (Correia et al., 2020). When compared to other cell hosts, the number of studies regarding the effect of culture pH on performance of insect cells is scarce. For instance, shifts in culture pH of mammalian cells have shown to influence protein expression and glycosylation (Borys et al., 1993) and biological activity (Zheng et al., 2018). Manipulation of culture pH can even be coupled with variations of culture temperature to optimize productivity (Yoon et al., 2005). The mechanisms underlying the change in cell culture performance at atypical values of culture

pH are not entirely understood. It is however important to note that protein conformation can be highly influenced by culture pH (Batys et al., 2020), and therefore manipulation of this culture parameter can be the key to achieve proper biological function.

Modifications in other cell culture parameters such as DO (Restelli et al., 2006) and osmolarity (Alhuthali et al., 2021) have been addressed to modulate cell performance.

3.3.2. Fed-batch and perfusion operation modes

Cell culture-derived vaccine production is usually operated in batch cultivation mode, however increasing vaccine demand resulted in more interest being given to process intensification approaches such as fed-batch or perfusion systems (Gallo–Ramírez et al., 2015). These processes are performed to avoid nutrients from being the limiting factor of cell culture performance, providing the culture with fresh medium or specific supplements as enhancers of certain biological processes; they mainly aim at increasing cell density and/or improve productivity.

Both linear and pulse fed-batch regimens can be implemented at specific cultivation time-points depending on limiting factors (e.g. depletion of nutrients, target cell density being reached, or change in growth rate). The design of tailor-made feeding strategies for insect cells has proven efficient in improving production of rBAC and recombinant proteins including VLPs (Monteiro et al., 2016), increasing cell density (Chiou et al., 2000), or a combination of both (Cao et al., 2019; Meghrous et al., 2009). Supplementation with specific metabolites shows promise in overcoming CDE (Sequeira et al., 2018) allowing to maximize productivity even in infections

performed at the typically non-ideal late-exponential phase of cell growth (Bédard et al., 1994).

Despite allowing supply of the required nutrients, fed-batch processes involve addition but not removal of components from the cell culture. Therefore, its use is limited by the bioreactor volume (hence concentrated feeds are usually used to avoid culture dilution) and can result in accumulation of byproducts throughout prolonged cell cultures (Gutiérrez-Granados et al., 2018). Perfusion processes overcome these drawbacks by allowing continuous culture media renewal without interfering with culture volume. Perfusion cultures are normally operated to assist cell growth until high cell density (HCD). While for stable insect cell lines it allows to extend and intensify production (Jardin et al., 2007), for IC-BEVS it may allow to perform infection at HCD without observing CDE (Chico and Jäger, 2000), since nutrient depletion can be one of the reasons of lower productivity at higher cell concentration (Maranga et al., 2003).

HCD can be achieved through perfusion by using cell retention devices such as acoustic filters and spin filter systems (Voisard et al., 2003). For instance, the BioSep ultrasonic filter was used to grow insect *Sf*9 cells at HCD (30 x10^6 cell/mL), and infection of these cells resulted in productivity (both protein of interest and rBAC) similar to that of low cell density batch cultures (Zhang et al., 1998). Membrane filtration-based approaches using hollow fibers (HF) have gained considerable interest. Despite being traditionally used for other filtration-based processes, HF have been successfully adjusted as cell retention devices placed outside of the bioreactor for perfusion purposes, allowing easy module replacement (Kelly et al., 2014) and process scalability (Tapia et al., 2016). These can be operated either in alternating tangential flow filtration (ATF) or tangential flow filtration (TFF) setups (Karst et al., 2016), the difference being a bidirectional or unidirectional flow in ATF and TFF, respectively; both

offer reduced filter clogging due to crossflow (Tapia et al., 2016). Depending on the application, one can play with the HF pore size to retain the product of interest in the bioreactor or continuously harvest it through the permeate.

Both methodologies have been used with considerable success to produce viruses such as influenza and MVA (Coronel et al., 2019; Genzel et al., 2014; Vázquez-Ramírez et al., 2019), and may facilitate production of difficult-to-express products (Särnlund et al., 2021). Recently, stable insect cells producing influenza VLPs were cultivated in perfusion using ATF until 100 $x10^6$ cell/mL (Fernandes et al., 2021). By using an HF with pore size of 0.2 µm, VLPs were retained in the bioreactor, and cell-specific productivity was maintained versus batch culture allowing to increase VLPs volumetric titer by up to 8-fold. This works reports the highest cell concentration reached with insect cells so far, however other studies show data regarding efficient cell growth at HCD (Elias et al., 2000). Perfusion can be incorporated in other process schemes such as continuous to support the culture with nutrient supply. Examples of operational setups using perfusion are depicted in Figure 3.

3.3.3. Continuous production and multi-stage bioreactors

Continuous production processes allow to minimize facility footprint which reduces operating costs significantly, as processes can be prolonged rather than scaled-up (Croughan et al., 2015). The production of unstable biopharmaceutical products is benefited in continuous process through continuous harvesting, as the longer periods of batch cultivation are commonly the driving factor of variability in product quality (Zydney, 2015). Continuous manufacturing can allow improved quality of the final product through more consistent glycosylation (Konstantinov and Cooney, 2015).

A typical process of continuous cell growth involves bioreactor bleeding and medium feeding at a certain flow to compensate cell growth, thus maintaining culture volume and cell concentration. This can be readily applied to cultures of stable insect cells, allowing continuous harvesting of the product of interest at constant titer, assuming that cell-specific productivity is efficiently maintained. Continuous cultures can be coupled with perfusion for higher level of medium replacement, which can be a critical factor when operating at HCD (Gutiérrez-Granados et al., 2018). If HF are used as cell retention devices for this purpose, the pore size should be carefully considered as it dictates whether the product of interest (if extracellular) is also harvested in the perfusion permeate or exclusively in the cell bleeding stream.

The continuous production of viral particles (viruses, VLPs, viral vectors) through processes involving lytic infection rather than stable production is not straightforward. The lytic nature of such systems hinders their use in a simple continuous bioreactor scheme since cell growth is usually arrested and the high rate of virus replication eventually results in wholesome cell lysis and consequently in process terminus. This is the case for processes using IC-BEVS, either aiming for rBAC amplification or recombinant protein production.

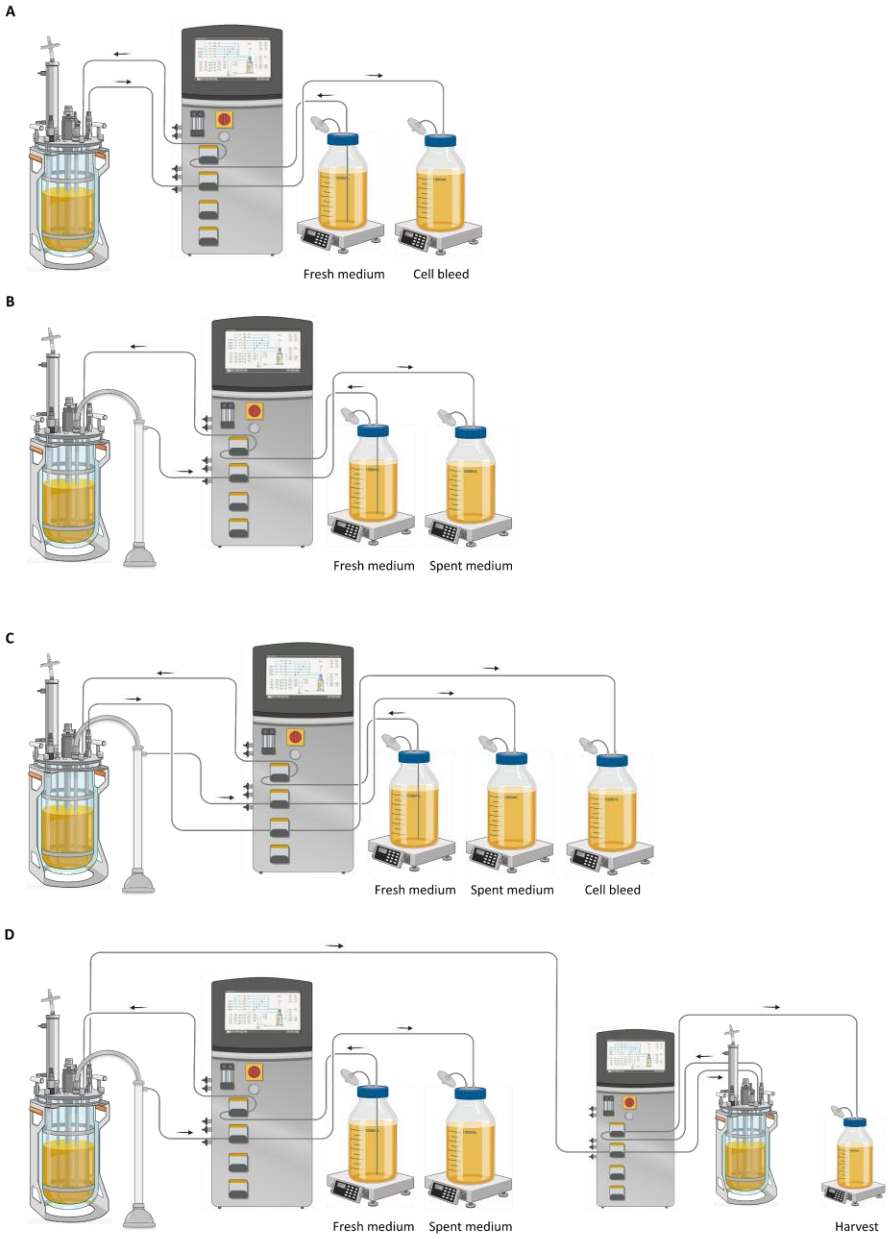

Figure 3 Examples of bioreactor schemes for process intensification. (A) Continuous. (B) Perfusion. (C) Continuous, coupled with perfusion. (D) Multi-stage continuous, coupled with perfusion in cell growth bioreactor.

To tackle this issue, multi-stage bioreactor schemes can be used (Tapia et al., 2016). These are composed of separate vessels connected in series: the first is usually dedicated to cell growth in continuous mode, and its cell bleeding is used as feed of non-infected cells to the second bioreactor (production bioreactor). The latter is infected and operates in continuous through culture bleeding with a flow equivalent to the flow of cells being fed, to maintain culture volume. This system allows constant renewal of the production bioreactor with new cells that are infected with the viruses being generated in this bioreactor. The residence time (RT) at which the production bioreactor is operated (i.e. the amount of time required to change one bioreactor volume) correlates with the rate at which cells and viruses are removed; higher RT means lower rate of cell renewal. Consequently, RT influences the kinetics of infection, with direct impact on the cell viability, cell concentration, virus titer, and recombinant protein concentration (Tapia et al., 2019). Like simpler continuous setups, perfusion can be coupled with either the cell growth or the production bioreactor to maintain higher nutritional levels and/or operate at HCD.

An important aspect to take into consideration in lytic processes such as IC-BEVS is the formation of defective interfering particles (DIPs), i.e. defective virus particles that require simultaneous infection with a helper (non-mutated) virus in order to replicate (Frensing, 2015; Kool et al., 1991). DIPs formation is a direct consequence of passage effect (Krell, 1996) on the generation of virus particles, as random and/or prevalent mutations tend to arise, resulting in loss of specific genomic regions including those coding for the gene of interest (Pijlman et al., 2003), which can be overcome through rBAC vector design (Pijlman et al., 2020, 2004). Processes operated at HCD and/or for prolonged periods can therefore result in variable patterns of DIP formation and

consequently ratios of infectious/total particles (Tapia et al., 2016), compromising the feasibility of continuous operation. As continuous multi-stage processes are to be operated for longer periods of time when compared to a batch cultivation, the formation of DIPs is more concerning as these may tend to periodically accumulate (Frensing et al., 2013), hence the importance of fine-tuning the RT to optimize the kinetics of virus propagation. Multi-stage bioreactor schemes with more than two vessels in series may allow to better play with RT to modulate virus production yields (Tapia et al., 2016) but can also result in earlier passage effect and consequently reduction of virus titer (van Lier et al., 1990).

Multi-stage bioreactor schemes have been employed for virus production using other cell hosts (Frensing et al., 2013; Tapia et al., 2019, 2017). Despite having allowed to generate groundbreaking knowledge, studies regarding continuous multi-stage bioreactor processes using insect cells and/or IC-BEVS are limited and/or outdated (Kompier et al., 1988; Pijlman et al., 2004; van Lier et al., 1996, 1994, 1992, 1990).

Examples of different operational setups for continuous operation, with or without perfusion implemented, are depicted in Figure 3.

3.3.4. Integrated processes

Integrated continuous operation (i.e. streamlining of continuous upstream and downstream) (Warikoo et al., 2012) is thought to be the next step of the expected trajectory of process evolution (Croughan et al., 2015). The increasing demand of drug products and the particularity of integrated processes in allowing cost reduction through reduced equipment footprint both at up- and downstream, motivate the shifting of paradigm towards these

processes. Integrated continuous biomanufacturing can reduce costs by up to 55 % when compared to a traditional batch process (Walther et al., 2015). In an integrated process, the advantages abovementioned for continuous production (e.g. stability of labile biologics, consistent glycosylation, timewise scalability) are also applied to downstream, as some unit operations such as hold steps are eliminated (Konstantinov and Cooney, 2015). For instance, a continuous process operated at HCD using perfusion (in the production bioreactor) can dismiss the need for clarification and concentration steps, commonly operated at the start of downstream processing (Tapia et al., 2016). A fully end-to-end integrated continuous biomanufacturing process involves direct connection and continuous processing of all unit operations, from upstream and downstream, with no hold steps (Fisher et al., 2019), and has shown promising improvements in overall antibody manufacturing process (Godawat et al., 2015).

Continuous operation in vaccine manufacturing has seen more advances in upstream than in downstream. While a bioreactor setup for continuous production is likely to be more easily adjusted to the production of various biologics (although requiring fine-tuning of operational and production conditions), establishment of a standard setup of continuous downstream processing is impaired by the vast array of purification techniques (Wolf and Reichl, 2011) inherent to the variability in product characteristics (e.g. size, charge, presence of purification tags, stability).

The development of continuous purification schemes for integration in a fully continuous process can take inspiration from the processes operated in batch mode. For instance, chromatography processes typically operated in batch can be converted into continuous multi-column chromatography (Mendes et al., 2021; Moleirinho et al., 2019), i.e. sets of chromatographic columns grouped

in parallel and/or in series. By using a series of smaller pre-packed columns operating at different stages of the process (e.g. loading, wash, elution, regeneration, equilibration), clarified culture supernatant can be continuously processed, reducing costs by shortening process duration and increasing column's resin utilization.

3.4. Vaccine storage and stability

Vaccines are typically stored at refrigerated conditions (2-8 °C) or even frozen to guarantee effectiveness. The hurdle of maintaining the vaccine cold chain (Ashok et al., 2017) can present a significant economic burden. Therefore, it is utterly important to invest in means of increasing vaccine stability (to cope with interruptions in vaccine cold chain) and in alternative formulation/storage options. For instance, with hydrolysis being one of the major degradation mechanisms, lyophilization is often used and allows higher shelf-life under refrigerated and non-refrigerated conditions than their aqueous counterparts (Chen et al., 2021; Hansen et al., 2015; Madan et al., 2018). Hydrolysis mechanisms can also be overcome with the development of water-free storage formulations (Correia et al., 2021).

Vaccines are usually formulated with adjuvants, with aluminum salts being the most used in human vaccines due to their exemplary safety track record (Kumru et al., 2014). However, adjuvants mainly aim at promoting vaccine immunogenicity and do not confer additional stability to adverse storage or transportation conditions. To target that, vaccines have been stabilized by using excipients like amino acids, antioxidants, proteins, sugars, polyols or surfactants in their storage formulation (Cardoso et al., 2017; Pelliccia et al., 2016). Stability screening protocols can be established to evaluate dozens of

excipients of various types to optimize vaccine formulation (Kumru et al., 2019). For instance, trehalose is generally a good stabilizer of enveloped viral particles (viruses, VLPs, vectors) by promoting strong hydrogen bonds with the envelope's lipid membrane (Toniolo et al., 2019), being successfully used for the stabilization of influenza VLPs (Kim et al., 2010).

The global vaccine availability would be significantly improved if technological advancements achieved for vaccine production would be combined with a similar degree of innovation in vaccine formulation, storage, and distribution.

4. Concluding remarks

Vaccine manufacturing is a multi-stage decision-making process, from the choice and design of the best-performing producer cells and vectors, through employment of the most suitable setup of production and purification and fine-tuning of expression conditions, and finally optimization of the formulation, storage, distribution, and administration route. Improvements in any of these stages contribute to facilitate the response towards the increasing vaccine demand and offer higher flexibility to cope with an ever-increasing unpredictability about the rise of novel pathogens and highly infectious strains. However, only a balanced development of the several stages of the manufacturing process is desired, to avoid the existence of limiting steps.

5. Aim of the thesis

Vaccine manufacturing platforms capable of meeting the vaccine demand are urgently needed as it was recently proved during the COVID-19 pandemic outbreak. This thesis aims to contribute to this goal by assessing the use of

atypical culture conditions (shift and ALE) to improve the production of vaccine candidates using insect cells and infection by baculovirus, and later combine with bioprocess strategies (continuous multi-stage bioreactors) to further intensify the production process. Additionally, an alternative formulation for water-free vaccine storage was assessed to improve the stability upon variations in storage temperature. The schematic representation of this thesis workflow is depicted in Figure 4. Two model vaccine candidates were explored, (i) an influenza VLP-based vaccine candidate (Chapters 2 and 3) composed of influenza matrix 1 (M1) and hemagglutinin (HA) proteins (hereon referred and HA-VLPs), and (ii) an antigenic protein-based subunit malaria vaccine candidate (Chapter 4) based on the PfRipr5 protein, the most immunogenic fragment of the blood-stage malaria vaccine candidate PfRipr. Two culture parameters were manipulated in order to improve productivity: pH (Chapter 2, to produce influenza HA-VLPs) and temperature (Chapter 4, to produce malaria PfRipr5 protein). A trehalose-glycerol natural deep eutectic solvent (NADES) system was used to improve the storage stability of influenza HA-VLPs (Chapter 3). Finally, the first steps for establishing a continuous multi-stage bioreactor production process using IC-BEVS were undertaken Chapter 5.

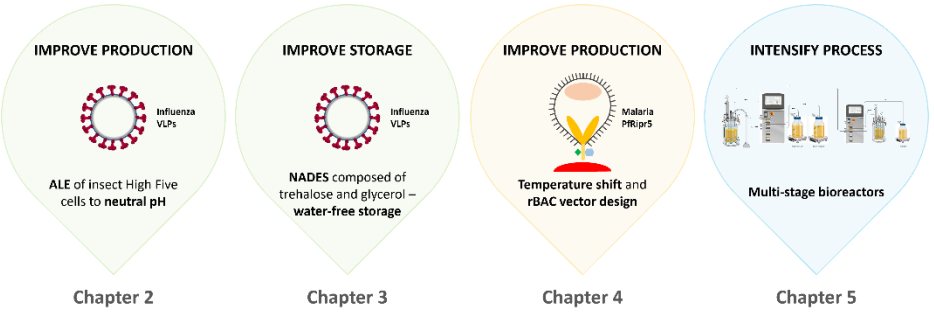

Figure 4 Schematic representation of the thesis workflow.

Current influenza vaccine supply still depends greatly on egg-based production platforms. Therefore, it relies on the constant supply of high-quality eggs, which is insufficient to meet the demand in pandemic events. Cell culture-based approaches of producing influenza vaccines offer higher flexibility, with VLPs being an attractive approach for allowing high level of antigen display to confer strong immune response, and insect cells being a powerful tool to produce these particles. Nevertheless, strategies to improve this platform are needed to reduce the production scale and/or increase the production load. In **Chapter 2**, insect High Five cells were adapted to grow at neutral culture pH (7.0) using ALE, in order to improve the productivity of influenza HA-VLPs using IC-BEVS. ALE was essential to maximize productivity (pH shift was inefficient in improving the process) allowing to increase volumetric and specific productivities by 4- a 3- fold, respectively. The upstream processing is just one step of the vaccine manufacturing pipeline that can be improved. For instance, a large parcel of the vaccine manufacturing cost is related with maintaining the vaccine cold chain, i.e. assuring refrigerated conditions during vaccine storage and transportation, with cold chain disruptions being responsible for a large percentage of vaccine wastage. Considering this, the storage stability of influenza HA-VLPs was assessed in **Chapter 3** using a NADES system composed of trehalose and glycerol (TGly), aiming at developing a water-free storage formulation. TGly was more efficient than a control water-based buffer formulation in storing HA-VLPs, maintaining particles integrity and reducing HA degradation during an accelerated study at high temperature, and looking promising for room temperature storage.

The pursue for an effective malaria vaccine took a step forward with the recent approval of the RTS,S/AS01 for wider experimental use. However, this vaccine targets only the pre-erythrocytic stage of the malaria parasite cycle, and

current scientific opinion suggests that a more efficient and broadly applicable vaccine could be achieved by combining antigens targeting all different stages. According to recent studies, the *Plasmodium falciparum* merozoite Rh5 interacting protein (PfRipr) is one of the less polymorphic yet high immunogenic blood-stage target antigens, with PfRipr5 being the most potent fragment. Therefore, the production of this vaccine candidate using IC-BEVS was established and optimized in **Chapter 4**. Specifically, temperature shift from the standard 27 to 22 °C was combined with optimization of design of the rBAC expression vector to enhance protein secretion, allowing to improve process yield post-purification by 5.2-fold.

Intensified processes such as perfusion at HCD or continuous allow to achieve higher titers without increasing production scale, and/or to reduce turnover times and batch-to-batch variability by focusing on expanding production on a time- rather than physical space-basis. However, no recent developments in establishing continuous operations using IC-BEVS have been reported. Similar to other lytic systems, employment of multi-stage bioreactor configurations is the most suitable approach to implement continuous production in IC-BEVS, with fine-tuning of the virus residence time being crucial. While other non-insect cell related studies focus on the continuous production of viruses, the interest in continuous processes using IC-BEVS would be on the continuous production of the target protein rather than the baculoviruses, therefore increasing the complexity and difficulty of establishing such process. The first steps in establishing a continuous process of influenza HA-VLPs production using IC-BEVS were explored in **Chapter 5**. A multi-stage bioreactor setup using a cell growth bioreactor to simultaneously feed three production bioreactors operated at different RT was implemented, showing that this parameter

strongly influences the kinetics of infection and quality/quantity of the viruses and product being produced.

Finally, in **Chapter 6**, an overall discussion of the achievements of this work is presented.

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Chapter 2

Insect cells adapted to neutral pH: improve production of influenza VLPs

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Author contribution

Ricardo Correia design and performed the experiments, analysed the data and wrote the chapter.

Abstract

The use of non-standard culture conditions has proven efficient to increase cell performance and recombinant protein production in different cell hosts. However, the establishment of high-producing cell populations through adaptive laboratory evolution (ALE) has been poorly explored, in particular for insect cells. In this study, insect High Five cells were successfully adapted to grow at a neutral culture pH (7.0) through ALE for an improved production of influenza hemagglutinin (HA)-displaying virus-like particles (VLPs). A stepwise approach was used for the adaptation process, in which the culture pH gradually increased from standard 6.2 to 7.0 (ΔpH=0.2-0.3), and cells were maintained at each pH value for 2–3 weeks until a constant growth rate and a cell viability over 95% were observed. These adapted cells enabled an increase in cell-specific HA productivity up to three-fold and volumetric HA titer of up to four-fold as compared to non-adapted cells. Of note, the adaptation process is the element driving increased specific HA productivity as a pH shift alone was inefficient at improving productivities. The production of HA-VLPs in adapted cells was successfully demonstrated at the bioreactor scale. The produced HA-VLPs show the typical size and morphology of influenza VLPs, thus confirming the null impact of the adaptation process and neutral culture pH on the quality of HA-VLPs produced. This work strengthens the potential of ALE as a bioprocess engineering strategy to improve the production of influenza HA-VLPs in insect High Five cells.

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1. Introduction

Influenza is a major burden to global healthcare systems, often causing pandemics with massive death-tolls (Cox and Subbarao, 2000; Simonsen, 1999). Prophylactic vaccination still remains the most efficient approach to reduce influenza disease (Houser and Subbarao, 2015). Due to the high tendency of influenza viruses to accumulate 3–4 amino acid substitutions per year and to genetically reassort amongst viruses in animal reservoirs (Smith, 2004), vaccine composition needs to be updated and new vaccines need to be formulated, produced and administrated on an annual basis, if not more often in the case of pandemics. Thus, the development of fast, economical and flexible vaccine production platforms to quickly cope with the need for high quantities of vaccine shots has become a worldwide key healthcare priority.

Vaccine manufacturing platforms based on cell culture allow superior process control and flexibility, potentially improving the responsiveness to influenza epidemics or pandemics (Milián and Kamen, 2015). Mammalian cells have been extensively explored and proven efficient for the production of influenza vaccines (Liu et al., 2009; Montomoli et al., 2012). Virus-like particles (VLPs) allow for a high level of antigen display, thus showing high potential to be used as vaccines. The insect cell-baculovirus expression vector system (IC-BEVS), which allows for a fast production of high protein titers, has proven to be efficient to produce complex VLPs (Fernandes et al., 2013), including influenza VLPs similar to those produced by mammalian cells (Thompson et al., 2015).

The use of non-standard values of cell culture parameters such as pH, temperature or dissolved oxygen concentration has often been shown to significantly impact the growth performance and recombinant protein production yields in different cell hosts (Lin et al., 2015; Reuveny et al., 1993;

Vergara et al., 2014). Most reports refer to the use of single or multiple shifts in such parameters as effective means to enhance cell performance (Oguchi et al., 2007; Rossi et al., 2012). Alternatively, the use of adaptive laboratory evolution (ALE), i.e., adaptation of cells to efficiently grow under such non-standard culture conditions, through consecutive sub-culturing under these selective pressures allows for the selection of cell populations with an enhanced fitness. Besides improving cell growth (Ibarra et al., 2002) by enabling beneficial mutations to arise (LaCroix et al., 2015), this process also leads to more efficient energy utilization, the use of non-conventional substrates (Lee and Palsson, 2010), tolerance to byproducts of interest (Atsumi et al., 2010; Lam et al., 2014), or even the development of thermo-resistant strains (Deatherage et al., 2017).

Importantly, ALE has been suggested as an approach to maximize recombinant protein titers in both prokaryotes and animal cells (Mundhada et al., 2017; Sunley et al., 2008). However, it has been poorly explored in insect cells. Recently, adaptation to hypothermic culture conditions has proven efficient to significantly increase the production of HIV1 Gag VLPs using stable insect cell lines (Fernandes et al., 2020). Additionally, ALE has proven to be efficient to increase the production of Chikungunya VLPs by more than 11-fold using IC-BEVS with insect Sf-21 cells adapted to a higher pH (Wagner et al., 2014). Thus, we have hypothesized that adaptation to higher culture pH could also have an impact on the production of other VLPs using insect cells.

In this work, High Five insect cells were adapted to grow at neutral pH and their capacity to produce influenza A hemagglutinin-displaying virus-like particles (HA-VLPs) was assessed, aiming at developing a platform for a faster production of influenza vaccine candidates.

2. Materials and Methods

2.1. Cell Line and Culture Media

High Five insect cells (Invitrogen, kindly provided by Redbiotec AG, Schlieren, Switzerland) were routinely sub-cultured at 0.3–0.5 × 10^6 cell.mL^{-1} every 2–3 days when cell concentration reached 2–3 × 10^6 cell.mL^{-1} in serum-free Insect-XPRESS^{TM} medium (Lonza, Basel, Switzerland) (herein mentioned as $CM_{pH6.2}$) using 125–500 mL shake flasks (Corning, New York, USA) with a 10% working volume, and maintained at 27 °C in a shaking incubator (Inova 44R – Eppendorf, Hamburg, Germany) set to 100 RPM and with 2.54 cm shaking diameter.

2.2. Adaptation of Insect Cells to Neutral pH

Culture medium containing a 1:1 mixture of Insect-XPRESS^{TM} medium and a chemically defined solution containing 50 mM HEPES, 124 mM Sucrose, 5 mM Glucose, 50 mM NaCl, 20 mM KCl, 3 mM $CaCl_2$, 10 mM $MgSO_4$ and 0.1% (w/v) Pluronic F-68 (Wagner et al., 2014) was used for the adaptation process of High Five cells to neutral pH (hereon referred to as CM_{pH7}). The pH was adjusted to the desired value by adding NaOH 1 M and sterile filtered using a 0.22 µm Stericup (Millipore, Burlington, Massachusetts, USA). A stepwise approach was used for the adaptation process, in which culture pH was gradually increased from standard 6.2 to 6.5, 6.8 and finally to 7.0, and cells maintained in each pH value for approximately 2–3 weeks until a constant growth rate and cell viability over 95% were observed. To circumvent medium acidification during cell growth, culture pH was monitored daily using a benchtop probe (Crison, Barcelona, Spain) and adjusted by aseptically adding sterile NaOH 1M at a

proportion of 15 µL/pH unit/mL of culture. Master cell banks were prepared prior to each pH increase by re-suspending cells in CryStore CS10 freezing medium (Sigma, St. Louis, Missouri, USA) and freezing at −80 °C using a Coolcell cell freezing container (Biocision, Larkspur, California, USA).

2.3. Baculovirus Amplification and Storage

Recombinant baculoviruses containing influenza M1 (from A/California/06/2009 H1N1 strain) and HA (from A/Brisbane/59/2007 strain) genes were kindly provided by Redbiotec AG (Schlieren, Switzerland). An amplification of baculovirus stocks was performed as described elsewhere (Vieira et al., 2005). Briefly, insect *Sf*-9 cells (cultivated in Sf-900^{TM} II medium (Gibco, Waltham, Massachusetts, USA)) were infected at a concentration of 1 × 10^6 cell/mL using a multiplicity of infection of 0.1 plate-forming units per viable cell (pfu/cell). When a cell viability of approximately 80% was reached, the supernatant was harvested by centrifugation at 200 g and 4 °C for 10 min and centrifugation at 2000 g and 4 °C for 20 min. The clarified supernatant was aliquoted appropriately and stored at 4 °C until further use.

2.4. Production of HA-Displaying VLPs

VLPs displaying HA were produced in 250 mL shake flasks (10% working volume) and in 0.5 L glass stirred-tank bioreactors.

In shake-flask cultures, cells were cultured in 500 mL shake flasks (Corning, New York, USA) with a 10% working volume in either $CM_{pH6.2}$ (for non-adapted cells) or CM_{pH7} (for adapted cells). Infection experiments were performed in 250 ml shake flasks (Corning, New York, USA), with a 10% working volume, and

at different cell concentrations at the time of infection (CCIs; 1×10^6, 2×10^6 and 3×10^6 cell.mL^{-1}) and multiplicity of infection (MOI; 0.1, 1 and 10 pfu.cell^{-1}). At the time of infection, a complete medium exchange was performed by centrifugation at 200 g at room temperature for 10 min.

Bioreactor runs were operated in computer-controlled BIOSTAT Qplus 0.5 L vessels (Sartorius, Göttingen, Germany) using adapted cells at generation 53 after the establishment of the master cell bank. Culture mixing was achieved by equipping the bioreactor with one Rushton impeller and varying the agitation rate from 70 to 270 rpm. Gas was supplied through a ring sparger at a flow rate of 0.01 vvm, and the percentage of O_2 in the gas mixture varied between 0 and 100%, to maintain the pO_2 (partial pressure of oxygen) setpoint at 15% of air saturation. The bioreactor headplate included multiple ports for temperature, pH and pO_2 probes as well as for additions (i.e., culture medium, cells and baculovirus) and the sampling/harvesting of the cell culture. Culture pH was controlled at 7.0 by adding NaOH 1 M. Bioreactors were operated at a working volume of 0.3 L, inoculated at a cell concentration of 2×10^6 cell.mL^{-1}, and infected immediately at an MOI of 1 pfu.cell^{-1}.

2.5. Purification of HA-Displaying VLPs

Culture bulk from bioreactor runs was harvested and centrifuged at 4 °C, 200 g, for 10 min (for cell removal) and at 4 °C, 2000 g, for 20 min (for further clarification). The clarified supernatant was filtered using a 0.22 µm Stericup (Millipore, Burlington, Massachusetts, USA), and HA-VLPs were concentrated/purified using a (scheme 1) polyethylene glycol (PEG) precipitation method followed by size-exclusion chromatography, or (scheme 2) anion-exchange chromatography. In scheme 1, proteins were precipitated

overnight with PEG (8.5% w/v) and NaCl (0.3 M). The precipitated proteins were centrifuged at 4500 g and 4 °C for 30 min, pellets were re-suspended in buffer containing HEPES and NaCl, and the concentrated protein content was purified by size-exclusion chromatography with a Superdex200 10/300 column (GE Healthcare, Chicago, Illinois, USA). The fractions corresponding to the HA-VLPs peak were pooled and sterile filtered using a Whatman cellulose regenerated membrane filter. In scheme 2, HA-VLPs were purified using a SartoBind Q capsule (Sartorius Stedim Biotech, Göttingen, Germany) according to manufacturer's instructions.

Specifically, elution buffer was composed by HEPES (50 mM) and NaCl (300 mM) at pH 7.4. The fraction corresponding to HA-VLPs was collected and sterile filtered using a Whatman cellulose regenerated membrane filter. Trehalose (pH 7.4) was added to the purified material to a final concentration of 15% (w/v) using a stock solution of 65% (w/v) previously prepared. The resulting purified material was stored at -80 °C (long-term storage) or at 4 °C (short-term storage).

2.6. Analytics

2.6.1. Cell Concentration and Viability

Cell counting was performed in a Fuchs–Rosenthal hemocytometer chamber (Brand, Wertheim, Germany) and viability was assessed using the trypan-blue exclusion method.

2.6.2. Hemagglutination Assay

The HA titer was determined using the hemagglutination assay as described elsewhere (Sequeira et al., 2018). Briefly, in-process and purified samples (25 µL) were serially diluted 1:1 with DPBS(-/-) 1X (Gibco, Waltham, Massachusetts, USA) in V-bottom 96-well plates (Thermo Scientific, Waltham, Massachusetts, USA) and gently mixed 1:1 with 1% chicken erythrocytes (Lohmann, Cuxhaven, Germany). Plates were incubated at 4 °C for 30 min. The HA titer was estimated as being the inverse of the highest dilution of sample that completely inhibited hemagglutination.

2.6.3. SDS-PAGE and Western Blot

In-process and purified samples were denatured by mixing with 1X LDS Sample Buffer (ThermoFisher Scientific, Waltham, Massachusetts, USA, stock solution at 4X) and 1X Sample Reducing Agent (ThermoFisher Scientific, Waltham, Massachusetts, USA, stock solution at 10X) and heating for 10 min at 70 °C. Denatured samples were loaded into a 4–12% NuPAGE Bis-Tris protein gel (ThermoFisher Scientific, Waltham, Massachusetts, USA), using MOPS running buffer (ThermoFisher Scientific, Waltham, Massachusetts, USA) and SeeBlue Plus2 Prestained Standard (ThermoFisher Scientific, Waltham, Massachusetts, USA) as a molecular weight marker. After electrophoresis (50 min at 200 V and 400 mA), proteins were transferred to a nitrocellulose membrane using the iBlot system (ThermoFisher Scientific, Waltham, Massachusetts, USA). Membranes were blocked for 1 hour at room temperature with a blocking solution composed of Tris-Buffered Saline (Sigma, St. Louis, Missouri, USA) with Tween-20 (Millipore, Burlington, Massachusetts, USA) and 5% (w/v) skim milk (Millipore, Burlington, Massachusetts, USA), and incubated overnight at

room temperature with primary antibodies diluted in blocking solution. For HA identification, a mouse monoclonal antibody (IRR, Manassas, Virginia, USA, FR-494—mouse monoclonal antibody to recombinant H1 HA from influenza A/Brisbane/59/2007 (H1N1)) was used at a dilution of 1:2000. M1 protein was identified using a goat polyclonal antibody (Abcam, Cambridge, UK, Cat# ab20910) at a dilution of 1:2000. Secondary anti-mouse or anti-goat IgG antibodies conjugated with alkaline phosphatase were used at a dilution of 1:2000 for identification of HA and M1, respectively. Protein band detection was performed by covering membranes with NBT/BCIP 1-Step (Thermo Scientific, Waltham, Massachusetts, USA) for 10 min; membranes were then scanned with a benchtop scanner device.

2.6.4. Baculovirus Titration

Baculovirus titers were determined using the MTT assay (Mena et al., 2003; Roldão et al., 2009) (for infectious particles) or qPCR (for total baculovirus genome copies) as described elsewhere (Carvalho et al., 2016). Briefly for qPCR, culture samples were firstly treated with DNAse (Roche, Basel, Switzerland) to eliminate free residual baculovirus DNA prior to quantification. Baculovirus DNA was extracted using the High Pure Viral Nucleic Acid Kit (Roche Life Science, Mannheim, Germany). For the qPCR, a master mix was prepared using the LightCycler 480 SYBR Green I Master (04707516001; Roche Life Science, Mannheim, Germany), a final concentration of 0.5 µM of reverse and forward primers for the ie1 baculovirus gene region, and PCR-grade water. The qPCR was performed in 96-well white plates (04729692001; Roche Life Science, Mannheim, Germany) using a LightCycler 480 Instrument II (Roche Life Science, Mannheim, Germany).

2.6.5. Metabolite quantification

The metabolite (glucose and glutamine) quantification was performed using Cedex Bio Analyzer 7100 (Roche, Mannheim, Germany) as described elsewhere (Fernandes et al., 2021).

2.6.6. Nanoparticle Tracking Analysis

The concentration and size distribution of HA-VLPs were measured using the NanoSight NS500 (Nanosight Ltd., Salisbury, UK). The samples were pre-diluted with DPBS(-/-) 1X (Gibco, Waltham, Massachusetts, USA) to cope with the instrument's range of analysis (10^8–10^9 particles.mL^{-1}). All measurements were performed at 22 °C. Sample videos (60 seconds) were analyzed with the Nanoparticle Tracking Analysis (NTA) 2.3 Analytical software. Capture settings (shutter and gain) were adjusted manually.

2.6.7. Transmission Electron Microscopy Analysis

The conformation and size of purified influenza HA-VLPs from bioreactor runs were assessed by negative staining TEM using a Hitachi H-7650 Transmission Electron Microscope (JEOL, Tokyo, Japan). For sample preparation, 10 µL of purified HA-VLPs was fixed for 1 min in a copper grid previously coated with Formvar-carbon (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA). Grids were then washed three times with water and finally stained with 1% (v/v) uranyl acetate for 2 min. Grids containing fixed samples were left to air dry and immediately analyzed.

2.7. Mathematical Equations

2.7.1. Mathematical Equations for Estimation of HA Production Rate

The specific HA production rate, r_{HA}, is given by:

$$r_{HA}(\text{HA titer}.10^6\ \text{cell}^{-1}.\text{h}^{-1}) = \frac{\Delta HA}{\int_0^t X\,dt}\ , 0 < t < t_f \quad (1)$$

where HA is the extracellular HA titer (HA titer.mL^{-1}) and X is the total cell concentration (10^6 cell.mL^{-1}) during the production phase (from t = 0 to t = t_f).

2.7.2. Mathematical Equations for Estimation of Reaction Rates

Cell growth rate, μ, is given by:

$$\mu(h^{-1}) = \frac{1}{X}\cdot\frac{dX}{dt}\ , 0 < t < t_e \quad (2)$$

where X is the concentration of viable cells (cell.mL^{-1}) and t is the culture time (h) during the exponential growth phase (from t = 0 and t = t_e).

During the cell growth phase, the specific rates of lactate formation (r_{Lac}), and glucose and glutamine consumption (r_{Glc} and r_{Gln}), are estimated by:

$$r_{Lac}(\text{nmol}.10^6\text{cell}^{-1}.\text{h}^{-1}) = Y_{X/Lac}\cdot\mu \quad (3)$$

$$r_{Glc}(\text{nmol}.10^6\text{cell}^{-1}.\text{h}^{-1}) = Y_{X/Glc}\cdot\mu \quad (4)$$

$$r_{Gln}(\text{nmol}.10^6\text{cell}^{-1}.\text{h}^{-1}) = Y_{X/Gln}\cdot\mu \quad (5)$$

where μ is the cell growth rate (h^{-1}, as described in Equation (2)), and $Y_{X/j}$ is the yield of metabolite j consumed (Glc or Gln) or produced (Lac) per biomass (X) formed, which is defined as follows:

$$Y_{X/Lac}(\text{nmol}.10^6\text{cell}^{-1}) = \frac{\Delta\,\text{Lac}}{\Delta\,X}\,, 0 < t < t_e \quad (6)$$

$$Y_{X/Glc}(\text{nmol}.10^6\text{cell}^{-1}) = \frac{-\Delta\,\text{Glc}}{\Delta\,X}\,, 0 < t < t_e \quad (7)$$

$$Y_{X/Gln}(\text{nmol}.10^6\text{cell}^{-1}) = \frac{-\Delta\,\text{Gln}}{\Delta\,X}\,, 0 < t < t_e \quad (8)$$

during the exponential growth phase (from t = 0 to t = t_e).

During the production phase (i.e., infection), the yield of product (HA) formed per substrate (Glc or Gln) consumed is defined as follows:

$$Y_{Glc/HA}(\text{HA titer}.\text{nmol}^{-1}) = \frac{\Delta\,\text{HA}}{-\Delta\,\text{Glc}}\,, 0 < t < t_f \quad (9)$$

$$Y_{Gln/HA}(\text{HA titer}.\text{nmol}^{-1}) = \frac{\Delta\,\text{HA}}{-\Delta\,\text{Gln}}\,, 0 < t < t_f \quad (10)$$

where HA is the extracellular HA titer (HA titer.mL^{-1}), Glc is the concentration of glucose (mM) and Gln is the concentration of glutamine (mM) during the production phase (from t = 0 to t = t_f).

2.8. Statistical Analysis

Data were expressed as the mean ± standard deviation from three independent biological replicates. Differences in cell-specific HA productivity between adapted and non-adapted cells were tested by a one-way ANOVA using Dunnett's multiple comparison analysis method (adjusted p-value < 0.05 was considered statistically significant). Pearson's correlation (r) was used to analyze the similarity between the shake-flask and stirred-tank bioreactor (r = 1 representing the best linear fit between the data) in terms of extracellular HA titer.

3. Results

3.1. Increased Production of Influenza HA-Vlps in Adapted Cells

Aiming at improving the production yields of influenza HA-VLPs, High Five insect cells were adapted to grow at a neutral culture pH (7.0) instead of a standard culture pH (6.2). Given the significant difference between the standard and neutral pH, a single step change in culture pH resulted in a loss of cell viability and incapacity to maintain cells in culture (data not shown). Thus, a stepwise approach was used for the adaptation process, in which the culture pH was gradually increased from standard 6.2 to 6.5, 6.8 and finally to 7.0. Upon each change in culture pH, a decrease in cell viability and growth rate was observed, but standard values were quickly re-established. Adaptation to each culture pH endured for 2–3 weeks. In approximately 8 weeks, cells were fully adapted to grow at a neutral pH, showing a growth rate similar to that of non-adapted cells cultured at a standard pH (Figure 1A). Importantly, adapted cells were able to maintain their growth rate (≈ 0.04 h^{-1}, i.e., ≈ 18 hours per generation) and viability for more than 100 generations (Figure 1B), thus demonstrating their stability when routinely cultured in such a non-physiological condition (i.e., neutral pH).

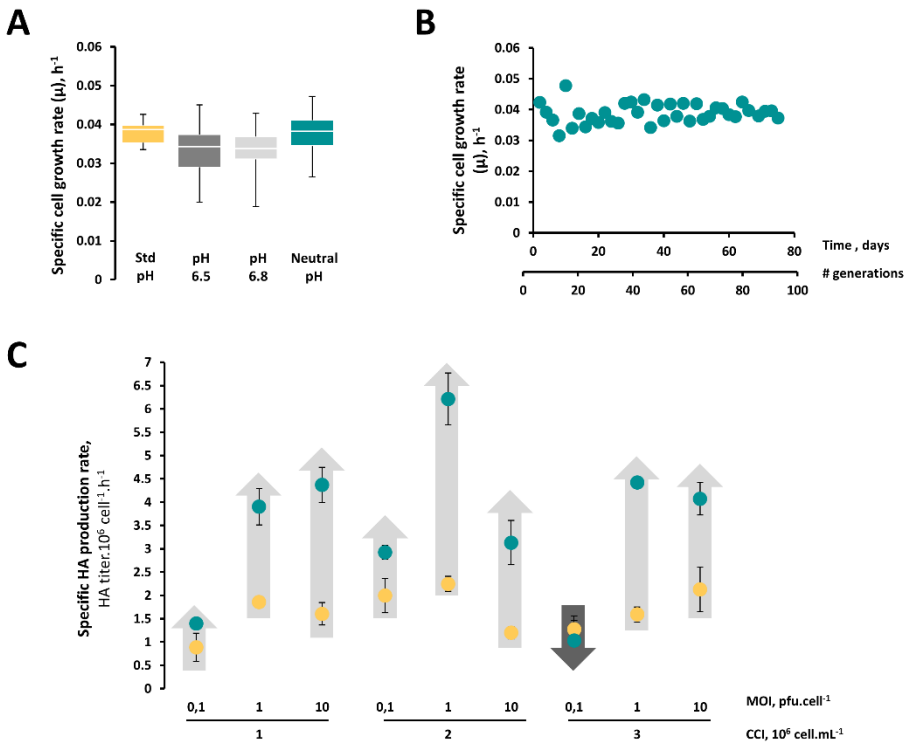

Figure 1 Adaptation of High Five insect cells to neutral culture pH for influenza hemagglutinin (HA)-displaying virus-like particle (VLP) production. (A) Box-plot diagrams showing the degree of dispersion and skewness in specific cell growth rate (μ, h−1) values during the adaptation process; horizontal lines are medians, boxes represent the interquartile range, and error bars show the full range of values. (B) Scatter graph representing the variation in growth rate of adapted cells over 100 generations after establishment of master cell bank. (C) Specific HA production rate of adapted (green circles) and non-adapted (yellow circles) cells at different cell concentration at the time of infection (CCI)/multiplicity of infection (MOI) combinations; arrows represent the variation in specific HA production rates from non-adapted to adapted cells, where light grey denotes an increase and dark grey denotes a decrease (cell-specific production rate was calculated as described in M&M section)—data represent one biological replicate (n = 1).

The capacity of adapted cells to produce HA-VLPs was evaluated at different combinations of MOIs (0.1, 1 and 10 pfu.cell^{-1}) and CCIs (1, 2 and 3 × 10^6 cell.mL^{-1}), and compared to that of non-adapted cells. Adapted cells performed better than their non-adapted counterparts in all combinations tested as

depicted by an increase in specific productivity (Figure 1C), the exception being the combination MOI of 0.1 pfu.cell^{-1} and CCI of 3 × 10^6 cell.mL^{-1}.

The best infection condition was achieved using a CCI of 2 × 10^6 cell.mL^{-1} and MOI of 1 pfu.cell^{-1} as it maximized the cell-specific HA production rate (6.2 HA titer.10^6 cell^{-1}.h^{-1}) concomitantly with the HA titer (768 HA titer.mL^{-1}) (see Figure S1). The specific HA productivity of adapted cells was similar at different generations after the establishment of the master cell bank using the best infection condition (6.7 ± 0.6, 5.3 ± 0.5 and 5.8 ± 0.5 HA titer.10^6 cell^{-1}.h^{-1} at generations 10, 40 and 80, respectively). These infection parameters were used in subsequent studies for the characterization of the adapted cell population.

3.2. The adaptation process (and not pH shift) is the element driving increased specific HA productivity.

To evaluate if a pH shift at the time of infection is sufficient to increase HA production in non-adapted cells, these were infected either in standard pH 6.2 culture medium ($CM_{pH6.2}$) or in neutral pH culture medium (CM_{pH7}). Similarly, adapted cells were infected either in CM_{pH7} or in $CM_{pH6.2}$.

Non-adapted cells performed similarly when producing in CM_{pH7} and $CM_{pH6.2}$ (Figure 2A), meaning that a pH shift to a neutral pH upon infection does not suffice for improving specific HA productivity. In fact, a pH shift at the time of infection can even be detrimental for HA production, as exemplified for adapted cells, with a 40% reduction in the cell-specific HA production rate and 25% decrease in the total number of cells during the production phase (i.e., integral of total cell concentration, ITCC) when infection of these cells was performed in $CM_{pH6.2}$ rather than their optimal $CM_{pH7.0}$. Overall, cell-specific HA

productivity was higher in adapted cells when compared to non-adapted cells, regardless of the culture medium pH. A Western blot analysis corroborates these findings, with HA protein expression at the time of harvest (i.e., at cell viability ≈ 30–45%) being superior in adapted cells (Figure 2B) as compared to non-adapted cells. These results demonstrate that the adaptation process, rather than the pH shift at the time of infection, is essential to achieve a higher specific HA productivity.

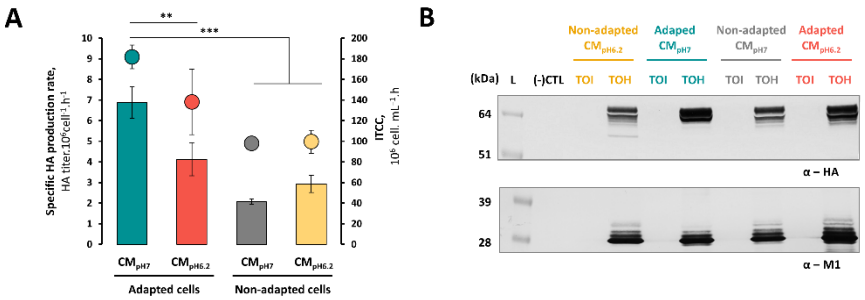

Figure 2 Impact of pH shift on HA production. (A) Bars represent specific HA productivity (HA titer.10^6 $cell^{-1}.h^{-1}$) and dots represent total number of cells during production phase (integral of total cell concentration, ITCC, 10^6 $cell.mL^{-1}.h$) of adapted and non-adapted cells in neutral pH culture medium (CM_{pH7}) or in standard pH 6.2 ($CM_{pH6.2}$); (B) identification of HA and M1 proteins by Western blot; samples were collected at the time-of-infection (TOI) and time-of-harvest (TOH) for adapted and non-adapted cells in neutral pH culture medium (CM_{pH7}) or in standard pH 6.2 ($CM_{pH6.2}$). Samples loaded are clarified supernatants, non-diluted; same volume was loaded in the gel for all samples. Relative band intensities for TOH samples (overproduction with non-adapted cells in $CM_{pH6.2}$) are 1.5:0.8:1.6 (for HA) and 1.1:0.8:1.5 (for M1) for adapted cells-CM_{pH7}: non-adapted cells-CM_{pH7}: adapted cells-$CM_{pH6.2}$, respectively. Uncropped membranes can be observed in Supplementary Figure S2. For Figure (A): data are relative to three biological replicates (n = 3); differences were tested with one-way ANOVA using Dunnett's multiple comparisons test (** = adjusted p-value < 0.005 and *** = adjusted p-value < 0.001 were considered statistically significant). For Figure (B): (-) CTL denotes negative control (supernatant of non-infected cells) and L denotes pre-stained protein standard SeeBlue® Plus2.

3.3. Delayed Cell Lysis and Higher Specific HA Production Rate of Neutral pH Adapted Cells

To investigate the impact of the adaptation process on infection kinetics (including baculovirus replication) and HA expression, adapted and non-adapted cells were cultured in their respective media (CM_{pH7} and $CM_{pH6.2}$, respectively) and infected under the best conditions identified above (CCI of 2 × 10^6 cell.mL^{-1} and MOI of 1 pfu.cell^{-1}).

Differences in infection kinetics (i.e., drop in cell viability) and HA expression (i.e., extracellular HA titer) were observed (Figure 3A). Adapted cells maintained higher cell viability values throughout the course of infection in comparison with non-adapted cells, delaying the onset of the cell viability drop by 20-40 h. In addition, extracellular HA titers are higher in adapted cells irrespective of the culture time; at the time of harvest, this difference reaches ≈ 4-fold. To ensure that titers are not being overestimated by the presence of baculovirus particles carrying HA in culture medium, infectious and total baculovirus titers were assessed (Figure 3B). The increase in infectious baculovirus titers (pfu.mL^{-1}) as cell viability decreases throughout the infection is similar in adapted and non-adapted cells. The same pattern was observed for total baculovirus particles (IE1 genome copies.mL^{-1}), thus suggesting that the increased extracellular HA titer obtained with adapted cells does not derive from baculovirus particles carrying HA but rather from the synergistic effect of a higher total number of cells during the production phase—i.e., ITCC, 10^6 cell.mL^{-1}.h (≈ 1.34-fold)—and specific HA productivity, i.e., r_{HA}, HA titer.cell^{-1}.h^{-1} (≈ 3-fold) (Figure 3C).

The consumption of glucose (Glc) and glutamine (Gln), and the production of lactate (Lac) were followed throughout the culture, and their specific

consumption (r_{Glc} and r_{Gln}) or production (r_{Lac}) rates were estimated accordingly (Figure 3D).

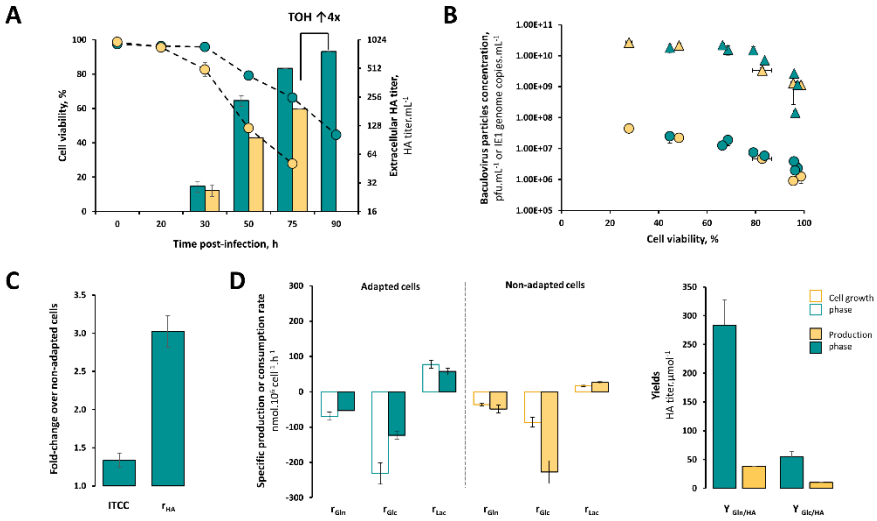

Figure 3 Infection kinetics, HA expression and cell metabolism read-outs of adapted and non-adapted High Five cells during production of influenza VLPs. (A) Cell viability (%) and extracellular HA titer (HA titer.mL^{-1}) profile upon infection; (B) infectious (circles, pfu.mL^{-1}) and total (triangles, IE1 genome copies.mL^{-1}) baculovirus titers upon infection; (C) fold-change (adapted/non-adapted) of total number of cells during production phase (ITCC, 10^6 cell.mL^{-1}.h) and specific HA productivity (r_{HA}, HA titer.cell^{-1}.h^{-1}); (D) specific consumption rate of glucose (r_{Glc}, nmol.10^6 cell^{-1}.h^{-1}) and glutamine (r_{Gln}, nmol.10^6 cell^{-1}.h^{-1}), specific production rate of lactate (r_{Lac}, nmol.10^6cell^{-1}.h^{-1}), and yields of product (HA) formed per substrate (Glc or Gln) consumed; i.e., $Y_{Glc/HA}$ and $Y_{Gln/HA}$. Color code: green represents data for adapted cells, and yellow represents data for non-adapted cells. Data are expressed as mean ± standard deviation (relative to three biological replicates, n = 3).

During the growth phase, the r_{Glc} (nmol. 10^6 cell^{-1}.h^{-1}) and r_{Gln} (nmol.10^6 cell^{-1}.h^{-1}) of adapted cells were 2.7- and 2.0-fold higher than those of non-adapted cells. Likewise, lactate was also produced at a higher rate in adapted

cells; nonetheless, this byproduct did not accumulate growth-impairing values as the concentration achieved was ≈ 3-fold lower than that reported in the literature as the maximum for insect cells (15 mM (Palomares and Ramirez, 1996)).

Upon infection, r_{Glc}, r_{Gln} and r_{Lac} changed in both adapted and non-adapted cells, with a reduction in all rates in adapted cells contrasting with an increase in non-adapted cells.

These differences in cell metabolism reflect a more efficient utilization of carbon and nitrogen sources for product formation in adapted cells as suggested by the higher yields obtained (i.e., $Y_{Glc/HA} \approx 5$ and $Y_{Gln/HA} \approx 7$-fold).

3.4. Proof-of-Concept at the 0.5 L Bioreactor Scale

The feasibility of adapted cells to produce influenza HA-VLPs in a computer-controlled and scalable system was confirmed in 0.5 L stirred-tank bioreactors (STBs). Adapted cells at generation 53 were infected in STBs and shake flasks (SFs) using previously optimized conditions (CCI = 2×10^6 cell.mL^{-1} and MOI = 1 pfu.cell^{-1}); the infection kinetics and HA titer obtained in both culture systems were compared.

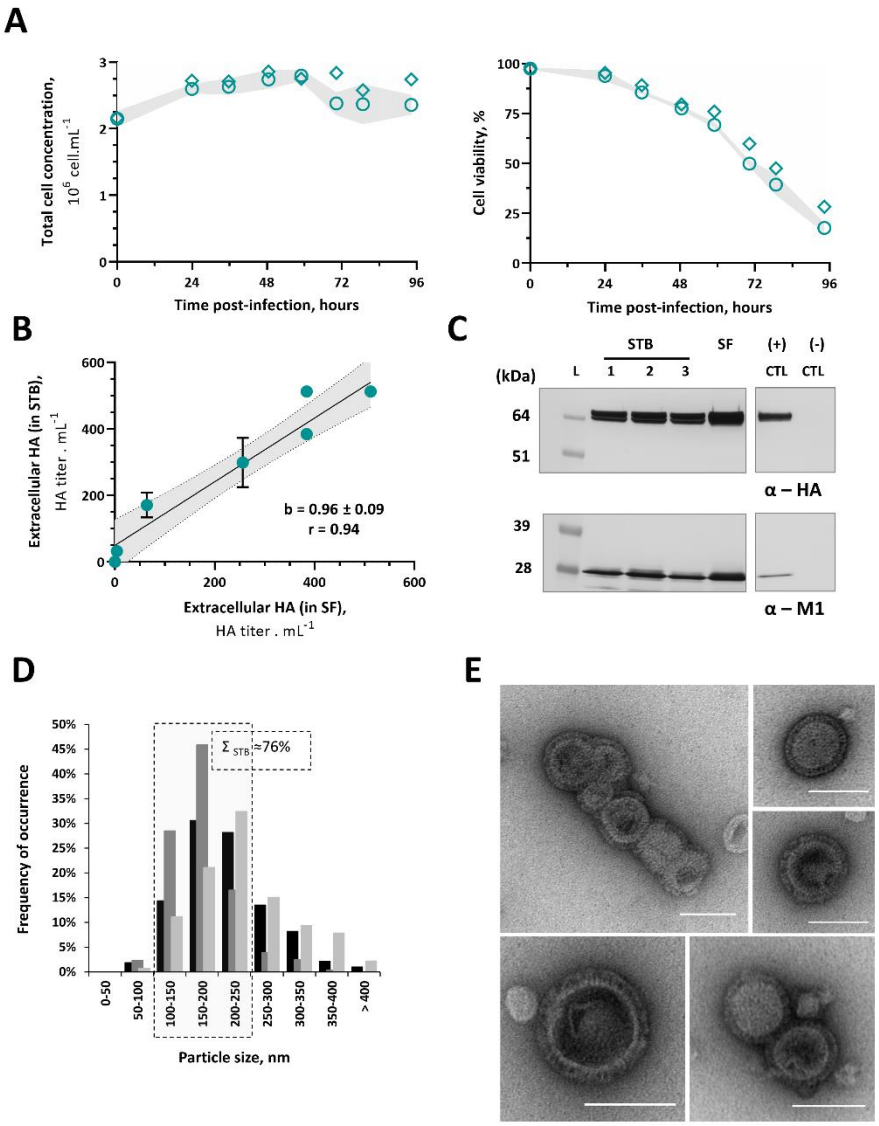

Figure 4 Production of influenza VLPs at a 0.5 L bioreactor scale. (A) Cell growth and viability kinetics upon infection in three stirred-tank bioreactors (STBs, circles) and one control shake flask (SF, diamonds), with the grey band representing standard deviation (relative to three biological replicates, n = 3) from STB cultures; (B) comparison of extracellular HA titers throughout infection between STB and SF, with straight line representing the best linear fit to the data (r—Pearson's correlation, m—regression slope) and the grey band representing a 95% confidence level. Data are expressed as mean ± standard deviation (relative to three biological replicates,

n = 3) from STB cultures. (C) identification of HA and M1 proteins by Western blot in in-process samples at the time of harvest from three STB cultures and one control SF culture. Samples loaded are clarified supernatants, non-diluted; same volume was loaded in the gel for all samples. (+) CTL (positive control): master virus seed stock used for influenza VLPs production. (-) CTL (negative control): supernatant of non-infected cell culture. L: Pre-stained protein standard SeeBlue® Plus2; Relative band intensities (overproduction in SF) for STB1: STB2: STB3 are 0.8: 0.9: 0.9 (for HA) and 0.8: 1.1: 0.8 (for M1). Uncropped membranes can be observed in Supplementary Figure S3. (D) Histogram showing the size distribution profile of HA-VLPs obtained by nanoparticle tracking analysis (binned by 50 nm intervals from 0 to >400 nm) in purified HA-VLP samples. Overlapping bars represent the three bioreactor runs. The average percentage of HA-VLPs in the expected size range of 100–250 nm is highlighted; (E) negative-staining transmission electron microscopy of purified HA-VLPs. Scale bar represents 100 nm.

Cell growth and viability kinetics in STBs and SFs were similar, showing traditional profiles of insect cells upon infection with an MOI of 1 pfu.cell^{-1} (Figure 4A). Likewise, extracellular HA titers throughout infection were similar between STBs and SFs, with a regression coefficient (b) and Pearson's correlation (r) close to 1 (Figure 4B). Both HA and M1 proteins could be identified at the time-of-harvest in supernatant samples from STB and SF cultures by Western blot (Figure 4C), showing similar level of HA expression. Influenza HA-VLPs produced in STB were concentrated and purified as mentioned in M&M, and then analyzed by nanoparticle tracking analysis (Figure 4D) and negative staining TEM (Figure 4E). A normalized frequency histogram of size distribution confirms the presence of a single peak corresponding to the normal-size range of M1-HA-VLPs (≈ 100–250 nm) (Figure 4D). The presence of particles resembling HA-VLPs, both in size and morphology, was confirmed by TEM (Figure 4E). These results confirm the scalability of the strategy herein proposed (i.e., High Five insect cells adapted to grow at neutral culture pH instead of standard pH 6.2) for the production of influenza HA-VLPs.

4. Discussion

In this work, we adapted the High Five insect cell line to grow at neutral culture pH (7.0) and evaluated its potential to be used for the production of influenza HA-VLPs.

High Five cells were efficiently adapted to grow at neutral culture pH (7.0), showing similar specific growth rates (≈ 0.04 h^{-1}) and morphologic characteristics (round shape with diameter ≈ 16–17 µm (Huynh et al., 2015; Sander and Harrysson, 2007)) to non-adapted cells. In addition, adapted cells were efficiently maintained in culture for over 100 population doublings, thus demonstrating their stability to be routinely cultured in such a non-standard condition. The capacity of these cells for the improved production of HA-VLPs was demonstrated at different combinations of CCIs and MOIs, with CCI = 2 × 10^6 cell.mL^{-1} and MOI = 1 pfu.$cell^{-1}$ maximizing the specific HA production rate (6.2 HA titer.10^6 $cell^{-1}$.h^{-1}) and extracellular HA titer (768 HA titer.mL^{-1}). Under this optimal condition, adapted cells not only expressed more protein (up to 4-fold higher HA titer.mL^{-1}) but also showed a delayed onset of cell viability drop (up to 20-40 h) when compared to non-adapted cells, thus enabling (i) harvesting the same amount of HA protein at a higher cell viability, which facilitates downstream processing and prevents HA degradation by proteases, or (ii) harvesting significantly higher amounts of HA proteins, which reduces working volumes and increases production load. Further process optimization may be possible through the enrichment of an adaptation medium in essential nutrients (e.g., glucose and glutamine) to values similar to those of a standard medium, given that adapted cells show a more efficient utilization of carbon and nitrogen sources for product formation.

Shifts in culture parameters to non-physiological values have been shown to impact the growth performance and recombinant protein production yields of prokaryotes (Çalık et al., 2010; Jazini and Herwig, 2013; Wang et al., 2014) and other higher level organisms (Lin et al., 2015; Reuveny et al., 1993; Rossi et al., 2012; Seo et al., 2013; Shao-Hua et al., 1998). In this study, shifting the culture pH from standard 6.2 to 7.0 was not sufficient for improving HA production in parental, non-adapted cells. In adapted cells, a pH shift (from 7.0 to standard 6.2) was even detrimental for HA production. Osmolarity could possibly play a role on productivity (Olejnik et al., 2003). However, our data show that culture media at pH 6.2 and 7.0 have similar osmolarities, i.e., 344 and 363 mOsm, respectively, suggesting this is not contributing to the higher productivity of adapted cells. Therefore, it is possible to conclude that the adaptation process is essential to improve productivity and that infection at a neutral pH is required to maximize titers. Adaptation rather than culture parameter shifts have also been reported elsewhere as necessary to improve the production of recombinant proteins (Fernandes et al., 2020; Wagner et al., 2014; Yoon et al., 2006).

The production of HA-VLPs using adapted cells was scaled-up from the SF to the 0.5 L STB. Cell growth and HA production kinetics were similar between SFs and STBs, thus resulting in similar specific production rates. HA and M1 proteins were identified in both STB and SF cultures. The TEM analysis of purified HA-VLPs revealed a multi-component organization and the presence of HA molecules (spikes) uniformly displayed on VLPs' surfaces, similar to HA-VLPs shown in other reports (McCraw et al., 2018). Overall, these results confirm (i) the null impact of adaptation process and neutral culture pH on the quality of HA-VLPs produced, and (ii) the feasibility of the strategy herein

proposed (i.e., adaptation of High Five cells to neutral culture pH) for an improved production of HA-VLPs.

The mechanism(s) underlying the higher capacity of neutral pH-adapted cells to produce HA remain(s) unclear. Importantly, the proteolytic activity of each particular cell or viral protease varies differently depending on culture pH, potentially involving a loss of the product of interest (Ikonomou et al., 2003). Moreover, it has been shown that changes in the extracellular pH of mammalian cell cultures induces fluctuations in intracellular pH (Michl et al., 2019), with varying impacts on cell metabolism (Fellenz and Gerweck, 1988), enzyme activity (Ikonomou et al., 2003) and protein synthesis (Madshus, 1988). Unlike mammalian cells, insect *Sf*-9 cells have a strong buffering capacity, maintaining intracellular pH at a neutral value even upon variations in culture pH of up to 6.8 (from standard culture medium pH 6.2) or infection with recombinant baculoviruses (Medina et al., 1995). Assessing the intracellular pH of adapted and non-adapted cells during growth and HA-VLPs production would clarify if these values differ and, if so, help dissect its impact on cell behavior including metabolism and enzyme activity (cellular and viral) and thus protein production. Regardless, the smaller difference between the extracellular and intracellular pH of adapted cells could possibly result in less energy waste to maintain homeostasis, thus benefiting recombinant protein production.

Different cell lines and/or expression systems have been employed for the production of influenza HA-VLPs, yielding 128 HA titer.mL^{-1} (Sequeira et al., 2018), 16 HA titer.mL^{-1} (Thompson et al., 2015), or even above 300 HA titer.mL^{-1} (Krammer et al., 2010; Lai et al., 2019; Park and Song, 2017). Noteworthy, the production process herein developed was shown to yield an extracellular HA titer similar to those reported when using non-adapted cells

(192 HA titer.mL^{-1}) or higher when using adapted cells (768 HA titer.mL^{-1}). Regardless, the process yield can be significantly influenced by factors such as the culture medium, quality of virus stock, or even the HA strain being produced.

5. Conclusion

This work demonstrates the use of ALE as a valuable approach to increase the production yields of influenza HA-VLPs in High Five insect cells. A new cell population efficiently adapted to grow at a neutral pH, which induced higher cell-specific HA productivity and extracellular HA titers, and a showed delayed onset of the cell viability drop, thus enabling users to anticipate harvesting or to harvest larger amounts of protein. It is noteworthy that the adaptation process (and not pH shift) is essential for increased specific HA productivity.

To our knowledge, this is the first report exploring ALE in Trichoplusia ni insect cell lines and its impact on the production of an influenza vaccine candidate. The work here encourages the use of ALE as an alternative bioprocess strategy to improve production yields of other insect-derived biologics.

6. Supplementary Material

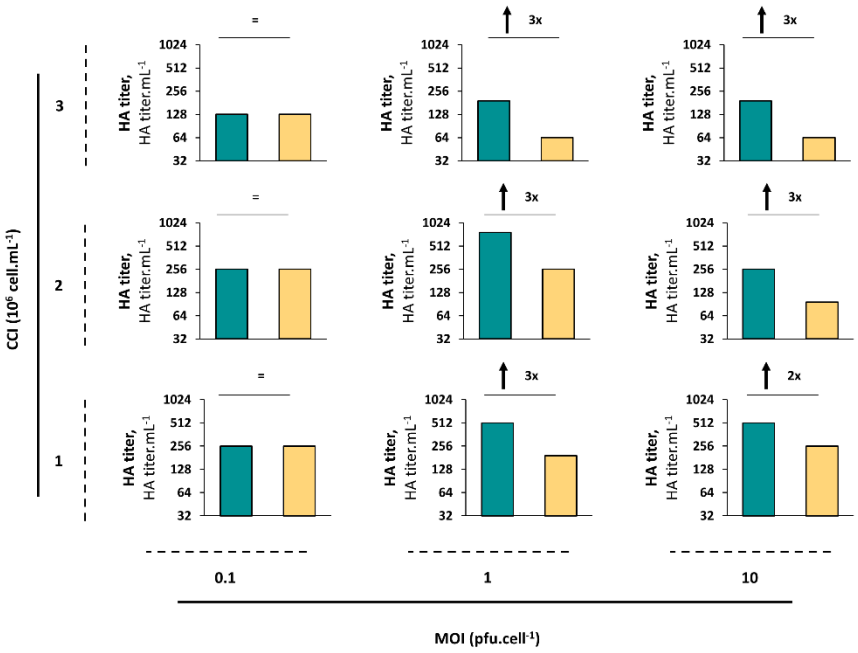

Figure S1 Extracellular HA titer (HA titer. mL^{-1}) of adapted cells (dark grey) and non-adapted cells (light grey) at different CCI/MOI combinations. Arrows and symbols next to each graph represent the variations in HA titer at the time-of-harvest from non-adapted to adapted cells for each CCI/MOI combination.

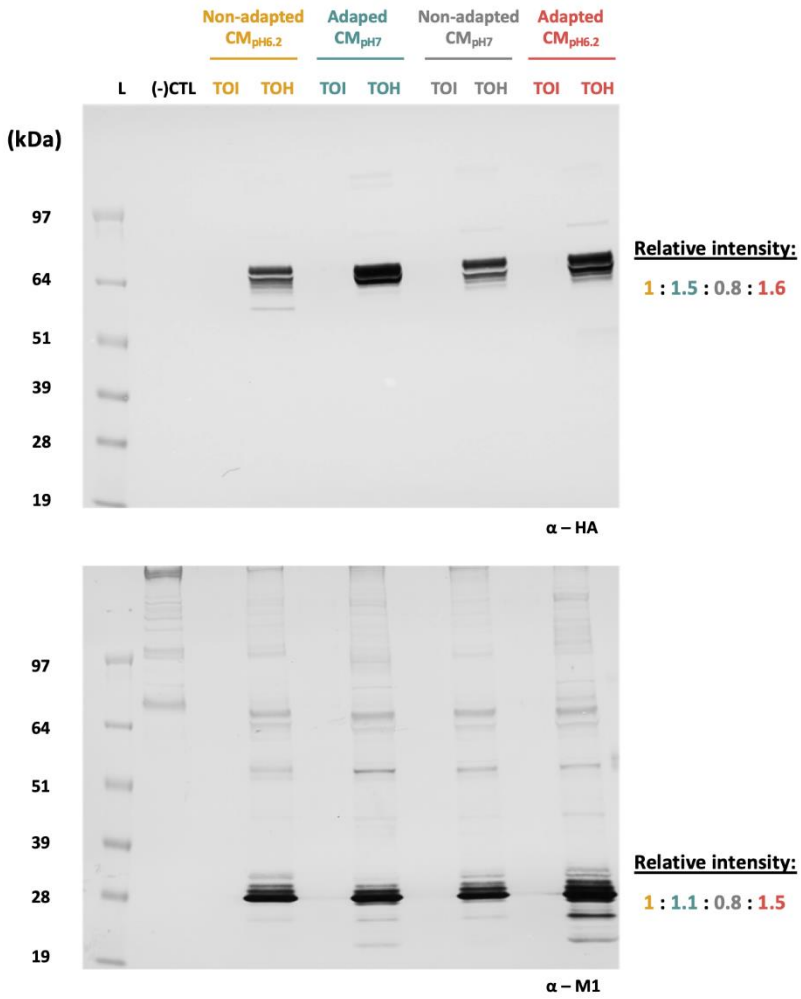

Figure S2 Identification of HA and M1 proteins by western blot; In-process samples were collected at the time-of-infection (TOI) and time-of-harvest (TOH) for adapted and non-adapted cells in neutral pH culture medium (CMpH7) or in standard pH 6.2 (CMpH6.2). Samples loaded are clarified supernatants, non-diluted; same volume was loaded in the gel for all samples. Band intensities for TOH samples are shown and are normalized to production performed with non-adapted cells in CMpH6.2.

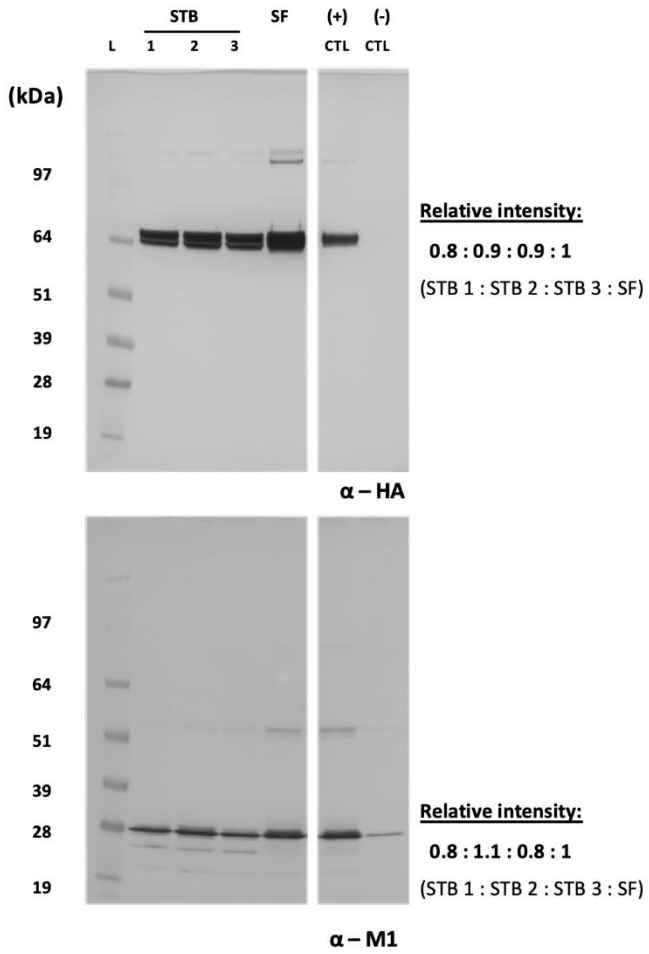

Figure S3 Identification of HA and M1 proteins by Western blot in in-process samples at the time of harvest from three STB cultures and one control SF culture. Samples loaded are clarified supernatants, non-diluted; same volume was loaded in the gel for all samples. (+) CTL (positive control): master virus seed stock used for influenza VLPs production. (-) CTL (negative control): supernatant of non- infected cell culture. L: Pre-stained protein standard SeeBlue® Plus2. Band intensities are normalized to production performed in SF.

7. Acknowledgments

This work was supported by EU-funded project “EDUFLUVAC” (FP7-HEALTH-2013-INNOVATION−1, GA n. 602640) and by the Portuguese “Fundação para a Ciência e a Tecnologia” (FCT) through the following initiatives: “Investigador FCT” Program (IF/01704/2014), Exploratory Research and Development Project (IF/01704/2014/CP1229/CT0001), and PhD fellowships (Ricardo Correia — SFRH/BD/134107/2017, Bárbara Fernandes — FRH/BD/138937/2018). The authors wish to thank: Marcos Sousa for the useful discussion and support in bioreaction, Mafalda Moleirinho and Ana Sofia Moreira for the support in nanoparticle analysis and qPCR and to Sara Bonucci and E.M. Tranfield from the Electron Microscopy Facility at Instituto Gulbenkian de Ciência for technical support in TEM.

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Chapter 3

Improve storage of influenza VLPs using water-free formulations

This chapter was based on the following manuscript:

Ricardo Correia, Liane Meneses, Carolina Richheimer, Paula M. Alves, Manuel J.T. Carrondo, Ana Rita C. Duarte, Alexandre Paiva, António Roldão, **(2021)**, "Improved storage of influenza HA-VLPs using a trehalose-glycerol natural deep eutectic solvent system." Vaccine 39, 3279–3286.

Author contribution

Ricardo Correia design and performed the experiments, analysed the data and wrote the chapter.

Abstract

Vaccines are typically stored under refrigerated conditions (2-8 °C) to limit potency and efficacy loss. Maintaining the cold chain is costly and prone to vaccine wastage due to unexpected changes in the storage conditions. The development of formulations conferring enhanced stability can extend vaccine shelf-life and facilitate storage under non-refrigerated conditions, thus simplifying the distribution process and reducing vaccine wastage.

In this study, the suitability of a natural deep eutectic system (NADES) consisting of trehalose and glycerol (TGly) for storage of influenza hemagglutinin (HA)-displaying virus-like particles (VLPs) was successfully demonstrated. TGly was efficient in maintaining the activity and physical integrity of HA-VLPs for up to 4 h at 50 °C (accelerated stability study), with half-life values ≈ 20-34 h for the best TGly formulations. Importantly, improved storage is achieved at increasingly higher TGly concentrations, hence confirming the importance of TGly content in its protective capacity. In addition, HA-VLPs were stable in TGly for over one month at room temperature (short-term stability study), with no impact on HA titer or particle size distribution.

This work highlights the potential of NADES to improve stability of VLP-based vaccines, showing promising protective capacity under non-refrigerated conditions and short-term thermal stress, and thus having a notable impact on vaccine's cold chain.

TABLE OF CONTENTS

1. Introduction

Influenza virus infects nearly 1 in 8 people every year, resulting in 3-5 million hospitalizations and up to 650.000 cases of influenza related deaths (Girard et al., 2005; "World Health Organization. 'Up to 650 000 people die of respiratory diseases linked to seasonal flu each year.' Accessed: 1 July 2020," 2017). Prophylactic vaccination remains the recommended approach by the World Health Organization to reduce influenza illness, providing immunity against the currently circulating seasonal influenza virus strain, while being cost-effective (Gerdil, 2003; Preaud et al., 2014).

Influenza vaccines are generally stored under refrigerated conditions (2-8 °C) to limit potency and efficacy loss. This requirement entails a significant financial burden as 20-65 % of the vaccines' price accounts for maintaining the vaccine cold chain (Lydon et al., 2014). Interruptions in the cold chain frequently occur and are responsible for around 50 % of vaccine wastage as estimated by WHO ("World Health Organization. 'Monitoring vaccine wastage at country level : guidelines for programme managers.' Accessed 1 July 2020," 2005). Enhancing vaccines stability to reduce wastage and storage costs is thus of utmost importance. Aqueous solvents are usually utilized for vaccine storage, phosphate buffer saline being preferably used in influenza vaccines' formulations (White et al., 2016), including the recombinant influenza vaccine Flublok (Cox and Hollister, 2009). Cold chain interruptions and/or storage malpractices often expose vaccines to adverse conditions, favouring mechanisms of degradation such as hydrolysis. Additionally, over-refrigeration may lead to the formation of ice crystals, having significant impact on vaccines containing freezing-sensitive viruses. Removing water from vaccines storage formulations reduces the risk of hydrolysis, whereas the use of a solvent with a lower melting point minimizes the risk of undesired vaccine freezing.

Lyophilization is commonly used in the pharmaceutical industry for storage of drug products. As for, lyophilization increases shelf-life over aqueous solvent options (Saluja et al., 2010). Also, vaccine's active ingredients are often stabilized by using excipients like amino acids, antioxidants, proteins, sugars, polyols or surfactants in their storage formulation (Cardoso et al., 2017). These include trehalose, known to inhibit protein aggregation and to stabilize influenza virus-like particles (Kim et al., 2010), and glycerol, commonly used in commercial vaccine formulations to prevent aggregation and to maintain antigen structure and potency (Braun et al., 2009).

Natural deep eutectic systems (NADES) are mixtures of two or more compounds, such as the excipients above, that when combined at certain molar ratios become liquid at room or near room temperature (Domínguez de María, 2017). These systems have immense potential as solvents in many applications, from extraction and biocatalysis to clinical therapy. Recently NADES have been used for the storage of human interferon-α2 at room temperature (Lee et al., 2018) and as a stabilizer and carrier of live attenuated vaccines (Vermeij et al., 2019). By containing low amount of water, the use of NADES in storage formulations presents great promise to extend vaccines' shelf-life in non-refrigerated conditions thus facilitating storage, distribution, and administration processes. Additionally, vaccine storage in NADES at room temperature would have a significant economic impact by replacing the refrigerated vaccine storage and transportation facilities which commonly present a significant economic burden.

In this work, the potential of a NADES composed of trehalose and glycerol (TGly) at a molar ratio 1:30 (Castro et al., 2018) was assessed for the storage of influenza hemagglutinin (HA) displaying virus like-particles (VLPs), aiming at

establishing a storage formulation conferring improved vaccine protective capacity.

2. Materials and Methods

2.1. Production of influenza HA-VLPs

Influenza HA-VLPs composed of M1 (from A/California/06/2009 H1N1 strain) and HA (from A/Fujian/411/2002 H3N2 strain) were produced using insect High Five cells and the baculovirus expression vector system as described elsewhere (Sequeira et al., 2018). In particular, production was carried out in 10 L wave bioreactors, whereas midstream and downstream processes were carried out based in the purification steps described elsewhere (Carvalho et al., 2019b, 2019a, 2017) with minor changes. Specifically, the size-exclusion chromatographic step previously reported (Carvalho et al., 2017) was replaced by a combination of tangential flow filtration steps, as described (Carvalho et al., 2019a). Purified HA-VLPs were formulated with 50 mM HEPES, 300 mM NaCl, and trehalose 15 % (w/v) at pH 7.4 (from now on named “control storage buffer formulation”), and stored at -80 °C. These frozen HA-VLPs were used as starting material for all experiments herein performed.

2.2. Preparation of natural deep eutectic solvent systems (NADES)

The natural deep eutectic system (NADES) composed of trehalose and glycerol was prepared as described elsewhere (Castro et al., 2018). Briefly, the compounds were weighed and mixed in a trehalose:glycerol molar ratio of 1:30 (from now on named “TGly”) and heated at 70 ^{0}C until a clear liquid was

formed, which contained 1.32 ± 0.05 % of residual water as assessed by Karl Fisher (KF) titration.

2.3. Lyophilization and reconstitution of HA-VLPs

HA-VLPs were freeze-dried directly using a FreeZone Plus 4.5 L Cascade Freezer Dry System (Labconco). Temperature and pressure of the collector chamber reached a maximum of -84 °C and 0.133 mBar, respectively. Importantly, upon lyophilization only water is removed and thus the remaining compounds of the initial HA-VLPs' storage formulation (HEPES, NaCl, trehalose) are maintained in the lyophilized mixture. For stability studies, freeze-dried HA-VLPs were reconstituted in either water (to restore control storage buffer formulation) or TGly formulations (15, 50 and 90% TGly diluted in water).

2.4. Stability studies

Two stability studies were performed: (i) an accelerated stability study at 50 °C for 72 h, and (ii) a short-term stability study at room temperature (20-23 °C) and 4 °C for 36 days. All experiments were performed in triplicate (n = 3).

2.5. Analytics

2.5.1. Physicochemical characterization of TGly systems

The water content of pure TGly was determined by KF titration performed in an 831 coulometer with generator electrode without diaphragm, using Hydranal Coulomat AG as reagent. Viscosity of TGly systems 15, 50 and 90 % containing HEPES (50 mM), NaCl (300 mM) and trehalose 15 % (w/v), and pure

TGly was determined using an MCR 102 rheometer (Anton Paar), following the protocol described elsewhere (Craveiro et al., 2019). Prior to viscosity measurements, the following components were mixed with the different TGly formulations to mimic the storage conditions of HA-VLPs during the stability tests to be performed: HEPES, NaCl and trehalose (final concentration of 50 mM, 300 mM, and 15 % (w/v), respectively). A constant shear rate of 10 s^{-1} was used. All measurements were performed in triplicate (n = 3).

2.5.2. Hemagglutination assay

HA titer was determined using hemagglutination assay, as described elsewhere (Correia et al., 2020). Briefly, samples (25 µL) were serially diluted 1:1 with DPBS (-/-) 1X (Gibco) in V-bottom 96-well plates (Thermo Scientific) and gently mixed 1:1 with 1 % chicken erythrocytes (Lohmann). Plates were incubated at 4 ºC for 30 minutes. HA titer was estimated as being the inverse of the highest dilution of sample that completely inhibited hemagglutination.

2.5.3. Western Blot

Western blot was performed as described elsewhere (Correia et al., 2020). For HA identification, a mixture of two sheep polyclonal antibodies (anti-A/Johannesburg/33/94 serum and anti-A/Nanchang933/1995 serum, NIBSC UK) was used at a dilution 1:1000 each. 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 alkaline phosphatase was used at a dilution of 1:1000 and 1:2000,

respectively. Protein band detection was performed with NBT/BCIP 1-Step (Thermo Scientific) and membranes scanned with a benchtop scanner device.

2.5.4. Nanoparticle tracking analysis

Particle size distribution was measured using NanoSight NS500 (Nanosight Ltd, UK). Briefly, samples were pre-diluted with DPBS (-/-) 1 X (Gibco) to cope with the instrument's range of analysis (10^8–10^9 particles.mL^{-1}). All measurements were performed at 22 °C. Sample videos (60 seconds) were analysed with the Nanoparticle Tracking Analysis (NTA) 2.3 Analytical software. Capture settings (shutter and gain) were adjusted manually.

2.5.5. Single radial immunodiffusion (SRID)

SRID was performed as described elsewhere (Schild et al., 1975). Biological reference materials from NIBSC (UK) were used for SRID, namely the influenza antigen A/Wyoming/03/03 and anti A/Wyoming/03/03 serum.

2.6. Mathematical equations to assess HA denaturation kinetics

The conversion of native HA form (HA_N) into the denatured HA form (HA_D) (Kumru et al., 2014) can be described by the following reaction, in which $k_{D,HA}$ (h^{-1}) represents the HA decay rate constant:

$$\mathrm{HA_N} \xrightarrow{k_{D,HA}} \mathrm{HA_D} \qquad (\mathrm{Eq.}\ 1)$$

The differential equation describing first-order kinetics is given below, where [HA] is the titer at each time point:

$$-\frac{d[HA]}{dt} = k_{D,HA}[HA] \quad , \quad 0 < t < t_f \qquad (Eq.\ 2)$$

Upon integration, $k_{D,HA}$ can be estimated for the phase of linear decrease in HA titer using the following equation, where $[HA]_0$ is the initial HA titer (prior to degradation):

$$\ln\left(\frac{[HA]}{[HA]_0}\right) = -k_{D,HA}t \quad , \quad 0 < t < t_f \qquad (Eq.\ 3)$$

The half-life of HA-VLPs ($t_{1/2}$, h), i.e. when $[HA]=1/2.[HA]_0$, can be estimated using the following equation:

$$t_{\frac{1}{2}} = \frac{\ln(1/2)}{-k_{D,HA}} \quad , \quad 0 < t < t_f \qquad (Eq.\ 4)$$

2.7. Statistical analysis

Data is presented as mean ± standard deviation. Differences were tested by One-Way ANOVA with Dunnett's multiple comparison analysis method or using unpaired t-test (adjusted p-value < 0.05 was considered statistically significant).

3. Results

3.1. Impact of lyophilization and reconstitution in TGly formulations on HA-VLPs

3.1.1. Assessing HA-VLPs degradation during stability studies

The standard analytical methods to measure HA concentration are the SRID and the hemagglutination assay. To ascertain whether these techniques are

suitable to monitor HA potency (i.e. HA concentration or titer) during stability tests, HA-VLPs in control storage buffer formulation were incubated at 50 °C for 24 hours and HA concentration assessed by both methods. The influenza antigen A/Wyoming/03/03 (NIBSC, UK) and the Influvac 2018/2019 vaccine were used as controls.

A significant decrease in HA titer was observed in all preparations using hemagglutination assay, with values between 70-100 %, but not SRID (Figure 1A). In fact, using SRID, we could only observe the degradation of HA from Influvac vaccine, with values around 80 %. Therefore, estimation of HA titer by hemagglutination assay was considered more efficient to predict degradation of HA and thus it was used to assess activity of HA-VLPs in further stability studies performed.

3.1.2. Impact of lyophilization on HA-VLPs

HA-VLPs were lyophilized, reconstituted in water, and then analysed. Results show a slight increase in peak particles size (84 ± 8 nm) as compared to non-lyophilized HA-VLPs (70 ± 10 nm), with no apparent impact on particles size distribution profiles (Figure 1B). Importantly, the lyophilization process seems to have no impact on HA titer, with similar values pre- (average of 1707 HA titer/mL) and post-lyophilization average of 2048 HA titer/mL). In addition, HA and M1 proteins could be identified by western blot in both lyophilized and non-lyophilized samples, with no apparent differences in bands intensities. Therefore, the lyophilization process was shown to have no impact on HA-VLPs.

3.1.3. Reconstitution of lyophilized HA-VLPs in TGly formulations: impact on particles integrity and activity

To evaluate the impact of formulating HA-VLPs in TGly systems, these were lyophilized, reconstituted in different TGly formulations (15, 50 and 90 % (w/v)), and then analysed.

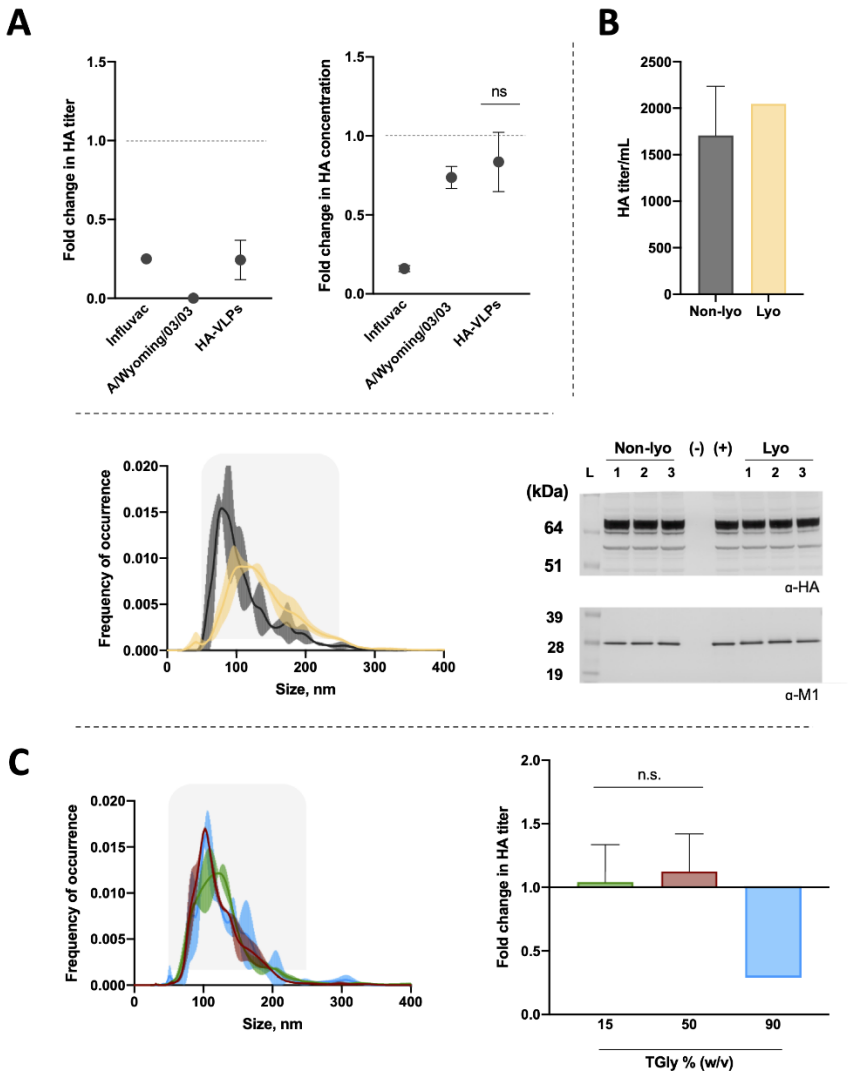


Figure 1 Assessment of HA activity and HA-VLPs physical integrity. (A) Degradation of HA from HA-VLPs, A/Wyoming/03/03 antigen and Influvac vaccine upon storage at 50 ºC for 24 hours, as

assessed by hemagglutination assay (left panel) and SRID (right panel). Bars represent mean fold-change over HA titer or concentration before exposure to high temperature, error bars represent standard deviation. (B) Activity and integrity of HA-VLPs pre- and post-lyophilization, as assessed by nanoparticle tracking analysis (left panel), hemagglutination assay (middle panel), and western blot (right panel). Non-lyo: purified HA-VLPs in control storage buffer formulation (non-lyophilized); Lyo: HA-VLPs lyophilized and reconstituted in water to restore control storage buffer formulation. (C) Activity and integrity of HA-VLPs reconstituted in TGly, as assessed by nanoparticle tracking analysis (left panel) and hemagglutination assay (right panel). Fold-change in HA titer is relative to non-lyophilized HA-VLPs. For nanoparticle tracking analysis: line represents mean and colored band represents standard deviation. Color code: black, non-lyophilized HA-VLPs; yellow, HA-VLPs lyophilized and reconstituted in water; green, HA-VLPs lyophilized and reconstituted in TGly 15 % (w/v); red, HA-VLPs lyophilized and reconstituted in TGly 50 % (w/v); blue, HA-VLPs lyophilized and reconstituted in TGly 90 % (w/v). Grey box represents expected size-range of HA-VLPs. For western blot, same volume was loaded in the gel for all samples; 1, 2 and 3 represent three biological replicates. (-) denotes negative control (water), (+) denotes positive control (viral stock used for HA-VLPs production) and L denotes pre-stained protein standard SeeBlue® Plus2. Data from three biological replicates (n = 3). Unpaired t-test (for (A)) or one-way ANOVA using Dunnett's multiple comparison test (for (C)) was used; ns = non-significant, adjusted p-value < 0.05 was considered statistically significant.

Upon reconstitution of the lyophilized HA-VLPs in TGly systems, peak particles size was observed at 90-98 nm, slightly above the 70 ± 10 nm observed for non-lyophilized HA-VLPs (Figure 1C). Nonetheless, particle size distribution profiles obtained in all preparations are similar and comparable to that of non-lyophilized HA-VLPs. Importantly, reconstitution of the lyophilized HA-VLPs in most TGly formulations was shown to have no impact on HA titer, the exception being TGly 90% (w/v). The approximate 70 % reduction in HA titer observed for this formulation can be explained by the incomplete re-suspension of HA-VLPs in such high viscosity preparation, and not by a degradation of the HA during the reconstitution process. In fact, viscosity decreases with increasing temperature and water content in TGly formulations (Supplementary Figure 1) and, importantly, TGly 90 % (w/v) showed a dynamic viscosity at room temperature above the recommended limit of 160 mPA.s for injectable vaccines (Cilurzo et al., 2011). This decay was considered when interpreting the data from the stability studies.

3.2. Accelerated stability study: impact of storage of HA-VLPs in TGly formulations in particles integrity and activity during exposure to high temperature

To evaluate the impact of TGly systems on preserving HA-VLPs integrity and activity, these were lyophilized, reconstituted in either water (control) or different TGly formulations (15, 50 or 90 % (w/v)), and then incubated at 50 °C for 72 h. HA titer and particle size distribution were assessed by hemagglutination assay and NTA, respectively, following 0, 2, 4, 24 and 72 h. The volume of each TGly formulation used for HA-VLPs reconstitution was adjusted to have a final glycerol content below the values reported for approved drug products as listed by FDA (Supplementary Figure 2).

HA titers were maintained at higher values following storage with TGly systems, and increasingly higher TGly content seems to be favourable (Figure 2A). Notably, a ≈ 60 % reduction in HA titer was observed after just 2 hours in control HA-VLPs, a value that is only reached at 24 h for TGly 50 % and 90 % (w/v). Regardless of the formulation used, only residual HA titer was quantified after 72 h. A first-order decay reaction, commonly used to describe the degradation of HA during initial assessment of vaccine stability (Kumru et al., 2014), was applied to estimate the HA degradation rate ($k_{D,HA}$) (Eq.3) and HA-VLPs half-life ($t_{1/2}$, Eq.4). $k_{D,HA}$ was higher in control storage buffer formulation (thus resulting in the lowest HA-VLPs half-life, $t_{1/2}$ = 3 h), and decreases with increasingly higher TGly content ($t_{1/2}$ = 12-34 h) . These results demonstrate that TGly is more efficient than control in preserving HA titre of HA-VLPs.

Particle size distribution suggests that HA-VLPs integrity is maintained for longer periods with increasingly higher TGly content, by showing a single and less disperse peak representing HA-VLPs (Figure 2B). Contrastingly, HA-VLPs in

control storage buffer formulation showed highly heterogeneous particle size distribution in the range of 0-400 nm even after short periods (2-4 hours) of incubation at 50 °C. In addition, a significant amount of particles with sizes below 50 nm was detected, suggesting disruption of HA-VLPs.

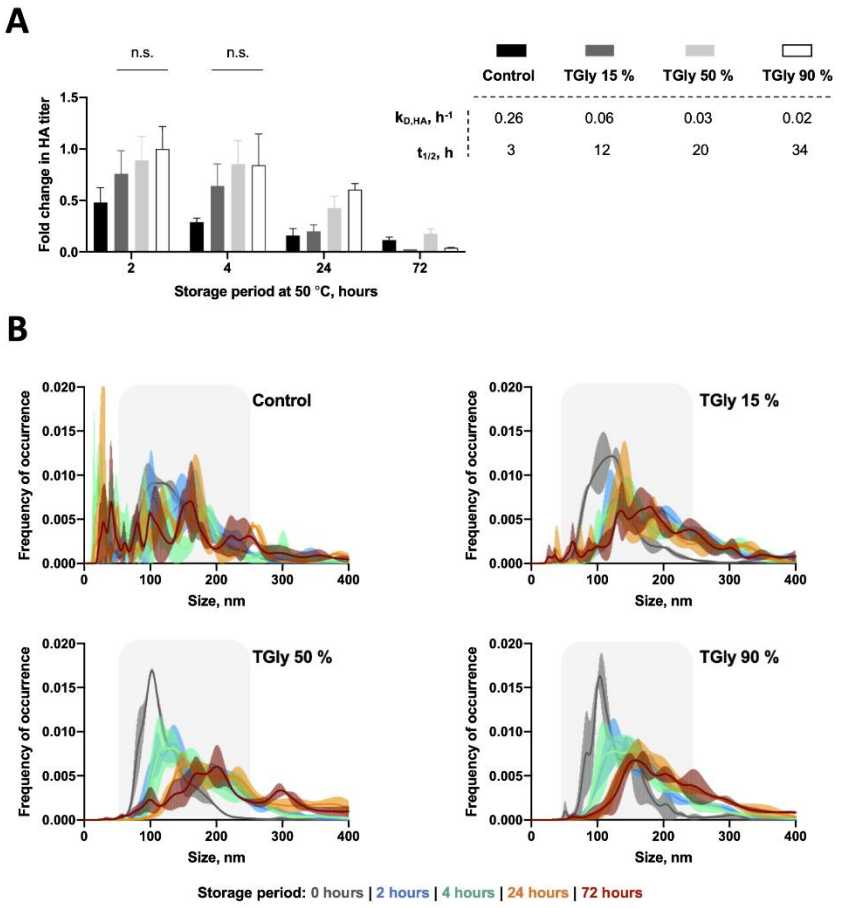


Figure 2 Potency and physical integrity of HA-VLPs throughout an accelerated stability study: storage at 50 ºC for 72 hours (n = 3). (A) fold-change in HA titer over 0 hours of storage time. (B) Size distribution profile of HA-VLPs obtained by nanoparticle tracking analysis. For figure (A), one-way ANOVA using Dunnett's multiple comparison test was used; ns = non-significant, adjusted p-value < 0.05 was considered statistically significant; HA decay constants were estimated for the phase of linear decrease in HA titer. For figure (B): line represents mean and colored band represents standard deviation. Grey box represents expected size-range of HA-VLPs.

Together, these results show that reconstitution of lyophilized HA-VLPs in TGly systems increases particles integrity and activity when compared to control storage buffer formulation, thus conferring better protective capacity upon exposure to high temperature for up to 24 hours.

3.3. Short-term stability study: stability of HA-VLPs in TGly formulations at room temperature

A short-term stability study was performed to assess the stability of HA-VLPs stored in TGly at room temperature (RT, 20-23 °C). HA-VLPs, lyophilized and reconstituted TGly 15 % (w/v), were incubated at RT, and assessed after 14 and 36 days. HA-VLPs incubated in storage buffer formulation (without TGly) were used as control.

HA titers do not vary significantly along time independently of the system used to reconstitute lyophilized HA-VLPs (i.e. control storage buffer formulation or TGly) (Figure 3A). Likewise, particle size distribution profiles were similar for HA-VLPs reconstituted in TGly and the control after over one month of storage at RT, showing a single peak in the expected size range of HA-VLPs and the absence of particles below 50 nm (Figure 3B). Importantly, when comparing storage at the standard refrigerated conditions (Supplementary Figure 3) and at RT, TGly formulation was equally efficient in preserving HA-VLPs.

Together, these results confirm that TGly systems are at least as efficient as control storage buffer formulation for short-term storage of HA-VLPs at RT, maintaining HA titer and particle integrity for over one month.

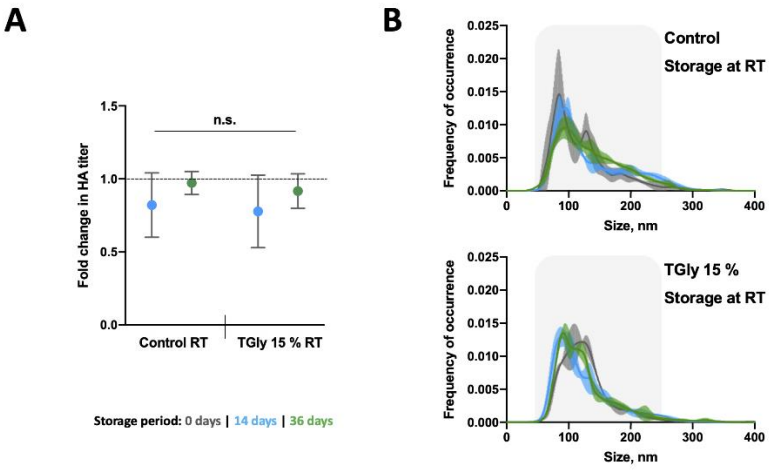


Figure 3 Potency and physical integrity of HA-VLPs throughout a short-term stability study: storage at room temperature (RT) for 36 days (n = 3). Color code: blue: 14 days of storage, green: 36 days of storage. (A) fold-change in HA titer over 0 hours of storage time. (B) size distribution profile of HA-VLPs obtained by nanoparticle tracking analysis. For figure (A), dots represent mean fold-change upon storage for 14 days (blue) and 36 days (green), error bars represent standard deviation. Differences were tested with one-way ANOVA using Dunnett's multiple comparison test; ns = non-significant, adjusted p-value < 0.05 was considered statistically significant. For figure (B): line represents mean and colored band represents standard deviation. Grey box represents expected size-range of HA-VLPs.

4. Discussion

This work aims to evaluate the impact of a NADES composed of trehalose and glycerol at molar ratio 1:30 (TGly systems) on the integrity and activity of influenza HA-VLPs, assessed through (i) an accelerated stability study (72 h at 50 °C) and (ii) a short-term stability study (36 days at room temperature, i.e. approximately 21 °C).

Incubation at high temperature (50 °C) even for short periods (24 hours or less) resulted in a significant decrease in HA titer of influenza virus (Jonges et al., 2010), as assessed by SRID. However, despite this being the gold-standard analytical tool to assess potency of influenza vaccines, hemagglutination was

more efficient to track HA degradation in HA-VLPs following exposure to high temperatures. Unlike SRID, which depends on multiple antibody-antigen interaction sites due to the use of a polyclonal serum, hemagglutination assay depends on a specific interaction site between red blood cells' sialic acids and HA, and thus it is more likely that conformational changes in HA during stability studies result in incapacity to perform agglutination. The hemagglutination capacity of HA-VLPs confirms that they maintain functionally active trimeric forms of the HA protein during storage in this system (Holtz et al., 2014), thus not compromising their efficiency to protect against influenza infection and disease (Loeffen et al., 2011). Hemagglutination assay was selected as the most efficient method to assess quality of HA-VLPs during stability studies.

HA-VLPs integrity and activity were maintained upon lyophilization and reconstitution in either water or TGly, confirming the null impact of these operations on HA-VLPs. The presence of trehalose in the HA-VLPs storage buffer formulation (pre-lyophilization) may have proven critical for this outcome. Stabilizers are commonly used in industrial processes requiring freeze-drying, these typically being sugars like trehalose or inulin. These are known to form a matrix inhibiting mobility of molecules and isolated particles, thus preventing aggregation and degradation of vaccine components (Amorij et al., 2008; Saluja et al., 2010). The lyophilization of influenza subunit and virosome vaccines, without compromising the immunogenicity of HA, has been reported elsewhere (Amorij et al., 2007; Jonges et al., 2010).

Accelerated stability studies are important to assess the impact of short-term excursions outside pre-defined storage conditions, that might occur during shipping and transportation. Here, HA titers of HA-VLPs were maintained at considerably higher values during storage at 50 °C using TGly systems when compared to control. Notably, higher concentrations of TGly showed increased

protective capacity; 0-10 % decrease in HA titer was observed after up to 4 hours when using TGly 90 % (w/v) as compared to over 70 % decrease when using the control storage buffer formulation, for the same time period. This improved protective capacity was corroborated by particle size distribution data which suggests that the use of TGly systems promote HA-VLPs physical integrity avoiding their aggregation and disruption. Electrostatic interaction between glycerol molecules and proteins have been shown to reduce the contact area between these proteins and the solvent (Vagenende et al., 2009). Thus, glycerol, the main component of TGly, could have potentially contributed for a protective layer around HA-VLPs (McCraw et al., 2018) conferring protection from disruption upon thermal stress.

HA-VLPs were stable in TGly 15 % (w/v) for over one month at RT, with minor variations in HA titer and particles size distribution profiles. Importantly, HA-VLPs were mostly identified in the expected size range (McCraw et al., 2018) confirming that TGly is an efficient storage system to maintain physical integrity of HA-VLPs by avoiding their aggregation and disruption. The possibility of vaccine storage at RT rather than refrigerated conditions would significantly reduce the cost and logistics for maintaining the vaccine cold chain.

Whilst higher concentration of TGly provided better protection, it also significantly hindered the reconstitution process of lyophilized HA-VLPs due to increasingly higher viscosity. Syringeability and injectability are two important properties to be considered when designing an injectable vaccine formulation (Cilurzo et al., 2011). Recommended viscosity for injectable vaccines ranges between 10 and 200 mPa·s (Zhang et al., 2018). The use of TGly 90 % (w/v) for storage and direct injection would be impaired by their high viscosity at room temperature, and thus a higher water content is expected to be required in the

final formulation. In fact, it is common practice to add water to NADES to facilitate its use in other applications such as extraction processes (Islamčević Razboršek et al., 2020). We observed that viscosity of NADES is highly influenced by the addition of water even at small amounts, which has also been reported elsewhere (Dai et al., 2013; Savi et al., 2019). Whether the properties of this DES are maintained after adding different amounts of water remains a question that can be pursued in a follow-up study. While water-water and DES-water interactions might be favoured when mixing at lower DES concentrations (Hammond et al., 2017), mixtures containing water:DES molar ratio of 6:1 can still be considered a deep eutectic solvent (Smith et al., 2019). A compromise between TGly content and improvement in HA-VLPs stability must be thoroughly considered.

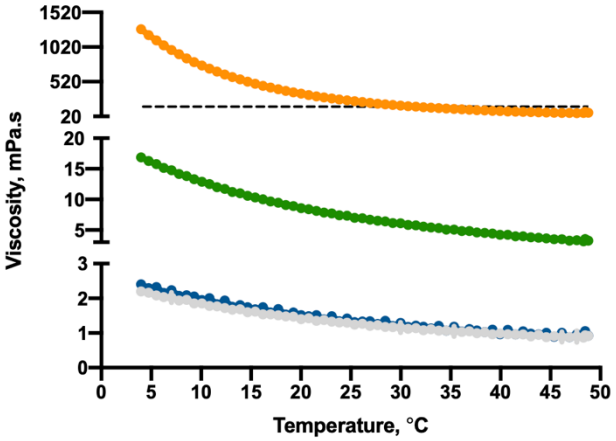
Further stability studies exploring the use of other NADES composed of more cost-effective compounds such as sucrose or with lower viscosities could potentially facilitate the formulation process aiming to achieve a complete water-free storage system, despite their use being usually dependent on the addition of water. Additionally, a long-term storage test (i.e. 1 year or more) of HA-VLPs in TGly systems would be valuable to conclude on the possibility of storage at room temperature during the entire vaccine's shelf-life. Finally, further studies exploring the impact of TGly or other NADES on the stability of other types of vaccines (e.g. subunit or inactivated viruses) would allow to confirm the broad applicability of this storage system.

5. Conclusion

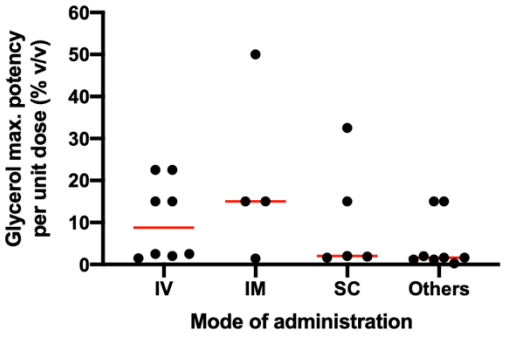
This work demonstrates that NADES composed of trehalose and glycerol (TGly) is a potential formulation for storage of influenza HA-VLPs. We show that TGly

preserves activity and physical integrity of HA-VLPs for up to 4 h at 50 °C (accelerated stability study) as opposed to a commonly used water-based storage buffer formulation, thus showing its promising protective capacity upon thermal stress that might occur during vaccines shipping and transportation. In addition, TGly is able to maintain HA-VLPs activity and integrity for over one month at room temperature (short-term stability study). This opens a new window of possibilities for vaccine storage under non-refrigerated conditions, with significant economic impact and potentially prolonging vaccine shelf-life. Further stability studies exploring the impact of TGly (at different concentrations) and/or other NADES (with different viscosity profiles) on the stability of other vaccine modalities (e.g. DNA, r-subunit, r-vector) would allow to confirm the broad applicability of this storage system.

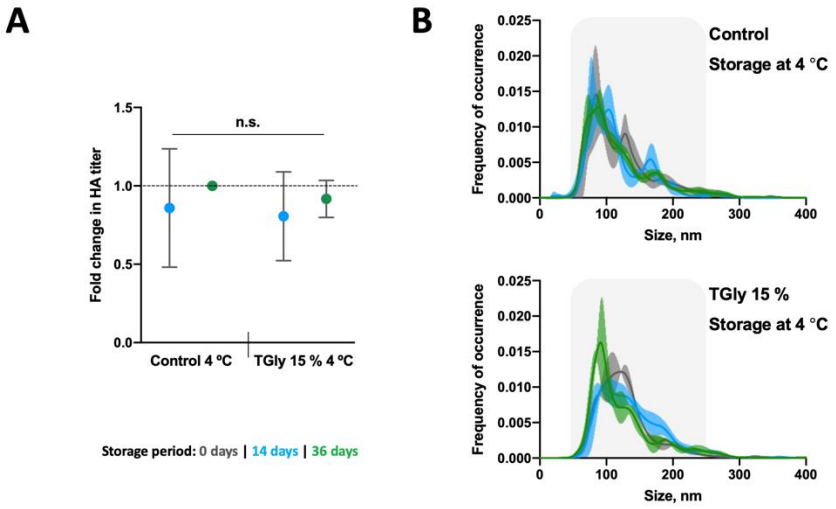
6. Supplementary Material



Supplementary Figure 1 Viscosity of storage formulations at the temperature range of 4-50 °C (n = 3). Color code: grey, control storage buffer formulation (50 mM HEPES, 300 mM NaCl, and trehalose 15 % (w/v) at pH 7.4); blue, TGly 15 % (w/v); green, TGly 50 % (w/v); orange, TGly 90 % (w/v). Dashed line represents the maximum viscosity limit recommended for injectable vaccines (Cilurzo et al., 2011).



Supplementary Figure 2 Glycerol content in approved drug products as listed by FDA, clustered according to administration method. IV: intravenous; IM: intramuscular; SC: subcutaneous; Others: include drugs to be administered via IM/IV, IM/SC, IV/SC, IM/IV/SC, oral or intradermal. Horizontal red lines represent medians.



Supplementary Figure 3 Activity and physical integrity of HA-VLPs throughout a short-term stability study: storage at 4 ºC for 36 days (n = 3). Color code: blue: 14 days of storage, green: 36 days of storage. (A) fold-change in HA titer over 0 hours of storage time. (B) size distribution profile of HA-VLPs obtained by nanoparticle tracking analysis. For figure (A), dots represent mean fold-change upon storage for 14 days (blue) and 36 days (green), error bars = standard deviation. One-way ANOVA using Dunnett's multiple comparison test was used; ns = non-significant, adjusted p-value < 0.05 was considered statistically significant. For figure (B): line represents mean and coloured band represents standard deviation. Grey box represents expected size-range of HA-VLPs.

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Chapter 4

Shift to low culture temperature and rBAC vector design: improve production of malaria PfRipr5 protein

This chapter was based on the following manuscript:

Ricardo Correia, Bárbara Fernandes, Rute Castro, Hikaru Nagaoka, Eizo Takashima, Takafumi Tsuboi, Akihisa Fukushima, Nikola K. Viebig, Hilde Depraetere, Paula M. Alves, António Roldão **(2022)**. "Asexual blood-stage malaria vaccine candidate PfRipr5: enhanced production in insect cells". Accepted for publication in Frontiers in Bioengineering and Biotechnology.

Author contribution

Ricardo Correia design and performed the experiments, analysed the data and wrote the chapter.

Abstract

The malaria asexual blood-stage antigen PfRipr, as well as its most immunogenic fragment PfRipr5, have recently arisen as promising vaccine candidates against this infectious disease. Continued development of high-yielding, scalable production platforms is essential to advance malaria vaccine research. Insect cells have supplied the production of numerous vaccine antigens in a fast and cost-effective manner; further improving this platform could prove key for its wider use.

In this study, insect (*Sf*9 and High Five) and human (HEK293) cell hosts as well as process optimizing strategies (new baculovirus construct designs and culture temperature shift to hypothermic conditions) were employed to improve the production of the malaria asexual blood-stage vaccine candidate PfRipr5. Protein expression was maximized using High Five cells at CCI of 2 x10^6 cell/mL and MOI of 0.1 pfu/cell (production yield = 0.49 mg/mL), with high purity PfRipr5 binding to a conformational anti-PfRipr monoclonal antibody known to hold GIA activity and parasite PfRipr staining capacity. Further improvements in PfRipr5 expression were achieved by designing novel expression vector sequences and performing culture temperature shift to hypothermic culture conditions. Addition of one alanine (A) amino acid residue adjacent to the signal peptide cleavage site and a glycine-serine linker (GGSGG) between the PfRipr5 sequence and the purification tag (His_6) induced a 2.2-fold increase in the expression of secreted PfRipr5 over using the expression vector with none of these additions. Performing a culture temperature shift from the standard 27 °C to 22 °C at the time of infection improved PfRipr5 expression by up to 1.7-fold. Notably, a synergistic effect was attained when combining both strategies, enabling to increase production yield post-

purification by 5.2-fold, with similar protein quality (i.e. purity and binding to anti-PfRipr monoclonal antibody).

This work highlights the potential of insect cells to produce the PfRipr5 malaria vaccine candidate and the importance of optimizing the expression vector and culture conditions to boost expression of secreted proteins.

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1. Introduction

Development of a highly effective malaria vaccine is urgently needed for the control and eventual eradication of this disease. Currently only the RTS,S/AS01 vaccine against Plasmodium falciparum has been recommended by the WHO for wider use, with modest efficacy (Vogel, 2021). While the RTS,S/AS01, the novel R21/Matrix-M (Datoo et al., 2021), as well as whole inactivated sporozoites, focus on the pre-erythrocytic stage of the parasite's life cycle, additional asexual blood-stage vaccines are desired. However, asexual blood-stage vaccine development efforts have been significantly hampered by target antigen polymorphism (Takala et al., 2009), and redundancy of merozoite invasion pathways into erythrocyte (Wright and Rayner, 2014). Recently, a heterotrimeric protein complex consisting of three highly conserved proteins (PfRh5, PfRipr and CyRPA) was identified from P. falciparum merozoites as necessary to invade erythrocytes (Volz et al., 2016, p. 5; Wong et al., 2019, p. 5) presenting a promising vaccine target (Ragotte et al., 2020). Vaccine development efforts have mostly advanced regarding PfRh5, showing for the first time a significant reduction in parasite growth rate upon controlled human malaria infection (Minassian et al., 2021). Recently, another component of the complex, the *P. falciparum* merozoite Rh5 interacting protein (PfRipr), has been shown to be highly conserved (Ntege et al., 2016). In particular, its most potent fragment PfRipr_5 (aa 720-934) (hereon referred as PfRipr5), induces the generation of antibodies with merozoite invasion inhibitory capacity similar to that obtained when vaccinating with the full length PfRipr (Nagaoka et al., 2020).

Manufacturing relevant vaccine amounts demands robust, scalable, and cost-effective expression systems. Production of malaria vaccine candidate antigens has been widely explored using different cell hosts (Crosnier et al., 2013; Jones

et al., 2013). Mammalian cells are an attractive option to produce therapeutic products requiring complex processing (Dumont et al., 2016), but its use to produce recombinant protein vaccines can be more costly and yield lower expression levels than other expression systems (Cid and Bolívar, 2021). Noteworthy, insect cells allow high production yields of mammalian-like recombinant proteins at reduced costs in short-time frames (Mena and Kamen, 2011), having been successfully used to produce Rh5 (Hjerrild et al., 2016; Patel et al., 2013).

Producing recombinant protein antigens as secreted products is of upmost importance to facilitate purification. Trafficking of proteins to the secretory pathway requires the use of a signal peptide (SP) at the N-terminus of the peptide sequence. The choice of the correct SP is essential for efficient recombinant protein secretion in insect cells (Olczak and Olczak, 2006; Soejima et al., 2013). Addition or substitution of amino acid residues with different characteristics to the N- and/or C-terminal of the SP sequence is often the key to improve protein secretion (Futatsumori-Sugai and Tsumoto, 2010; Ohmuro-Matsuyama and Yamaji, 2018). The use of a SP of insect origin may be necessary to drive efficient expression of heterologous proteins (Tessier et al., 1991). In addition, efficient SP cleavage is required for further protein processing in the endoplasmic reticulum (ER) and through the secretory pathway; prediction of the SP cleavage site (von Heijne, 1986) can give insights on whether a protein will be efficiently secreted or not, and suggest the addition of extra residues to the SP's C-terminus to improve the probability of cleavage and consequent secretion.

Manipulation of cell culture conditions has proven efficient in modulating cell phenotype towards improved growth and/or recombinant protein production (Vergara et al., 2018). Adaptive laboratory evolution approaches have shown

great potential to develop high producing cell lines (Yoon et al., 2006) or cell lines with more efficient nutrient utilization (Lee and Palsson, 2010). Single or multiple time-point shifts in process parameters such as pH (Wang et al., 2011), dissolved oxygen concentration (Dick et al., 1994), temperature (Vergara et al., 2014), or a combination thereof (Paul et al., 2019; Tang et al., 2009), have also shown to significantly improve recombinant protein production and/or activity. Specifically for insect cells, the manipulation of culture temperature has been used to modulate cell growth (Reuveny et al., 1993), glycosylation potential (Donaldson et al., 1999), baculovirus replication (Shao-Hua et al., 1998), and recombinant protein production (Rossi et al., 2012). Lowering culture temperature reduces metabolic activity and prolongs cell viability in culture, promoting higher protein expression (Fernandes et al., 2020).

In this work, the production of the malaria vaccine candidate PfRipr5 using insect cells was improved by optimizing the recombinant baculovirus (rBAC) expression vector and by performing temperature shift to hypothermic conditions (27 → 22 °C) at the time-of-infection (TOI).

2. Materials and Methods

2.1. Cell lines and culture media

Insect *Sf*9 (Invitrogen) and High Five (Invitrogen) cells were routinely sub-cultured at 0.4-1 $\times 10^6$ cell/mL every 3-4 days when cell density reached 2-3 $\times 10^6$ cell/mL in Insect-XPRESSTM (Sartorius) and Sf-900TM II SFM (Thermo Fisher Scientific) medium, respectively. HEK293-E6 cells (NRC) (Durocher et al., 2002) were routinely sub-cultured at 0.5-0.6 $\times 10^6$ cell/mL every 3-4 days, when cell density reached 2-3 $\times 10^6$ cell/mL in Free Style F17 (Thermo Fisher Scientific) medium supplemented with 4 mM GlutaMAX™ (Thermo Fisher

Scientific), 0.1 % Pluronic™ F-68 (Life Technologies) and 25 µg/mL of Geneticin (Thermo Fisher Scientific). Cells were cultured using 125-500 mL shake-flasks (Corning) with 10-20 % working volume. Insect *Sf*9 and High Five cells were maintained at 27 °C in a shaking incubator (Inova 44R – Eppendorf) set to 100 RPM and with 2.54 cm shaking diameter. HEK 293 cells were maintained in a similar incubator at 37 °C with 5 % CO2 and stirring rate set to 75 RPM.

2.2. Expression vectors

PfRipr5 nucleotide sequence (Nagaoka et al., 2020) was codon optimized for expression in insect or human cells, with an N-terminal gp67 SP sequence to allow secretion into the culture medium, a C-terminal His_6-tag to allow purification, and a Kozak consensus sequence (GCCACT or ACC for human or insect expression plasmid, respectively) as translation initiation site (synthetized by GenScript, Leiden, Netherlands). Sequences were cloned into the pTT5 vector for expression in human HEK293 cells or into the pOET3 vector for expression in insect *Sf*9 and High Five cells. For PfRipr5 production optimization in insect cells, five additional expression vectors based on the original vector (hereon named rBAC $_{gp67}$) were synthesized, comprising a different SP and/or additional amino acid residues: rBAC $_{BVM}$ (replace gp67 SP sequence by the bee venom melittin, BVM, SP sequence), rBAC $_{A-}$ (addition of one A spacer residue between the gp67 SP sequence and the PfRipr5 sequence), rBAC $_{-GG}$ (addition of a GG linker between the PfRipr5 sequence and the His6-tag), rBAC $_{A-GG}$ (addition of one A spacer residue between the gp67 SP sequence and the PfRipr5 sequence and addition of a GG linker between the PfRipr5 sequence and the His6-tag), and rBAC $_{A-GGSGG}$ (addition of one A spacer residue between the gp67 SP sequence and the PfRipr5 sequence and addition

of a GGSGG linker between the PfRipr5 sequence and the His6-tag). Schematics of the constructs are depicted in Figure 3.

2.3. Baculovirus generation, amplification and storage

Six independent rBAC master viral stocks (MVS) were generated from the pOET3 expression vectors described above using the flashback ULTRATM system (Oxford Expression Technologies) according to manufacturer's instruction. Amplification of MVS was performed as described elsewhere (Vieira et al., 2005). Briefly, insect *Sf*9 cells were infected at a cell concentration at infection (CCI) of 1 $\times 10^6$ cell/mL using a multiplicity of infection (MOI) of 0.1 plaque-forming units per viable cell (pfu/cell). When a cell viability of approximately 80 % was reached, supernatant was harvested by centrifugation at 200 ×g and 4 °C for 10 min and centrifugation at 2000 ×g and 4 °C for 20 min. Clarified supernatant was aliquoted appropriately and stored at 4 °C until further use.

2.4. Production of PfRipr5

2.4.1. Insect cells as host

PfRipr5 was produced in 500 mL shake-flask (SF) with 10 % working volume and in 2 L glass stirred tank bioreactors (STB).

In SF cultures, cells were seeded at 0.4-0.6 $\times 10^6$ cell/mL and infections performed (i) at different combinations of CCI (1 and 2 $\times 10^6$ cell/mL) and MOI (0.1 and 1 pfu/cell) for the initial assessment of PfRipr5 production using rBAC$_{gp67}$, or (ii) at CCI = 2 $\times 10^6$ cell/mL and MOI = 0.1 pfu/cell for the optimization of PfRipr5 production using rBAC$_{BVM}$, rBAC$_{A-}$, rBAC$_{-GG}$, rBAC$_{A-GG}$ and rBAC$_{A-GGSGG}$.

STB cultures were performed in computer-controlled BIOSTAT B-DCU 2 L vessels (Sartorius) equipped with two Rushton impellers, a sparger for gas supply, a water recirculation jacket for temperature control, and multiple ports for temperature, pH and pO2 probes as well as for additions (e.g. culture medium) and sampling/harvesting of cell culture. Cells were seeded at 0.4-0.6 $\times 10^6$ cell/mL and infections performed at CCI = 2 $\times 10^6$ cell/mL and MOI = 0.1 pfu/cell, using rBAC $_{gp67}$ (initial assessment of PfRipr5 production) or rBAC $_{A\text{-}GGSGG}$ (optimization of PfRipr5 production). The pO2 was set to 30 % of air saturation and was maintained by varying the agitation rate from 70 to 270 rpm and the percentage of O2 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 (initial assessment of PfRipr5 production) or changed to 22 °C at TOI (optimization of PfRipr5 production). Bioreactors were operated with an initial working volume of 2 L.

2.4.2. Human cells as host

PfRipr5 was produced in 500 mL SF with 20 % working volume and in 2 L glass STB.

For productions in SF, cells were seeded at 0.5 $\times 10^6$ cell/mL and transfected at ≈ 1.6 $\times 10^6$ cell/mL with a mixture of pTT5-PfRipr5 plasmid (pDNA) and polyethylenimine (PEI, Polysciences) cationic polymer prepared in 10 % (v/v) of total volume. Different combinations of pDNA concentration ([pDNA] = 0.5 and 1 mg/L) and pDNA:PEI ratio (1:2 and 1:1.5, w/w) were tested.

STB culture was performed in a 2 L STB set-up similar to insect cells. Cells were seeded at 0.5 $\times 10^6$ cell/mL and transfected at ≈ 1.6 $\times 10^6$ cell/mL with [pDNA] = 0.5 mg/L and pDNA:PEI ratio = 1:2. Culture pH was maintained at 7.4 using NaHCO3. The pO2 was set to 40 % of air saturation and was maintained by

varying the agitation rate from 70 to 270 rpm and the percentage of O2 in the gas mixture from 0 to 100 %. The gas flow rate was set to 0.01 vvm and temperature was kept at 37 °C. Bioreactors were operated with an initial working volume of 2 L.

2.5. Purification of PfRipr5

Purification of secreted PfRipr5 produced in STB was carried out on ÄKTA Explorer 100 systems (Cytiva). Cell culture bulk was harvested, filtered through 0.45 and 0.22 µm Sartopore 2 Midicap filter cartridges 10 (Sartorius), concentrated with a Sartocon disposable polyethersulfone (PES) membrane 2x 0.1 m2 10 kDa (Sartorius), and filtered through a Nalgene cup 0.2 µm (Thermo Scientific). PfRipr5 was purified by immobilized metal ion affinity chromatography (IMAC) on a Histrap HP column (5 mL volume; Cytiva) equilibrated with 50 mM NaP, 500 mM NaCl, 5 mM DTT, pH 8.0. After washing the column with the same buffer containing 500 mM Imidazole, the protein was eluted with a linear Imidazole gradient over 10 column volumes. The eluate was concentrated using AmiconUltra 15 Centrifugal filter unit 10 kDa (Merck Milipore), filtered through 0.2 µm and loaded into a HiLoad 26/60 Superdex 75 gel permeation column (SEC) (Cytiva equilibrated with 16 mM NaP, 250 mM NaCl, 5mM DTT, pH 8.0. The eluate was concentrated using AmiconUltra 15 Centrifugal filter unit 10 kDa and loaded in a HiPrep desalting 26/10 column (Cytiva) equilibrated with 16 mM NaP, 250 mM NaCl, pH 8.0 for removal of DTT. The eluate was concentrated using AmiconUltra 15 Centrifugal filter unit 10 kDa and filtered through 0.2 µm. The final sample was stored in 16 mM NaP, 250 mM NaCl, pH 8.0 buffer formulation, aliquoted and stored at -80 °C.

2.6. Analytics

2.6.1. Cell concentration and viability

Cell concentration and viability were assessed using a Cedex HiRes Analyzer (Roche).

2.6.2. SDS-PAGE and Western blot

SDS-PAGE and Western blot analyses were performed as reported previously (Correia et al., 2020). (39). In particular, reduced (R) samples were mixed with NuPAGE Sample Reducing Agent 1X and heated at 70 °C for 10 minutes; non-reduced samples (NR) were mixed with water in replacement of Reducing Agent. Both samples were run in the same gel (4-12 % Bis_Tris, NuPAGE). For PfRipr5 identification by Western blot, anti-PfRipr5 antiserum generated in rabbit (Nagaoka et al., 2020) was used at a dilution 1:1000. As secondary antibody, an anti-rabbit IgG antibody conjugated with alkaline phosphatase was used at a dilution of 1:5000. Protein band detection was performed with NBT/BCIP 1-Step (Thermo Scientific) for 10 min and membranes were scanned with a benchtop scanner device. Densitometry analysis of SDS-PAGE gels (to assess PfRipr5 protein purity) and Western blot membranes (to assess relative productivity) was performed using FIJI software (Schindelin et al., 2012). PfRipr5 antigen generated by wheat germ cell-free system (WGCFS) (Nagaoka et al., 2020; Tsuboi et al., 2010) was used in all membranes as positive control for PfRipr5 detection and as normalizing factor (i.e. band intensity of control = 1.0) for relative band intensity between samples in separate membranes.

2.6.3. Baculovirus titration

Baculovirus infectious titers were determined using the MTT assay (Mena et al., 2003; Roldão et al., 2009).

2.6.4. Protein concentration

Protein concentration was determined by spectrophotometry at 280 nm using the mySPEC equipment (VWR).

2.6.5. Dynamic light scattering

The size distribution of the purified PfRipr5 was analysed by dynamic light scattering (DLS) on a Spectro Light 600 (Xtal Concepts).

2.6.6. ELISA

Ninety-six well enzyme-linked immunosorbent assay (ELISA) plates (Greiner Bio-One, Kremsmünster, Austria) were coated with 50 µL of mouse anti-PfRipr mAb (clone 29B11) (produced as described in supplementary information), diluted to 2.5 µg/mL in catcher buffer (25 mM carbonate buffer pH 9.6) and incubated at 4 °C overnight. The plates were washed with phosphate-buffered saline (PBS) with 0.1 % (v/v) Tween-20 (PBS-T) and then blocked with 200 µL 3 % skimmed milk in PBS-T for 1 hour at RT. Purified PfRipr5 was diluted in PBS, added to antibody-coated plates in triplicate, and incubated for 1 hour at RT. After washing, the plates were incubated with 100 µL of anti-PfRipr5 rabbit antiserum diluted 1:2000 in PBS for 1 hour at RT. After incubation, the plates were washed and incubated with HRP-conjugated anti-rabbit IgG (Cytiva) diluted 1:1000 in PBS-T for 1 hour at RT. The plates were washed and incubated

for 15 min at RT with TMB substrate solution (Thermo Scientific). The reaction was stopped with 2 M sulfuric acid, and optical density (OD) was determined at 450 nm using a precision microplate reader (Molecular Devices, Sunnyvale, CA).

2.6.7. Surface plasmon resonance

Surface plasmon resonance (SPR) was performed with Biacore X100 (Cytiva). All the reagents and sensor chip CM5 were purchased from Cytiva. Biacore X100 evaluation software was used for the analysis. Kinetic analysis was done with mouse antibody capture kit according to the manufacturer's instructions. Fresh HBS-EP+ (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% (v/v) surfactant P20) was used as the running buffer. The anti-PfRipr mouse mAb 29B11 (25 µg/mL) was injected into the anti-mouse antibody coated CM5. The purified PfRipr5 protein (18.8, 37.5, 75, 159 and 300 nM) was then injected. After subtracting the reference, individual association (ka) and dissociation (kd) rate constants were obtained by global fitting.

2.7. Statistical analysis

Data are expressed as mean ± standard deviation. Differences were tested by unpaired t-test analysis method (p-value < 0.05 was considered statistically significant, ns denotes non statistically significant). Pearson’s correlation (r) was used to analyze the correlation between infection kinetics in SF and STB. One-way ANOVA was conducted by GraphPad Prism (Ver. 8.4.3).

3. Results

3.1. PfRipr5 production in insect and human cells

3.1.1. Optimizing production conditions

Production of PfRipr5 was assessed in small-scale SF cultures using (i) insect (High Five and *Sf*9) cell-baculovirus expression vector system (IC-BEVS) and (ii) PEI-mediated human (HEK293) cell transfection. Aiming at finding the best production conditions, insect cells were infected at different combinations of MOI (0.1 and 1 pfu/cell) and CCI (1 and 2 $\times 10^6$ cell/mL) while human cells were transfected at different combinations PfRipr5 [pDNA] (0.5 and 1 mg/L) and pDNA:PEI ratios (1:2 and 1:1.5).

Infection kinetics in IC-BEVS were in accordance with the different conditions tested, characterized by higher cell growth and delayed onset of cell viability drop when using lower MOI (Figure 1A). Similarly, different transfection conditions resulted in distinct cell growth and viability kinetics (Figure 1B). PfRipr5 expression varied with the cell line and condition tested, this being favored in insect cells and when using low MOIs (Supplementary Figure 1). Time-of-harvest (TOH) for each cell line and condition tested was defined based on PfRipr5 expression and cell viability, aiming for the later to be above 65-70 % to avoid purification issues caused by overaccumulation of cell debris and intracellular content. For insect cells, TOH was set at days 2-3 post-infection while for human cells the TOH was set at days 4-5 post-transfection. At the selected TOH, the concentration of PfRipr5 produced in insect cells was up to 17.2-fold higher than that obtained with the best-performing transfection condition in human cells (Figure 1C).

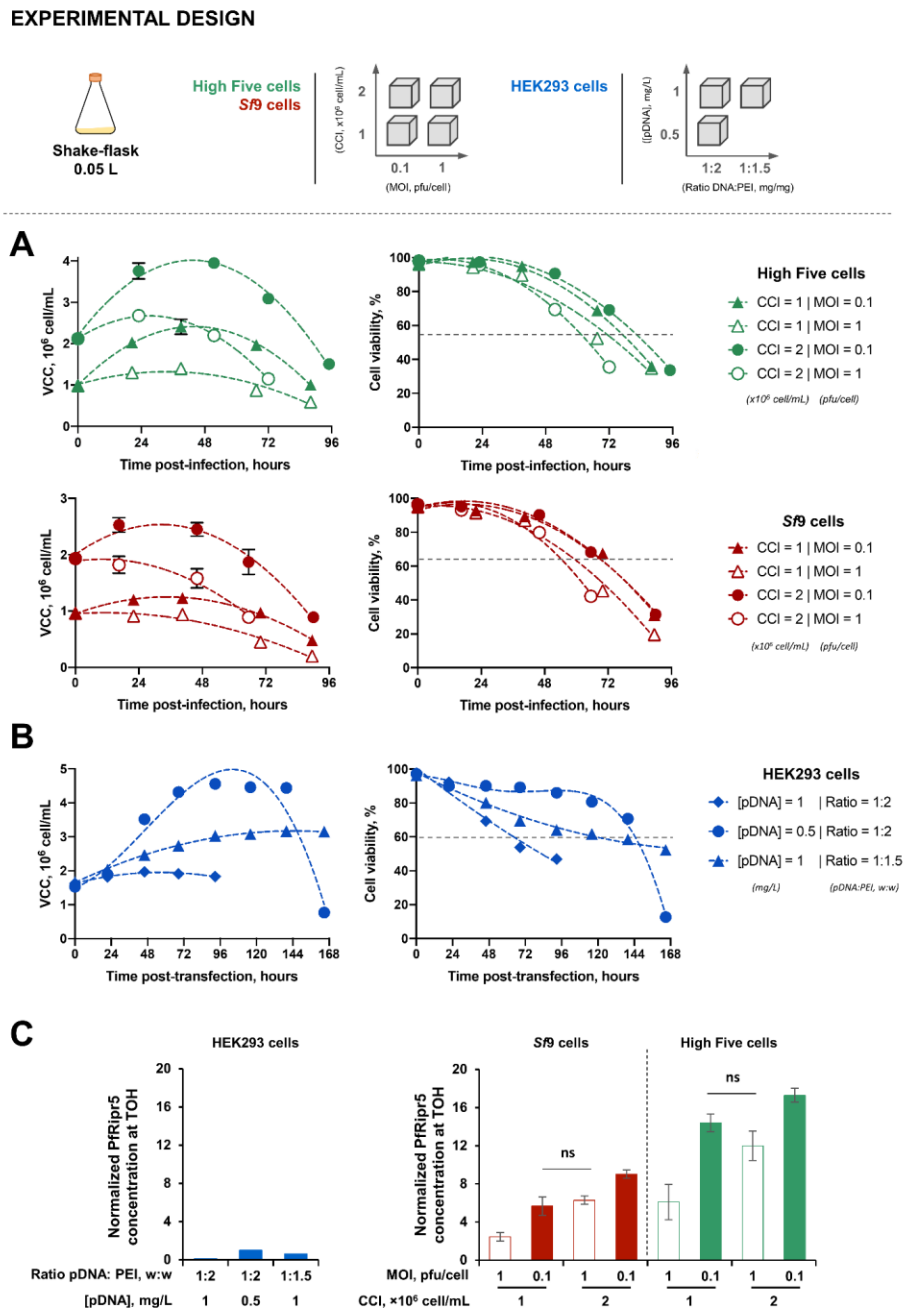


Figure 1 Production of PfRipr5 recombinant protein. (A) Kinetics of cell growth and viability upon infection of insect High Five (green) and *Sf*9 (orange) cells at different combinations of cell concentration at infection (CCI) and multiplicity of infection (MOI). (B) Kinetics of cell growth and viability upon transfection of human HEK293 cells at different combinations of PfRipr5 plasmid

DNA (pDNA) concentration ([pDNA]) and ratio pDNA:PEI. (C) Relative PfRipr5 concentration at the time-of-harvest (TOH) of each production condition. Data is expressed as mean ± standard deviation. For insect cells, data is relative to three biological replicates (n = 3). For human cells, data is relative to one biological replicate (n = 1). For figure (A) and (B), VCC denotes Viable Cell Concentration, dashed line represents lower limit of cell viability to define TOH. For figure (C), relative PfRipr5 expression was assessed by densitometry analysis of Western blot (Supplementary Figure 1) as described in the M&M section; graph is normalized at 1 for the best condition using human cells (i.e. [pDNA] = 0.5 mg/L and ratio pDNA:PEI = 1:2 (w:w)).

Overall, the production conditions maximizing PfRipr5 concentration at TOH in insect and human cells were CCI = 2 $\times 10^6$ cell/mL and MOI = 0.1 pfu/cell, and [pDNA] = 0.5 mg/L and pDNA:PEI ratio = 1:2, respectively, and thus used in subsequent studies.

3.1.2. Production at bioreactor scale

Production of PfRipr5 in insect (High Five and *Sf*9) and human (HEK293) cells was demonstrated in computer-controlled 2 L STB, using previously optimized process conditions; SF cultures were used as control. Purification was identical for all three production runs, and production yields and quality of purified PfRipr5 were assessed and compared.

Insect cells concentration and viability profiles were similar between STB and SF cultures, contrary to what was observed in human cells where higher cell concentration and viability were achieved in STB when compared to SF during the initial days of transfection (Supplementary Figure 2A). PfRipr5 expression in all cell lines was confirmed by Western blot analysis (Supplementary Figure 2B), with relative PfRipr5 concentration at TOH being maximized in insect cells (Figure 2A), consistent with SF data.

Insect and human cells derived PfRipr5 proteins were successfully purified, all of them being identified by SDS-PAGE (Figure 2B) and Western blot

(Supplementary Figure 2C) analysis; nonetheless, insect cells derived proteins presented higher purity (Table 1). Despite this difference, similar degree of polydispersity was observed in all purified PfRipr5 proteins as assessed by dynamic light scattering (Figure 2C). Binding of purified PfRipr5 proteins to anti-PfRipr mAb 29B11, which has previously shown to hold growth inhibition assay (GIA) activity and parasite PfRipr staining capacity (Supplementary Figure 3A-B), was assessed by ELISA and SPR as a proxy for confirming protein´s biological activity. In ELISA, PfRipr5 produced in insect cells showed improved binding (i.e. higher absorbance at similar protein concentrations) than that produced in human cells (Figure 2D). In SPR, the KD values could not be estimated for the protein produced in human cells because of its poor fitness with the model equation (Table 1), thus corroborating its poor binding as assessed by ELISA. The KD values obtained from the PfRipr5 produced in both insect cell lines showed no statistical difference (One-way ANOVA, P = 0.3885), suggesting they have similar functionality. Noteworthy, the production yields after purification achieved in insect cells were 4-5-fold higher than in human cells (Table 1).

Based on the results mentioned above, further production optimization was only performed in insect cells.

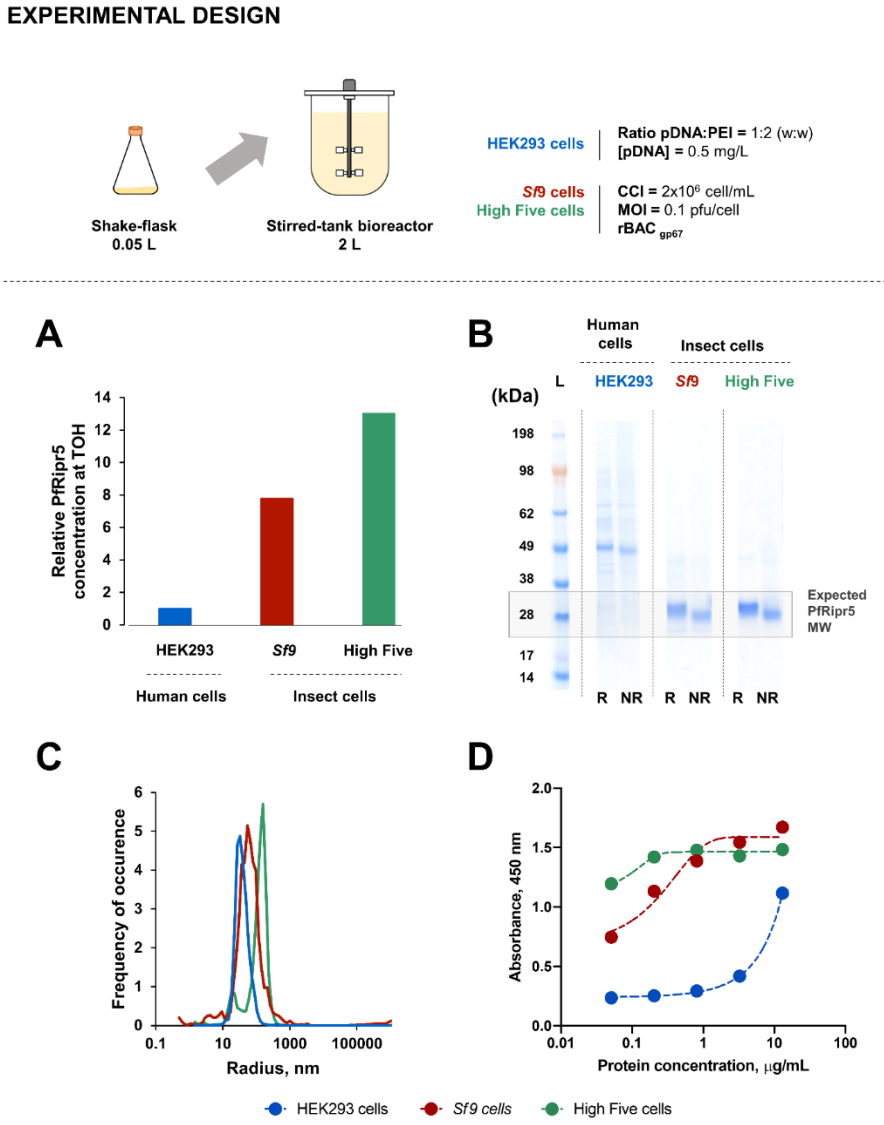


Figure 2 Production of PfRipr5 at 2 L stirred-tank bioreactor (STB) scale. (A) Relative PfRipr5 concentration at the TOH of each cell line. (B) SDS-PAGE of purified PfRipr5. (C) Size distribution profile of purified PfRipr5 assessed by dynamic light scattering. (D) ELISA of purified PfRipr5 using an anti-*P. falciparum* PfRipr mouse monoclonal antibody (mAb) 29B11. For figure (A): relative PfRipr5 expression was assessed by densitometry analysis of Western blot (Supplementary Figure 2B) as described in the M&M section. For figure (B): L denotes pre-stained protein standard SeeBlue® Plus2, R denotes reduced sample, NR denotes non-reduced sample. Data is relative to one biological replicate (n = 1).

3.2. Optimizing PfRipr5 production in insect cells: exploring different baculovirus constructs and culture conditions

Aiming to improve PfRipr5 production in insect cells, three different strategies were devised (Figure 3A): Strategy I – design new rBAC constructs; Strategy II – culture temperature shift at TOI; Strategy III – combine new rBAC construct with culture temperature shift at TOI. The best process conditions identified before were herein used (CCI = 2 ×10^6 cell/mL and MOI = 0.1 pfu/cell); cultures were performed in small-scale SF. Fold-change improvement in PfRipr5 expression is relative to non-optimized infection conditions, i.e. infection with $rBAC_{gp67}$ construct at the standard culture temperature (27 °C).

In strategy I, rBAC constructs seem to have an impact on PfRipr5 expression but not on the infection kinetics (Supplementary Figure 4A). Indeed, PfRipr5 expression could be maximized using $rBAC_{A\text{-}GGSGG}$ (addition of the A spacer with the GGSGG linker), leading to a 2.2 and 1.6-fold improvement in protein concentration in High Five and *Sf*9 cells, respectively (Figure 3B). In strategy II, lowering culture temperature at TOI reduced cell growth after infection and delayed the onset of cell viability drop (Supplementary Figure 4B), and consequently TOH was set at days 4-5 post-infection; most importantly, this strategy induced a 1.7 and 1.6-fold increase in PfRipr5 concentration in High Five and *Sf*9 cells, respectively (Figure 3B). In strategy III, cell growth and viability kinetics mimic those observed in previous strategy (Supplementary Figure 4C vs 4B). Noteworthy, a synergistic effect of combining new rBAC construct with culture temperature shift at TOI was observed, allowing to improve PfRipr5 expression by 3 and 4-fold in High Five and *Sf*9 cells, respectively (Figure 3B).

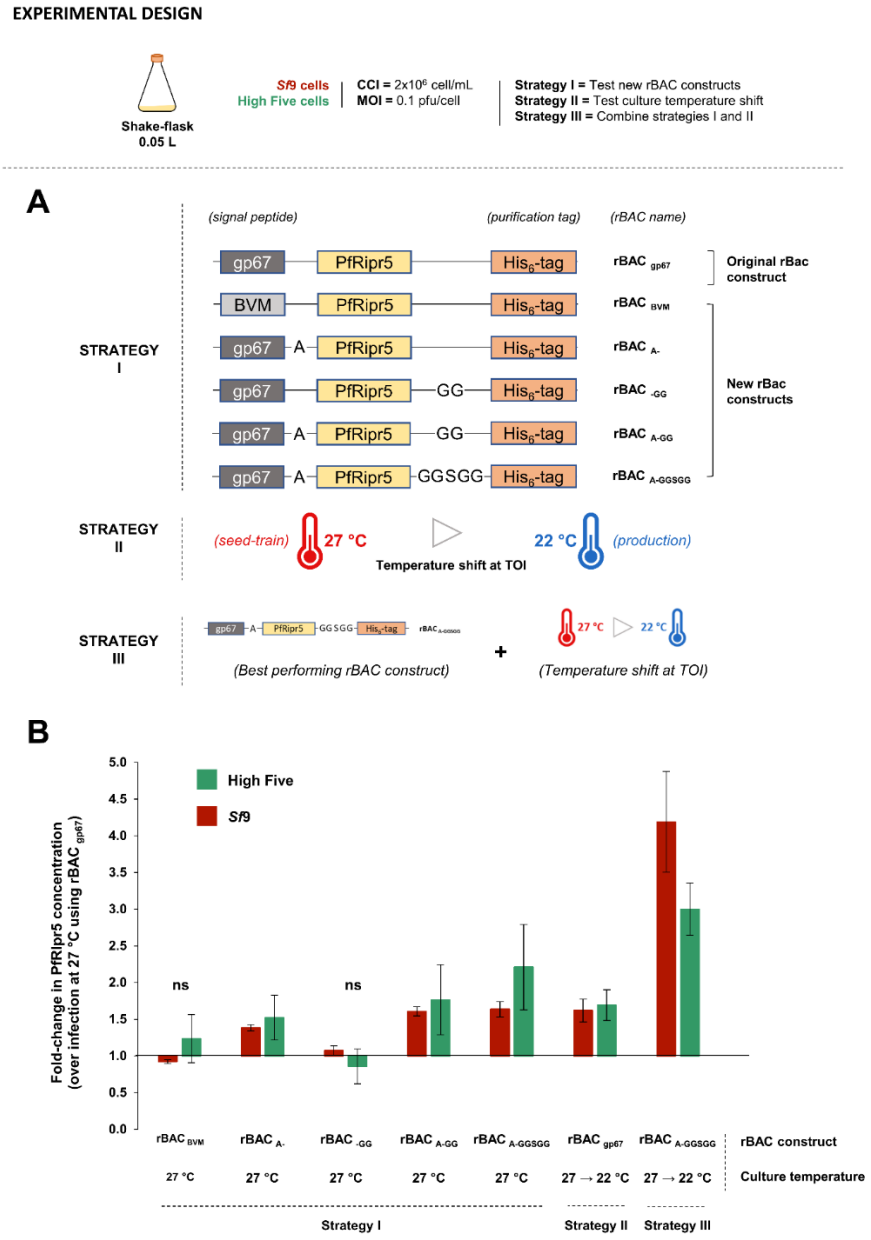


Figure 3 Optimization of PfRipr5 production using insect cells. (A) Optimization strategies devised. (B) Relative PfRipr5 concentration at the TOH between each optimization condition and the baseline production setup (infection with rBAC $_{gp67}$ without culture temperature shift). A denotes alanine, G denotes glycine and S denotes serine. Infections were performed using CCI = 2 ×106 cell/mL and MOI = 0.1 pfu/cell. Data is expressed as mean ± standard deviation and is relative to three biological replicates (n = 3).

Although the fold-change increase in PfRipr5 production (over non-optimized infection conditions) was higher in *Sf*9 cells, PfRipr5 concentration still remained superior in High Five cells. Thus, High Five cells were selected for the subsequent proof-of-concept production at bioreactor scale.

3.3. Proof-of-concept at 2 L bioreactor scale

PfRipr5 was produced in computer-controlled 2 L STB using the optimization strategy established above, i.e. infection of insect High Five cells at CCI = 2×10^6 cell/mL and MOI = 0.1 pfu/cell with rBAC $_{A\text{-}GGSGG}$ and performing temperature shift (from 27 °C to 22 °C) at the TOI. Purification was performed identically to previous STB runs, and the quality of purified PfRipr5 assessed by Western blot, SDS-PAGE, DLS, ELISA and SPR.

Cell growth and viability kinetics were identical to those previously observed in SF, allowing to harvest the culture at the expected TOH, i.e. 4 days post-infection (Figure 4A). Bands corresponding to PfRipr5 were identified by Western blot both in bulk and purified material (Figure 4B). Production yield achieved following purification was 2.55 mg/L, representing a 5.2-fold increase over the non-optimized STB production (Figure 4C and Table 1). Purity of purified PfRipr5 was > 95 % as assessed by SDS-PAGE, similar to that from previous STB runs performed using insect cells (Figure 4D and Table 1). Dynamic light scattering data resembles that from previous non-optimized STB runs, with the identification of an extra peak at smaller radius corresponding to the expected size range of monomeric PfRipr5 (Figure 4E).

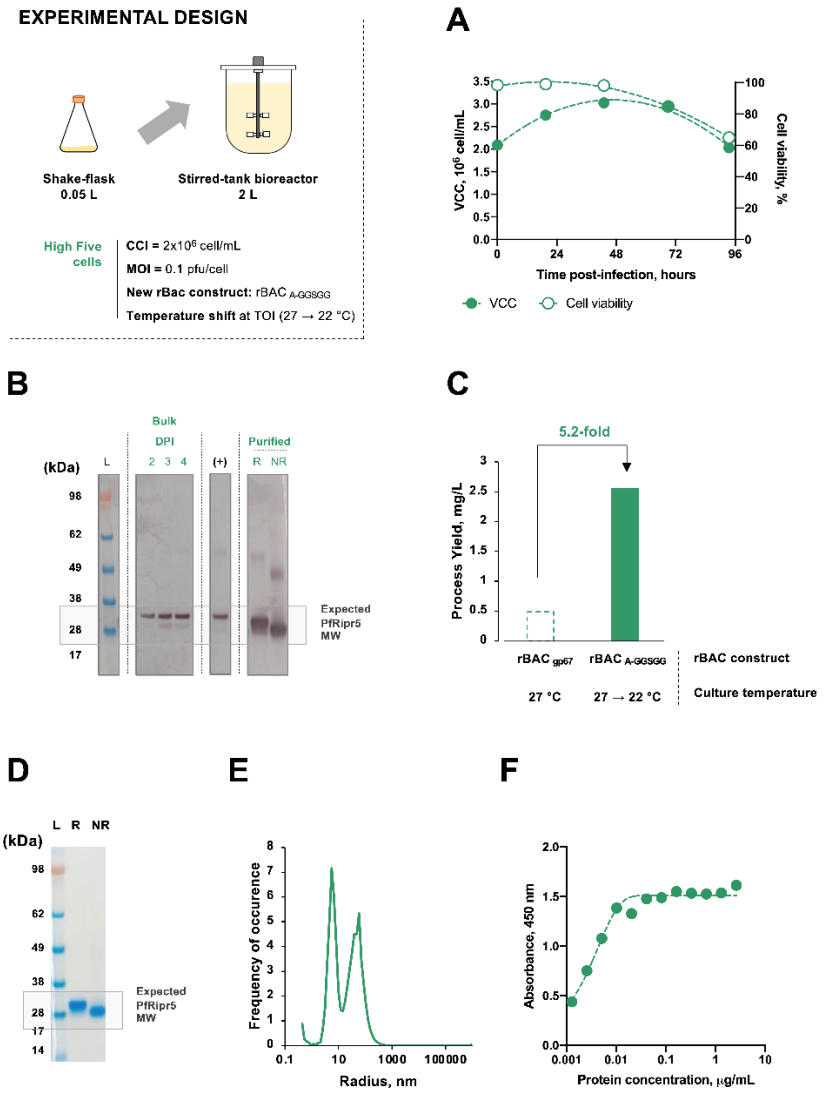


Figure 4 Production of PfRipr5 at 2 L STB scale using the optimized production strategy (infection of insect High Five cells with rBAC $_{A\text{-}GGSGG}$ and culture temperature shift from 27 °C to 22 °C at TOI). (A) Kinetics of cell growth and viability throughout infection. (B) Western blot identification of PfRipr5 in bulk and purified samples. (C) Production yield following purification. (D) SDS-PAGE of purified PfRipr5. (E) Size distribution profile of purified PfRipr5 assessed by dynamic light scattering. (F) ELISA of purified PfRipr5 using an anti-*P. falciparum* PfRipr mouse mAb 29B11. For figure (A), VCC denotes Viable Cell Concentration. For figure (B), DPI denotes day post-infection, (+) denotes the positive control (PfRipr5 produced by WGCFS). For figure (B) and figure (D), L denotes pre-stained protein standard SeeBlue® Plus2, R denotes reduced sample, NR denotes non-reduced sample. Infection was performed using CCI = 2 ×10^6 cell/mL and MOI = 0.1 pfu/cell. Data is relative to one biological replicate (n = 1).

Finally, the binding of purified PfRipr5 to anti-PfRipr mAb 29B11 was confirmed by ELISA (Figure 4F) and SPR (Table 1), with similar binding kinetics and KD values to those observed with purified PfRipr5 from previous STB runs.

Table 1 Summary of production runs at 2 L STB scale. ND denotes not determined.

Cell Line	Process	Production yield, mg/L	Purity, % [a]	KD, M [b] (mAb 29B11)
HEK 293	Non-optimized [c]	0.11	19	ND
*Sf*9	Non-optimized [c]	0.53	91	$3 \pm 4 \times 10^{-8}$
High Five	Non-optimized [c]	0.49	95	$1.5 \pm 0.1 \times 10^{-9}$
High Five	Optimized [d]	2.55	97	$8 \pm 4 \times 10^{-9}$

[a] **Purity** assessed by densitometry analysis of SDS-PAGE

[b] **KD**: equilibrium dissociation constant assessed by surface plasmon resonance. The values were derived from at least 3 independent experiments.

[c] **Baseline process**: Production at 27 °C using recombinant baculovirus construct $rBAC_{gp67}$

[d] **Optimized process**: Production with temperature shift at time of infection (27 → 22 °C), using recombinant baculovirus construct $rBAC_{A\text{-}GGSGG}$

4. Discussion

In this work, we developed an optimized process for production of PfRipr5 malaria vaccine candidate using IC-BEVS by combining new rBAC construct design with culture temperature shift (27 → 22 °C) at the TOI.

Insect (High Five and *Sf*9) cells were identified as a better host for PfRipr5 expression than human (HEK293) cells, with High Five cells outperforming *Sf*9 cells as commonly seen for other recombinant proteins (Wilde et al., 2014). In addition, expression in insect cells was highest using MOI of 0.1 virus per cell, reducing volumes being handled and wastage of commonly expensive, certified master virus stocks in comparison to higher MOI. Purified PfRipr5 derived from insect cells presented high purity and strong binding to anti-PfRipr mAb 29B11, an antibody shown to hold GIA activity and parasite PfRipr staining capacity and thus herein used as a proxy for confirming protein´s biological activity. Contrastingly, HEK293 cells expressed nearly undetectable levels of PfRipr5 in the culture bulk, subsequently compromising the purification process, product purity and ultimately its biological activity. Increase in production scale would be required to bring the human-based system close to the productivities and product quality achieved with IC-BEVS. We have also observed that in all expression systems the percentage of PfRipr5 protein being secreted was similar (data not shown), suggesting that the lower productivity attained with HEK293 cells was a translation rather than secretion-related limitation, hence the decision of not pursuing with further strategies (e.g. using a signal peptide of mammalian origin) for improving expression in human HEK293 cells.

Despite being reported elsewhere as capable of increasing protein secretion up to five times (Tessier et al., 1991), the use of BVM did not induce higher PfRipr5 expression. Additional amino acid residues at the upstream positions of the SP cleavage site are commonly used as spacers; in fact, the addition of A residues at the +1 and +1/+2 positions of the SP cleavage site was shown to be essential to increase recombinant protein expression levels in human cells (Güler-Gane et al., 2016). We have explored this hypothesis in our study,

adding one A residue at the +1 position of the SP cleavage site to rBAC constructs. Notably, all constructs with this modification induced higher PfRipr5 expression. With the objective of increasing availability and/or flexibility of the purification tag (Chen et al., 2013) to aid downstream processing, linkers composed of G and S residues have been included between the PfRipr5 sequence and the His6-tag. The construct comprising both an A spacer and a GGSGG linker induced the highest PfRipr5 expression. The flexibility conferred by this longer linker may have facilitated PfRipr5 folding by reducing the interference of the purification tag in protein´s tertiary structure during protein processing in the ER, therefore reducing the chances of impairing protein secretion. These results are in good agreement with the previous reports in which flexible G-rich linkers have been used to improve the folding and function of tagged proteins (Reddy Chichili et al., 2013; Sabourin et al., 2007).

To modulate recombinant protein production, culture temperature shift at TOI from standard 27 °C to 22 °C was performed, prolonging infection by delaying the onset of cell viability drop and, most importantly, improving PfRipr5 expression in both insect cell lines. Prolonging cell viability in IC-BEVS is an efficient approach to improve recombinant protein production (Steele et al., 2017). PfRipr5 is a cysteine-rich fragment of PfRipr (contains 31 cysteine residues) and thus shows high propensity to form disulfide bonds, which are part of the post-translational modifications (PTMs) that are facilitated within the secretory pathway of eukaryotic cells (Robinson and Bulleid, 2020) and are essential to maintain tertiary structure. Strategies facilitating protein processing in the ER allowing improved folding capacity are thus beneficial to improve protein secretion. Lowering culture temperature in IC-BEVS can slow down metabolic activity allowing structural protein processing

(Somasundaram et al., 2016) and allow proteins to have more time for going through the ER (Donaldson et al., 1999), facilitating folding and processing of complex PTMs including disulfide bonds, and therefore may promote PfRipr5 processing and secretion.

By combining new rBAC construct design with lowering culture temperature at TOI, a synergistic effect was observed and production yields could be improved by over 5-fold. Improved availability and/or flexibility of the purification tag as well as PfRipr5 stability and/or folding, promoted by the added spacers and linkers and by lower culture temperature, may have facilitated the affinity (i.e. IMAC) and concentration/polishing (i.e. SEC) steps during purification while limiting protein aggregation, thus explaining the improvements observed in protein expression and quality. Importantly, the purified insect cells derived PfRipr5 continue to show strong binding to anti-PfRipr mAb 29B11 in both ELISA and SPR analyses, thus suggesting that biological activity was not impacted by the optimizations herein devised.

5. Conclusion

This work demonstrates the optimization of expression and purification of the asexual blood-stage malaria vaccine candidate PfRipr5 while maintaining its biological activity. To our knowledge, this is the first report of using the insect *Sf*9 and High Five cell lines to produce an asexual blood-stage malaria vaccine candidate. These findings motivate the use of atypical culture conditions such as lower culture temperature to improve recombinant protein production using IC-BEVS and highlight the importance of optimizing the expression vector when producing secreted proteins.

6. Supplementary Material

Material and Methods

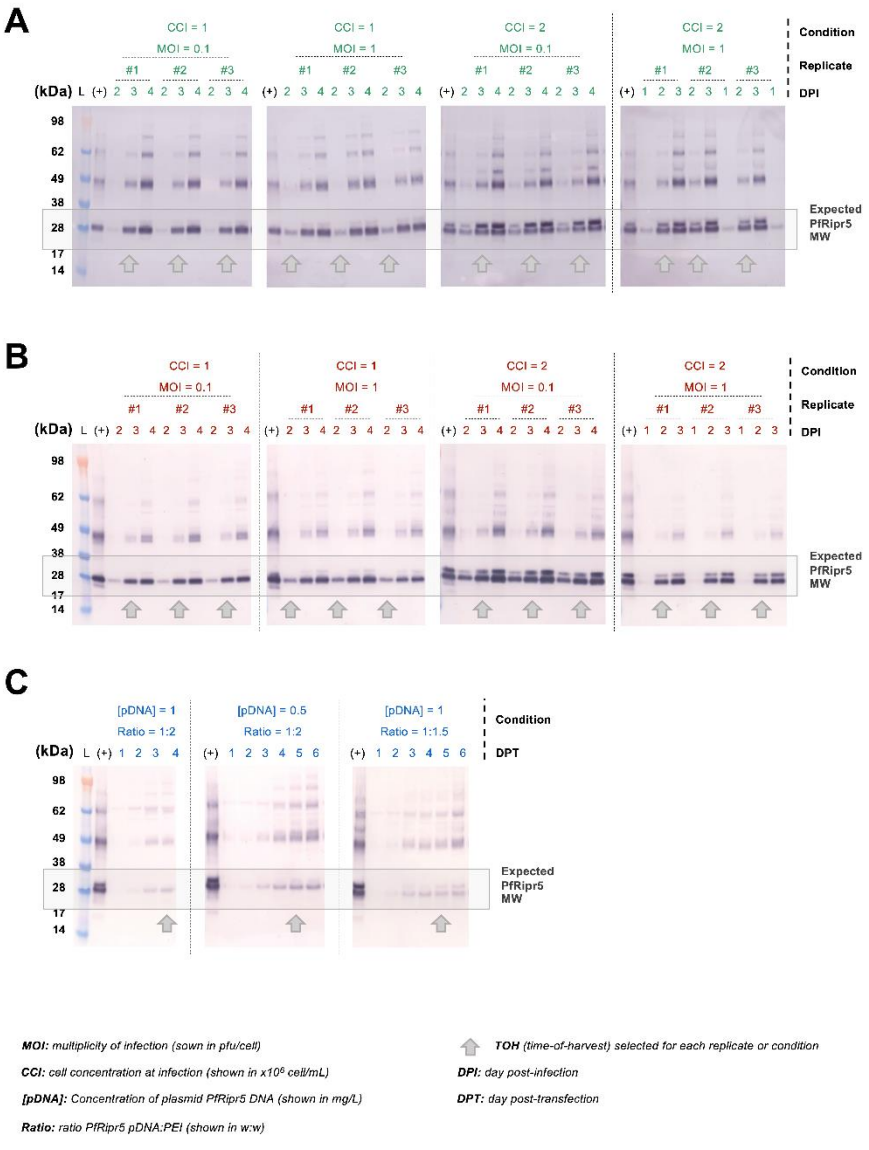
Production of Anti-PfRipr monoclonal antibody (mAb)

Mouse monoclonal antibodies (mAbs) against PfRipr were purchased from BEX Co., Ltd. Specifically, BALB/c mice were immunized three times with antigen in TiterMax® adjuvant. Lymphocytes from the spleen and the lymph nodes were used to fuse with P3U1 myeloma cells to produce hybridoma cells. Culture supernatants from hybridomas were initially screened for reactivity against immunogen by ELISA and secondarily with indirect immunofluorescence assays (IFA) using mature P. falciparum schizonts as antigen for the reactivity to parasite native PfRipr. Hybridoma cells producing positive signals by both ELISA and IFA were cloned by two rounds of limiting dilution and the antibody isotypes were determined (BEX Co., Ltd. Tokyo, Japan). The mAbs were further screened by western blot using WGCFS-produced PfRipr5 recombinant protein as an antigen (Nagaoka et al., 2020). Finally, a hybridoma clone, 29B11 (isotype: IgG1), producing mAbs recognizing recombinant PfRipr5 (Nagaoka et al., 2020) was expanded, purified and stored at –80˚C.

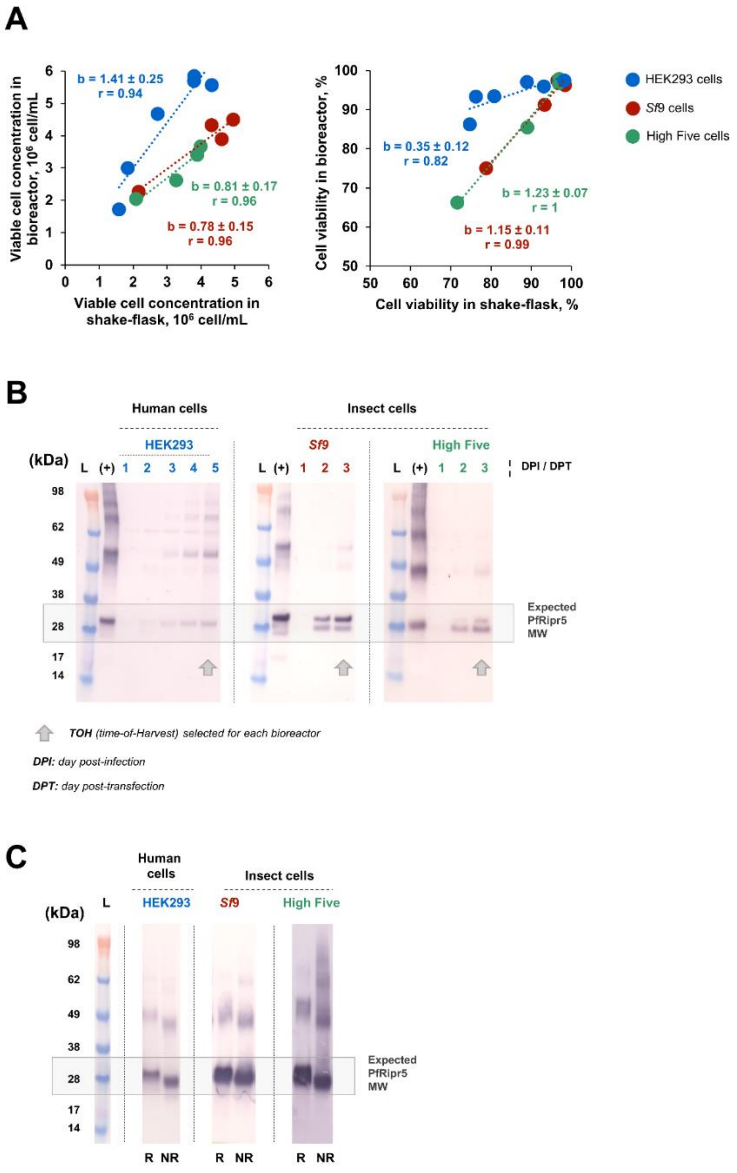
Growth inhibition assay

The inhibitory activity of the mAb29B11 on parasite invasion was tested over one cycle of parasite replication, and parasitemia was determined by flow cytometry (9,10). Briefly, twenty microliters of a late trophozoite-to-schizont stage -infected erythrocyte (pRBC) suspension (0.3% parasitemia and 2% hematocrit), 20 µl of serially diluted mAb, and 20 µl of 2× culture medium were seeded per well on half-area flat-bottom 96-well cell culture microplates (Corning, Corning, NY) and gently mixed. For a control, 20 µl of culture medium was added to the pRBC suspension. Cultures were incubated at 37 °C in

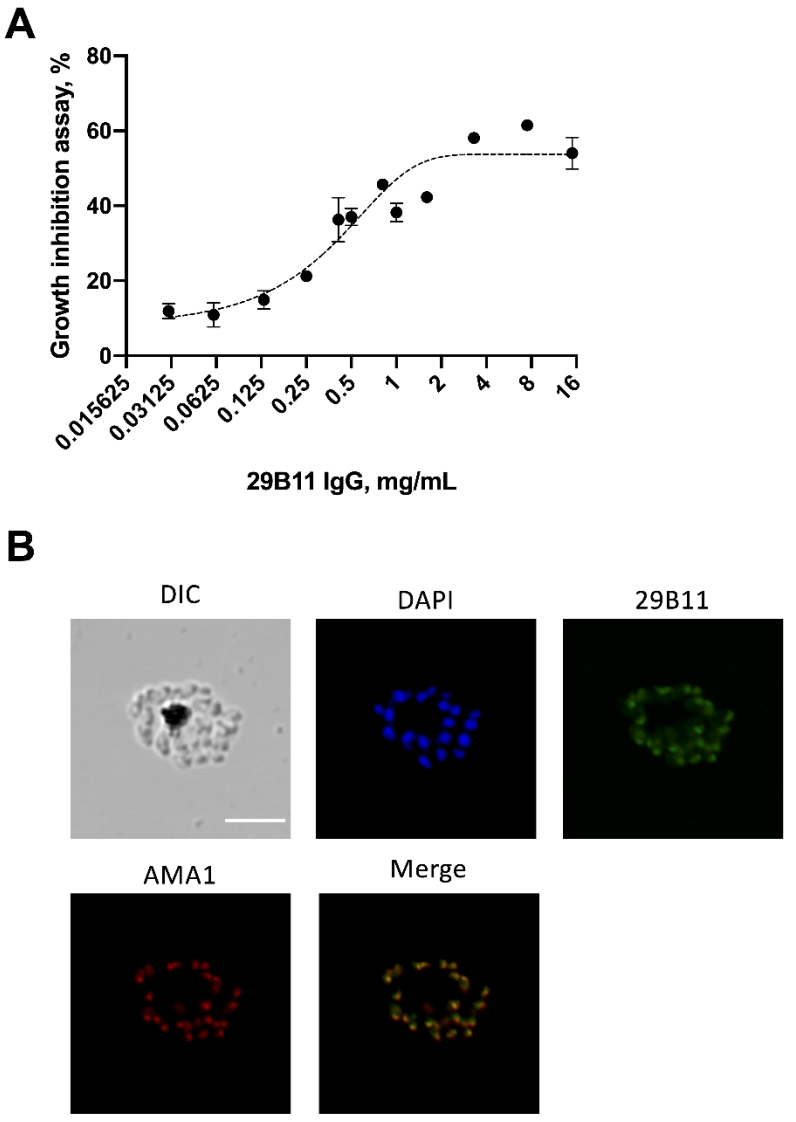
humidified airtight boxes, gassed with 90% N2, 5% O2, and 5% CO2. After 25 h of incubation, the pRBC were pelleted by brief centrifugation (1,300 × g for 5 min) and washed once in 100 µl PBS. The cells were then incubated with 50 µl of diluted (1:1,000 in PBS) SYBR green I (Invitrogen) for 10 min at RT and washed once in PBS. Parasitemia was measured by flow cytometry with a FACSCanto II (BD Biosciences, San Jose, CA) by the acquisition of 50,000 events per sample. Growth inhibition, expressed as a percentage relative to the maximal growth achieved in control wells, was calculated as: % inhibition = 100 - [(parasitemia (%) of infected RBCs with tested mAB - parasitemia (%) of normal RBCs)/ (parasitemia (%) of infected RBCs without any mAb - parasitemia (%) of normal RBCs) x 100].



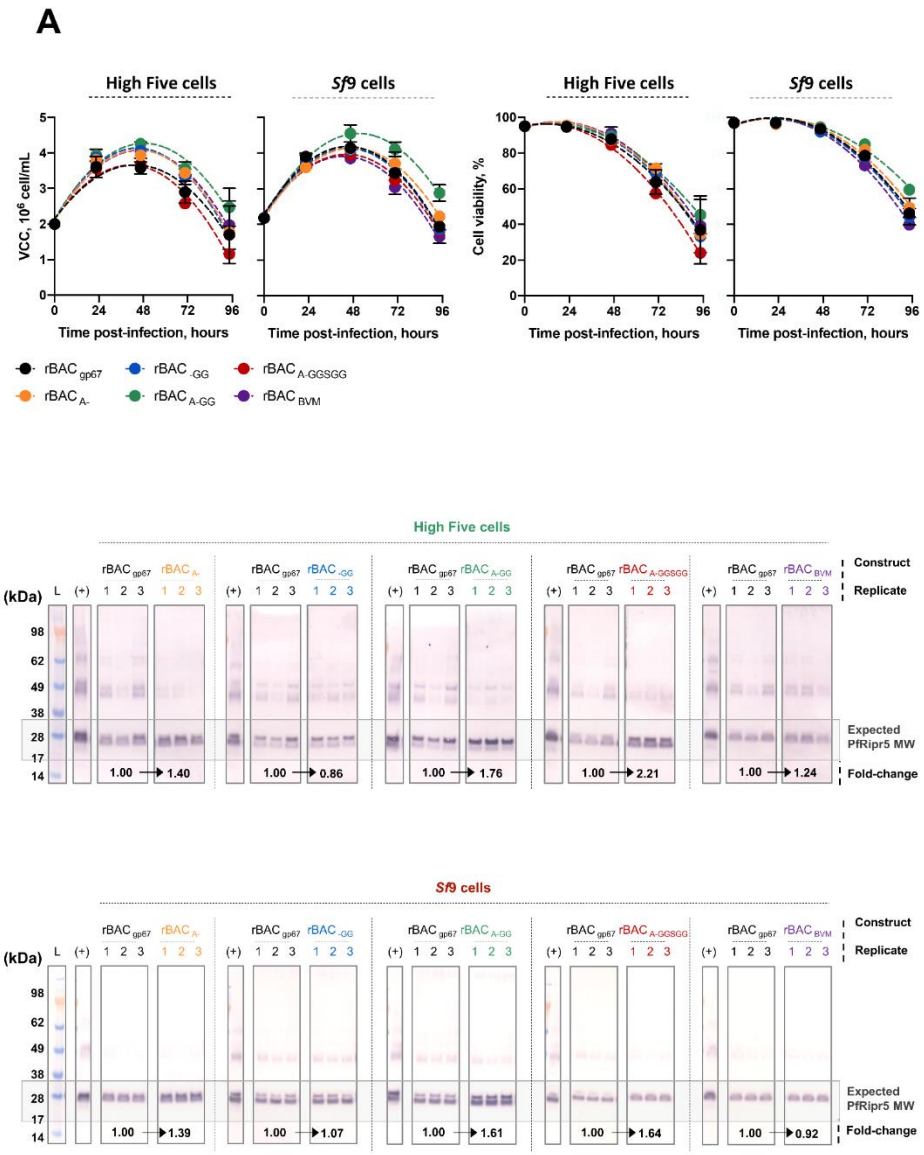
Supplementary Figure 1 Identification of PfRipr5 by Western blot. (A) Production using insect High Five cells. (B) Production using insect *Sf*9 cells. (C) Production using human HEK 293 cells. L denotes pre-stained protein standard SeeBlue® Plus2. Arrows represent the samples selected as TOH for each production condition/replicate, used to assess relative productivity (Figure 1C): (+) denotes the positive control (PfRipr5 produced by WGCFS) used as normalization factor to assess relative band intensity between samples in separate membranes.

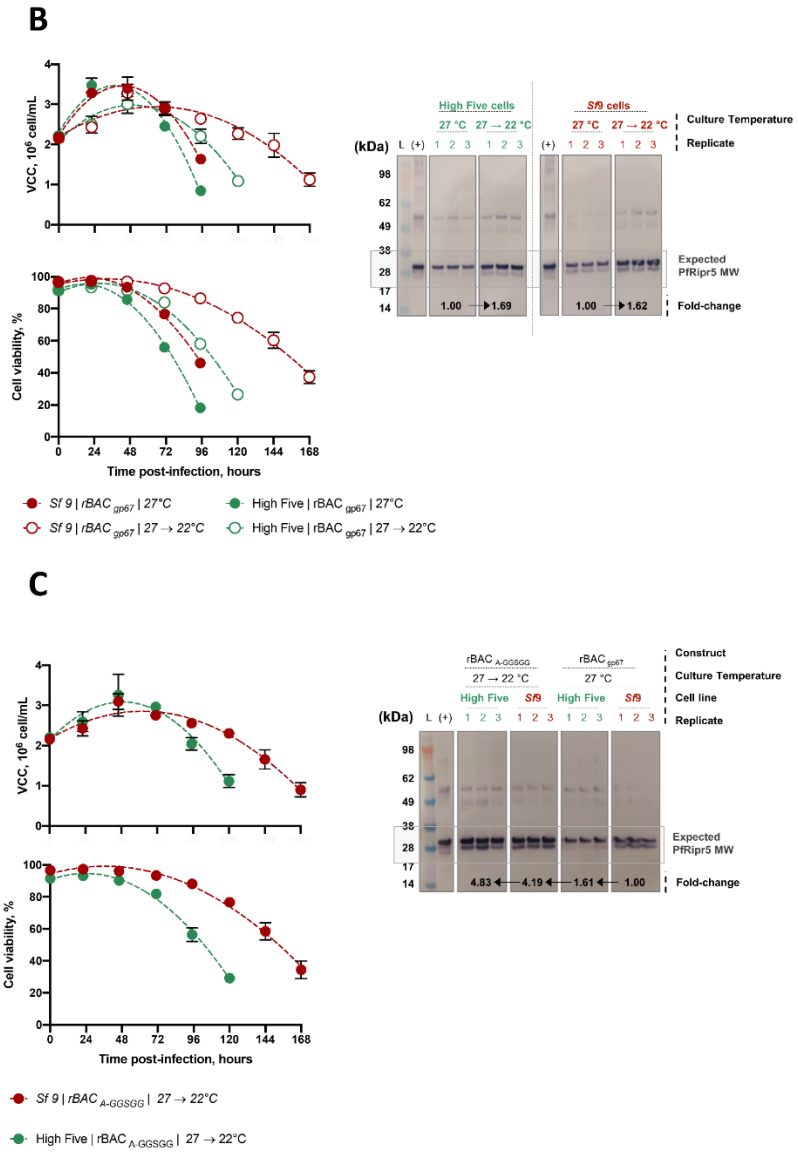


Supplementary Figure 2 Production of PfRipr5 at 2 L STB scale. (A) Correlation of kinetics of cell growth and cell viability between production in SF and STB. (B) Identification of PfRipr5 in culture bulk by Western blot. (C) Identification of purified PfRipr5 by Western blot. For figure (A), b represents the slope and r represents the Pearson correlation coefficient. For figure (B), arrows represent the TOH samples for each cell line, used to assess relative productivity: (+) denotes the positive control (PfRipr5 produced by WGCFS) used as normalization factor to assess relative band intensity between samples in separate membranes. For figure (C), R denotes reduced sample, NR denotes non-reduced sample. For figure (B) and figure (C), L denotes pre-stained protein standard SeeBlue® Plus2. Data is relative to one biological replicate (n = 1).



Supplementary Figure 3 Characterization of anti-PfRipr mouse mAb 29B11. (A) growth inhibition assay (GIA) activity against P. falciparum 3D7 parasite. (B) Staining of parasite PfRipr using mAb 29B11 in immunofluorescence assay (IFA). For figure (A), GIA was performed as described elsewhere (Nagaoka et al., 2020); data is expressed as mean ± standard deviation and is relative to three (n=3) independent GIA experiments. For figure (B), paraformaldehyde-fixed mature schizonts of P. falciparum 3D7 were probed with mAb 29B11 (29B11: green) and co-stained with rabbit antibodies to AMA1 (Nagaoka et al., 2020), a microneme marker (AMA1: red); the parasite nuclei were stained with DAPI (DAPI: blue); DIC denotes differential interference contrast, Merge denotes merged image of 29B11 and AMA1; scale bar = 3 µm.


A
High Five cells
Sf9 cells
High Five cells
Sf9 cells
VCC, 10^6 cell/mL
Cell viability, %
Time post-infection, hours
Time post-infection, hours
Time post-infection, hours
Time post-infection, hours
rBAC gp67
rBAC A-
rBAC -GG
rBAC A-GG
rBAC A-GGSGG
rBAC BVM
High Five cells
Sf9 cells
(kDa)
Construct
Replicate
Expected PfRipr5 MW
Fold-change
1.00 → 1.40
1.00 → 0.86
1.00 → 1.76
1.00 → 2.21
1.00 → 1.24
1.00 → 1.39
1.00 → 1.07
1.00 → 1.61
1.00 → 1.64
1.00 → 0.92



Supplementary Figure 4 Cell growth and viability kinetics and identification of PfRipr5 by Western blot, following optimization strategies herein devised. (A) Strategy I. (B) Strategy II. (C) Strategy III. Data is relative to three biological replicates (n = 3). For figure (B), full symbols represent infections performed at culture temperature of 27 °C, empty symbols represent infections performed with culture temperature shift (27 °C → 22 °C). For figures (A-C), only TOH samples are represented; VCC denotes Viable Cell Concentration; (+) denotes the positive control, L denotes pre-stained protein standard SeeBlue® Plus2; fold-change represents relative band intensity (assessed by densitometry analysis) between each condition (average of three replicates).

7. Acknowledgments

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Chapter 5

Continuous multi-stage production of influenza VLPs using IC-BEVS

Author contribution

Ricardo Correia design and performed the experiments, analysed the data and wrote the chapter.

Abstract

Traditional methods of cell culture-based vaccine manufacturing still rely on batch or fed-batch processes. With an increasingly higher demand of vaccines, the paradigm has started to shift towards intensified processes.

In this study, we implemented a continuous two-stage bioreactor production process using a previously validated high producer insect cell line (High Five cells adapted to neutral pH) and the insect cell-baculovirus expression vector system (IC-BEVS) to produce influenza hemagglutinin (HA)-displaying virus-like particles (VLPs). The system consists of one cell growth bioreactor connected to three production bioreactors operated in parallel at different residence time (RT) (18, 36 and 54 hours). The kinetics of cell concentration and viability, and virus and HA-VLPs titer, were shown to vary significantly depending on RT. Notably, higher RT allowed to achieve higher productivity. In all conditions, a strong virus passage effect was observed, with negative impact on the percentage of infectious particles (decrease of 50 % comparing to the virus seed stock after just 48 hours of infection) and resulting in loss of the genes of interest (influenza HA and M1) from the baculovirus genome (reduction of 80-95 % comparing to the virus seed stock after 14 days of infection). As a result, HA titer showed a decreasing tendency throughout most of the continuous culture. Nonetheless, continuous cultivation was successfully maintained for 14 days, with product quality being confirmed at the end of the process by the presence of particles resembling HA-VLPs.

This work demonstrates a first step for establishment of a continuous setup for influenza HA-VLPs production using IC-BEVS, serving as basis for further optimization towards process intensification and integration.

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1. Introduction

Vaccination is a powerful tool against major infectious diseases (Plotkina, 1999). The emergence of new virus strains demands the continued development of novel, flexible, and scalable platforms for vaccine production. Integrated continuous operation through streamlining of continuous upstream and downstream is projected as the next step of the expected trajectory of process evolution, moving away from traditional batch and fed-batch production processes (Croughan et al., 2015; Konstantinov and Cooney, 2015; Warikoo et al., 2012).

Continuous operation allows to reduce process cost and facility footprint, provides flexibility to produce a variety of products, and may result in higher quality products through continuous harvesting, eliminating batch-to-batch variability (Walther et al., 2015; Zydney, 2015).

Continuous production of certain viruses is hindered by their lytic nature, a burden that commonly limits its full implementation in an industrial setting. To tackle this issue, continuous multi-stage bioreactor schemes can be used (Tapia et al., 2016), i.e. cascades of bioreactors composed by a cell growth bioreactor feeding non-infected cells to an infected (production) bioreactor. This system allows constant renewal of the production bioreactor with new producer cells while mitigating overaccumulation of product/viruses. Multi-stage bioreactors have been used with great success for the continuous production of viruses (Frensing et al., 2013; Tapia et al., 2017, 2019).

Continuous processes of virus infection that are operated for longer periods of time tend to periodically accumulate defective interfering particles (DIPs) (Frensing et al., 2013), i.e. mutated virus particles that require simultaneous infection with a helper (non-mutated) virus in order to replicate (Frensing,

2015). To mitigate this, fine-tuning of operational conditions such as residence time (RT) is essential (Tapia et al., 2019). The RT at which the production bioreactor is operated influences the kinetics of infection, with direct impact on the cell viability and concentration, virus titer, and recombinant protein concentration.

Insect cells are a versatile hosts to produce vaccine candidates including complex virus-like particles (VLPs) (Fernandes et al., 2013), mainly using the insect cell-baculovirus expression vector system (IC-BEVS) (van Oers et al., 2015). VLPs allow high level antigen display allowing broad protection against diseases such as influenza (Schwartzman et al., 2015). Despite being part of the early-stage development of continuous multi-stage bioreactors, processes of recombinant protein production using IC-BEVS in these setups have not seen updates for almost two decades (Kompier et al., 1988; Pijlman et al., 2004; van Lier et al., 1996, 1994, 1992, 1990).

Establishment of high producing cell lines can substantially improve vaccine production process. Adaptative laboratory evolution (ALE) has been suggested as approach to select cells with improved phenotype (Dragosits and Mattanovich, 2013). ALE has been more widely applied for development of bacteria and yeast strains for specific biotechnological applications than for hosts of higher order, being particularly poorly explored in insect cells. Recently, adaptation of insect High Five cells to grow at neutral pH through ALE has resulted in 3-fold improvement in cell-specific productivity of influenza HA-VLPs using IC-BEVS (Correia et al., 2020).

In this work, we implemented a continuous multi-stage bioreactor setup to produce influenza HA-VLPs using neutral-pH adapted insect cells and IC-BEVS and assessed the impact of using three different RT on the dynamics of infection and virus/protein production.

2. Materials and Methods

2.1. Cell Line and Culture Media

High Five insect cells adapted to neutral pH from (Correia et al., 2020) were routinely sub-cultured at 0.3–0.5 × 10^6 cell.mL^{-1} every 2–3 days when cell concentration reached 2–3 × 10^6 cell.mL^{-1} in a culture medium containing a 1:1 mixture of Insect-XPRESS™ (Lonza, Basel, Switzerland) medium and a chemically defined solution containing 50 mM HEPES, 124 mM Sucrose, 5 mM Glucose, 50 mM NaCl, 20 mM KCl, 3 mM CaCl2, 10 mM MgSO4 and 0.1% (w/v) Pluronic F-68. Cells were cultured in 125–500 mL shake flasks (Corning, New York, USA) with a 10% working volume, and maintained at 27 °C in a shaking incubator (Inova 44R – Eppendorf, Hamburg, Germany) set to 100 RPM and with 2.54 cm shaking diameter.

2.2. Baculovirus Amplification and Storage

Recombinant bacmid-origin baculoviruses (rBAC) containing influenza M1 (from A/California/06/2009 H1N1 strain) and HA (from A/Brisbane/59/2007 strain) genes were kindly provided by Redbiotec AG (Schlieren, Switzerland). Amplification of baculovirus stocks was performed as described elsewhere (Vieira et al., 2005). Briefly, insect *Sf*-9 cells, cultivated in Sf-900™ II medium (Gibco, Waltham, Massachusetts, USA), were infected at a concentration at infection (CCI) of 1 × 10^6 cell.mL^{-1} using a multiplicity of infection (MOI) of 0.1 plate-forming units per viable cell (pfu.cell^{-1}). When a cell viability of approximately 80 % was reached, the supernatant was harvested by centrifugation at 200 g and 4 °C for 10 min and centrifugation at 2000 g and 4

°C for 20 min. The clarified supernatant was aliquoted and stored at 4 °C until further use.

2.3. Assessment of rBAC entry and release from insect cells

Insect High Five cells adapted to neutral pH were infected with the recombinant baculovirus mentioned above. Infection was performed in 500 mL shake flasks (Corning, New York, USA), with a 10% working volume, and at CCI of 1×10^6 cell.mL^{-1} and MOI of 1 pfu.cell^{-1}. Culture was sampled every 2 hours between the time of infection (TOI) and 8 hours post-infection, and later every 2 hours between 16 and 24 hours post-infection; supernatant was collected by centrifugation at 200 g and 4 °C for 10 min and stored at 4 °C until further use. Infectious baculovirus titer was assessed by MTT assay (described below). Experiment was performed in duplicate (n = 2).

2.4. Continuous production of influenza HA-VLPs in multi-stage bioreactors

Continuous production of influenza HA-VLPs was performed using insect High Five cells adapted to neutral pH in a two-stage bioreactor set-up composed of a cell growth bioreactor connected to three parallel infection bioreactors operated at different RT (18, 36 and 54 hours) (Figure 2).

For all bioreactors, culture mixing was achieved by varying the agitation rate from 70 to 270 rpm (cell growth bioreactor) or 70 to 300 rpm (production bioreactors). Gas was supplied through a ring sparger at a flow rate of 0.01 vvm, and the percentage of O_2 in the gas mixture varied between 0 and 100% to maintain the pO_2 (partial pressure of oxygen) setpoint at 70% of air

saturation. The bioreactor headplate included multiple ports for temperature, pH and pO_2 probes as well as for additions (i.e., culture medium, cells and baculovirus) and the sampling/harvesting of the cell culture. Cell growth bioreactor was operated in computer controlled BIOSTAT DCU 1 L vessel (Sartorius, Göttingen, Germany) equipped with 1 Rushton impeller. Production bioreactors were operated in computer controlled BIOSTAT Qplus 0.5 L vessels (Sartorius, Göttingen, Germany) equipped with 1 Rushton impeller.

Cells were seeded in cell growth bioreactor at 0.4 x 10^6 cell.mL^{-1} and cultivated in perfusion mode using alternating tangential filtration system (XCell™ ATF-2, Repligen) and an hollow fiber (0.2 µm) as cell retention device, to guarantee nutrient supply. When cell concentration reached approx. 2.1 x 10^6 cell.mL^{-1}, part of the cell culture was transferred to the production bioreactors for their inoculation at 1 x 10^6 cell.mL^{-1} (wv = 400 mL), and cell growth bioreactor was diluted to a final concentration of 1 x 10^6 cell.mL^{-1} (wv = 900 mL). At this point, the production bioreactors were infected at MOI 1 pfu.cell^{-1} and continuous operation was started after 3 hours. Continuous operation was performed by maintaining the cell growth bioreactor at constant cell concentration (1 x 10^6 cell.mL^{-1}) through continuous medium feeding and cell bleeding (used to feed non-infected cells to the 3 production bioreactors at different flow rates, depending on RT). Each production bioreactor was continuously bled at the same flow rate at which cells were fed from the cell growth bioreactor, to ensure RT and maintain culture volume. Cell growth bioreactor was kept in perfusion mode to provide additional medium renewal and maintain constant nutrient concentration.

2.5. Purification of influenza HA-VLPs

Culture bulk from production bioreactors was harvested in the last day of continuous operation and centrifuged at 4 °C, 200 g, for 10 min (for cell removal) and at 4 °C, 2000 g, for 20 min (for further clarification). The clarified supernatant was filtered using a 0.22 µm Stericup (Millipore, Burlington, Massachusetts, USA). HA-VLPs were purified using a SartoBind Q capsule (Sartorius Stedim Biotech, Göttingen, Germany) according to manufacturer's instructions. Specifically, elution buffer was composed by HEPES (50 mM) and NaCl (300 mM) at pH 7.4. The fraction corresponding to HA-VLPs was collected and sterile filtered using a Whatman cellulose regenerated membrane filter. Trehalose (pH 7.4) was added to the purified material to a final concentration of 15% (w/v) using a stock solution of 65% (w/v). The resulting purified material was stored at -80 °C (long-term storage) or at 4 °C (short-term storage).

2.6. Analytics

2.6.1. Cell Concentration and viability

Cell concentration and viability were assessed either using CEDEX HiRes (Roche, Mannheim, Germany) or a Fuchs–Rosenthal hemocytometer chamber (Brand, Wertheim, Germany).

2.6.2. Hemagglutination Assay

The HA titer was determined using the hemagglutination assay as described elsewhere (Sequeira et al., 2018). Briefly, in-process samples (25 µL) were serially diluted 1:1 with DPBS(-/-) 1X (Gibco, Waltham, Massachusetts, USA) in V-bottom 96-well plates (Thermo Scientific, Waltham, Massachusetts, USA)

and gently mixed 1:1 with 1 % chicken erythrocytes (Lohmann, Cuxhaven, Germany). Plates were incubated at 4 °C for 30 min. The HA titer was estimated as being the inverse of the highest sample dilution that completely inhibited hemagglutination.

2.6.3. SDS-PAGE and Western Blot

In-process and purified samples were denatured by mixing with 1X LDS Sample Buffer (ThermoFisher Scientific, Waltham, Massachusetts, USA, stock solution at 4X) and 1X Sample Reducing Agent (ThermoFisher Scientific, Waltham, Massachusetts, USA, stock solution at 10X) and heating for 10 min at 70 °C. Denatured samples were loaded into a 4−12% NuPAGE Bis-Tris protein gel (ThermoFisher Scientific, Waltham, Massachusetts, USA), using MOPS running buffer (ThermoFisher Scientific, Waltham, Massachusetts, USA) and SeeBlue Plus2 Prestained Standard (ThermoFisher Scientific, Waltham, Massachusetts, USA) as a molecular weight marker. After electrophoresis (50 min at 200 V and 400 mA), proteins were transferred to a nitrocellulose membrane using the iBlot system (ThermoFisher Scientific, Waltham, Massachusetts, USA). Membranes were blocked for 1 hour at room temperature with a blocking solution composed of Tris-Buffered Saline (Sigma, St. Louis, Missouri, USA) with Tween-20 (Millipore, Burlington, Massachusetts, USA) and 5% (w/v) skim milk (Millipore, Burlington, Massachusetts, USA), and incubated overnight at room temperature with primary antibodies diluted in blocking solution. For HA identification, a mouse monoclonal antibody (IRR, Manassas, Virginia, USA, FR-494—mouse monoclonal antibody to recombinant H1 HA from influenza A/Brisbane/59/2007 (H1N1)) was used at a dilution of 1:2000. M1 protein was identified using a goat polyclonal antibody (Abcam, Cambridge, UK, Cat# ab20910) at a dilution of 1:2000. Secondary anti-mouse or anti-goat IgG

antibodies conjugated with alkaline phosphatase were used at a dilution of 1:2000 for identification of HA and M1, respectively. Protein band detection was performed by covering membranes with NBT/BCIP 1-Step (Thermo Scientific, Waltham, Massachusetts, USA) for 10 min; membranes were then scanned with a benchtop scanner device.

2.6.4. Baculovirus Titration

Infectious baculovirus titers were determined using the MTT assay (Mena et al., 2003; Roldão et al., 2009).

2.6.5. qPCR

The qPCR analysis was performed to estimate the baculovirus genome copies (often referred as total particles) by assessing expression of IE-1 gene, and the quality of the baculovirus regarding presence of the genes of interest (by comparing expression of IE-1 with HA and M1 genes). Supernatant samples were treated with a DNAse (Roche, Basel, Switzerland) prior to quantification to eliminate overestimation due to free residual baculovirus DNA non assembled into full baculovirus particles. Baculovirus DNA was extracted using the High Pure Viral Nucleic Acid Kit (Roche Life Science, Mannheim, Germany). A master mix was prepared using LightCycler® 480 Probes Master (04707494001; Roche Life Science, Mannheim, Germany), reverse and forward primers (IDT) for the IE-1, HA, and M1 genes at a final concentration of 0.5 µM, PrimeTime® probes (IDT) for the IE-1, HA and M1 genes at a final concentration of 0.25 µM, and PCR-grade water. The qPCR was performed in 384-well plates

(047297490011; Roche Life Science, Mannheim, Germany) using a LightCycler 480 Instrument II (Roche Life Science, Mannheim, Germany).

2.6.6. Metabolite quantification

The metabolite (glucose and glutamine) quantification was performed using Cedex Bio Analyzer 7100 (Roche, Mannheim, Germany) as described elsewhere (Fernandes et al., 2021).

2.6.7. Transmission Electron Microscopy Analysis

The conformation and size of purified influenza HA-VLPs from bioreactor runs were assessed by negative staining TEM using a Hitachi H-7650 Transmission Electron Microscope (JEOL, Tokyo, Japan). For sample preparation, 10 µL of purified HA-VLPs was fixed for 1 min in a copper grid previously coated with Formvar-carbon (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA). Grids were then washed three times with water and finally stained with 1% (v/v) uranyl acetate for 2 min. Grids containing fixed samples were left to air dry and immediately analyzed.

3. Results

3.1. Baculovirus entry and release from insect cells

The kinetics of baculovirus entry and release from insect cells were assessed by infecting insect cells adapted to neutral pH (Correia et al., 2020) and quantifying the infectious baculovirus titer (through MTT assay) during the first day of infection. Virus titer in the culture supernatant was significantly

decreased shortly after infection, with a plateau being reached between at 2-4 hours post-infection (Figure 1). This was defined as the period of time allowing significant virus entry in adapted insect cells. Moreover, infectious baculovirus titer increased at 16 to 18 hours post-infection, this being defined as the period following the first virus replication cycle and further budding from cells.

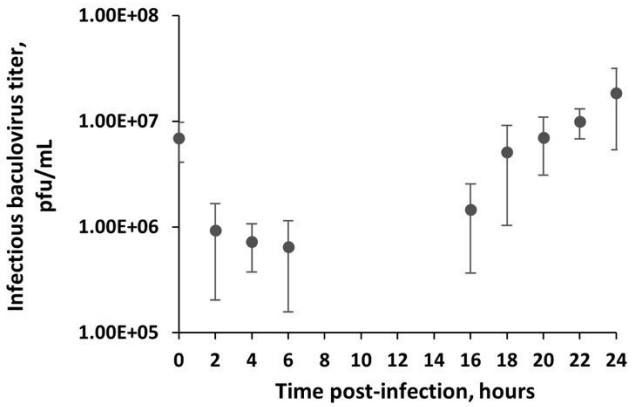


Figure 1 Kinetics of baculovirus entry and release from insect High Five cells adapted to neutral pH.

These results were used to aid on the fine-tuning of operational parameters for establishing the continuous production of influenza HA-VLPs in multi-stage bioreactors. The time required for virus entry in insect cells was defined as 3 hours and used as hold step following infection before operating the bioreactors in continuous mode, to avoid unnecessary virus washout. The time required for baculovirus release (defined as 18 hours) was used as criteria for the selection of the RT being tested.

3.2. Continuous production of influenza HA-VLPs in two-stage bioreactors using IC-BEVS

A two-stage bioreactor setup (i.e. a cascade of a cell growth bioreactor followed by a production bioreactor) was implemented for the continuous production of influenza HA-VLPs using insect High Five cells adapted to neutral pH and the IC-BEVS. Notably, the cell growth bioreactor was connected to three independent production bioreactors operated in parallel at different RT (Figure 2).

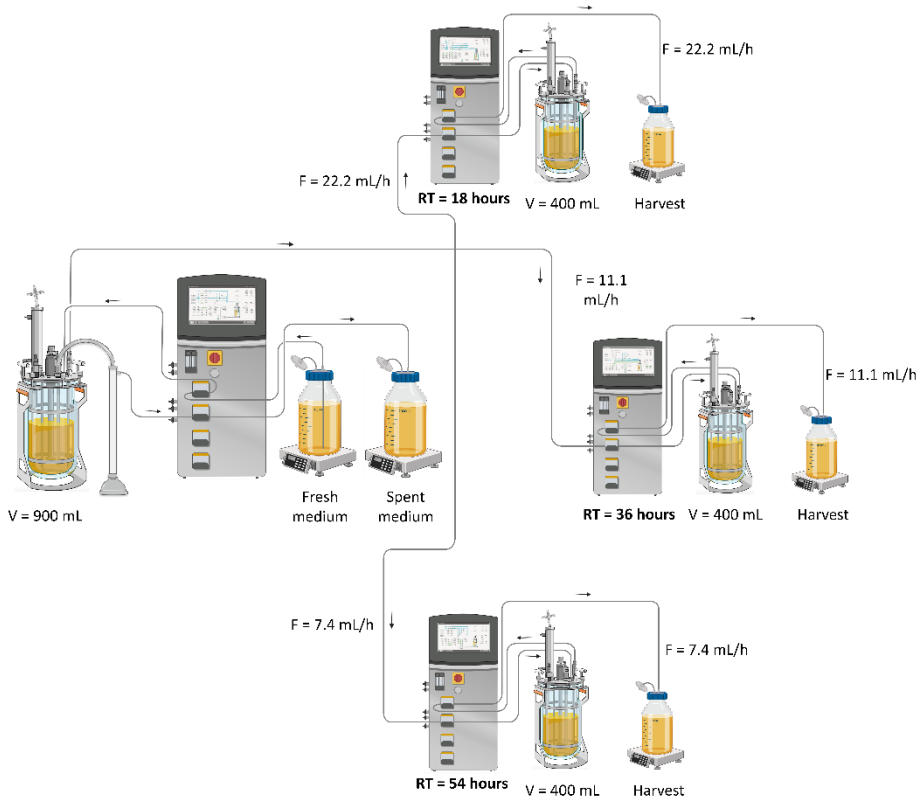


Figure 2 Schematic representation of the two-stage bioreactor setup implemented for continuous production of influenza HA-VLPs, using one cell growth bioreactor to simultaneously feed three parallel production bioreactors, operated at different residence time (RT).

3.2.1. Cell growth bioreactor

Following inoculation and infection of production bioreactors, the cell growth bioreactor was operated in continuous mode to feed non-infected cells to the production vessels. The cell concentration was maintained approx. constant throughout continuous operation (Figure 3A) and cell viability was maintained > 90 % (Figure 3B). By maintaining this bioreactor in perfusion in addition to continuous mode, extra nutrient supply was obtained allowing to maintain the concentration of glucose (Figure 3C) and glutamine (Figure 3D) at values similar to those of the start of the culture.

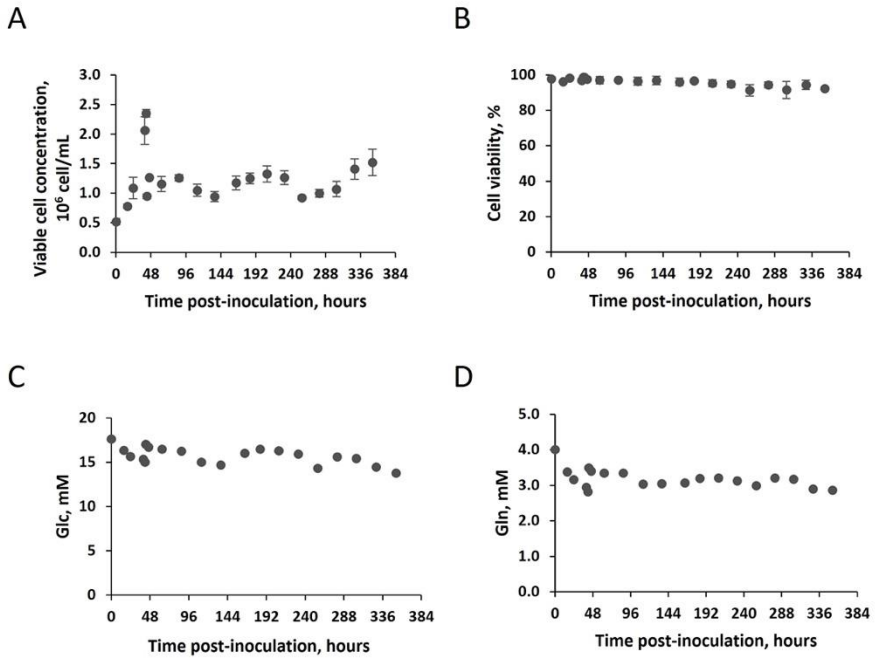


Figure 3 Kinetics of cell growth bioreactor. (A) Viable cell concentration. (B) Cell viability. (C) Glucose concentration. (D) Glutamine concentration. For (A), the peak in cell concentration (time post-inoculation = 42 hours) represents time of infection (TOI) (time post-infection = 0 hours) of the continuous production process, i.e. the point of production bioreactor inoculation and infection, and cell growth bioreactor re-dilution.

These results attest that a constant feeding of viable cells and medium with high nutritional level was provided to the production bioreactors.

3.2.2. Production bioreactors

Cell growth kinetics show a strong influence of RTs on the profiles of cell growth (Figure 4A) and cell viability (Figure 4B). As expected, at higher RT, which are characterized by a slower rate of cell renewal and removal of virus particles, cell growth arrest and cell viability drop were more pronounced. Likewise, the use of higher RTs resulted in lower concentration of glucose (Figure 4C) and glutamine (Figure 4D) throughout time.

The peak of HA titer was observed at 48 hours post-infection (Figure 4E), after which a continuous decrease was observed until the end of the culture. A similar profile of expression of HA and M1 over time was observed by western blot (Supplementary Figure 1A), and the peak in HA titer correlated with a peak in infectious baculovirus titer (Supplementary Figure 1B). In line with previous observations, RT strongly affected the titers achieved, with higher RTs resulting in higher HA titer. Importantly, the ratio of infectious/total baculovirus particles showed a decreasing tendency throughout most of the process (Figure 4F), being more evidence in the first 48 hours, therefore being likely to correlate with the observations for HA titer.

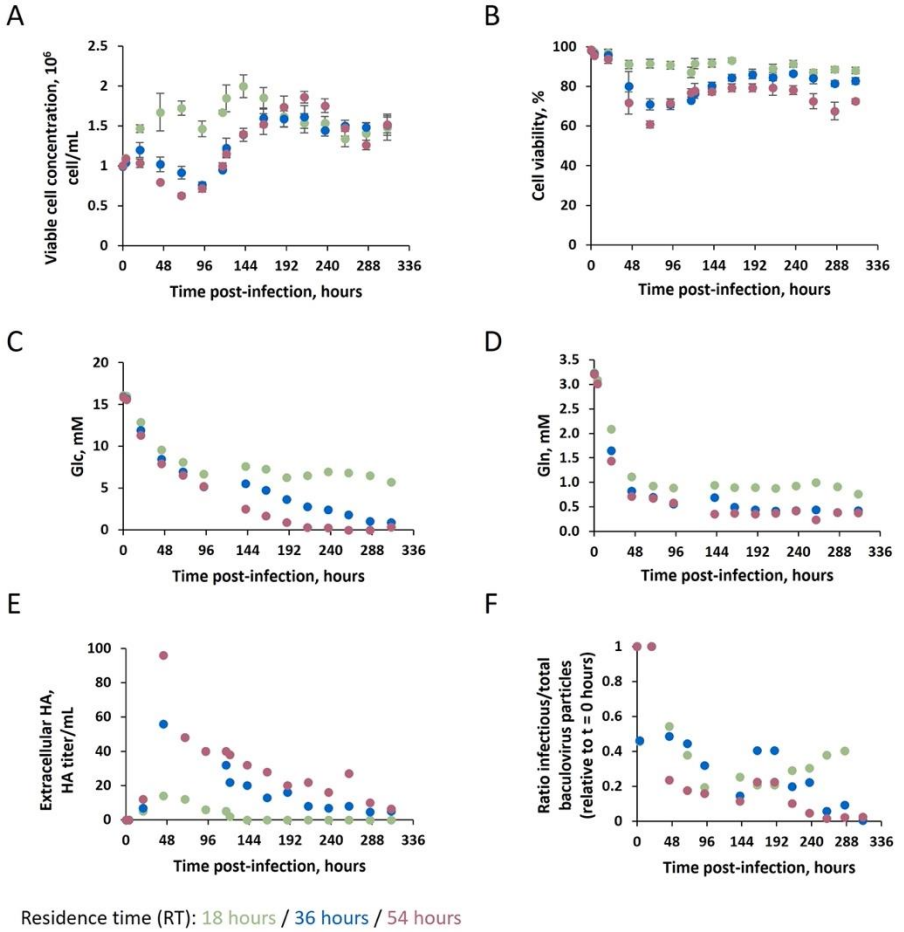

A
Viable cell concentration, 10^6 cell/mL
Time post-infection, hours
B
Cell viability, %
Time post-infection, hours
C
Glc, mM
Time post-infection, hours
D
Gln, mM
Time post-infection, hours
E
Extracellular HA, HA titer/mL
Time post-infection, hours
F
Ratio infectious/total baculovirus particles (relative to t = 0 hours)
Time post-infection, hours
Residence time (RT): 18 hours / 36 hours / 54 hours
G
RT = 18 hours
RT = 36 hours
RT = 54 hours
Ratio of genome copies (normalized to t=0 hours)
Time post-infection, hours
M1 / IE1
HA / IE1
HA / M1
H
RT = 36 hours
RT = 54 hours

Figure 4 Continuous production of influenza HA-VLPs in two-stage bioreactors. (A) cell growth kinetics; (B) cell viability kinetics; (C) profile of glucose concentration; (D) profile of glutamine concentration; (E) extracellular HA titer; (F) ratio of infectious/total baculovirus particles (G) ratio of genome copies; (H) negative-staining transmission electron microscopy of purified HA-VLPs. For (E), HA titer was assessed by hemagglutination assay. For (F), infectious baculovirus titer was assessed by MTT assay (pfu/mL) and total baculovirus titer was assessed by qPCR (IE1 genome copies/mL), data is normalized to TOI. For (G), data is normalized to TOI. For (H), culture bulk harvested at end of continuous culture was harvested and purified, scale bar = 100 nm.

The quality of the baculoviruses being produced was further analyzed regarding the propensity to lose the genes of interest for influenza VLPs production (HA and M1) from their genome. The results demonstrate that both genes of interest are lost from the baculovirus genome (as confirmed by the ratios of genome copies HA/IE-1 and M1/IE-1), with a 50 % loss being observed as early as approx. 5 days post infection for RT = 18 hours (Figure 4G). These results suggest that the production process was significantly compromised by virus passage effect, with implications on virus infectivity and quality (presence of the genes of interest), and thus on HA titer.

Nonetheless, the culture bulk of the production bioreactors operated at RT of 36 and 54 hours was harvested and purified, and particles resembling influenza HA-VLPs were observed by electron microscopy (Figure 4H).

Altogether, these results demonstrate a strong impact of the RT at which the production bioreactor is operated on the dynamics of infection. Amongst the RTs tested, and considering profiles of infectious virus titer, HA titer, glucose and glutamine concentration, cell viability, and loss of the genes of interest from baculovirus genome, a RT of 36 hours is most likely the best option for further optimization of this continuous operation.

4. Discussion

In this work, we implemented a two-stage bioreactor continuous production scheme to produce influenza HA-VLPs using insect High Five cells adapted to neutral pH and IC-BEVS.

The influence of RT on the dynamics of virus and protein production was assessed. Higher RTs are characterized by a slower rate of cell and medium renewal, as well as removal of virus and product, within the production bioreactor. As a result, cell viability drop and cell growth arrest were more evident, and nutrient levels were lower. Moreover, the maintenance of cells and virus particles for longer periods in the vessel benefited production of HA-VLPs. On the other hand, when using a RTs similar to the duration of the virus replication cycle (approx. 18 h), we cannot profit from the very late-stage expression characteristic of the polyhedrin promotor (Lu and Miller, 1997; Yang and Miller, 1999) here used in the baculovirus vector for controlling expression of both HA and M1 genes, with the coexistence of cells and viruses in the vessel being greatly reduced thus compromising recombinant protein production. In addition, it resulted in the highest rate of loss of the gene of interest, suggesting it could be the condition with highest propensity to form DIPs. Fine-tuning RT is crucial for implementing continuous two-stage bioreactor production using lytic systems (Tapia et al., 2019).

In this study, regardless of the RT, we observed a continuous decrease in HA and virus titers after an initial peak at 48 hours post-infection. An oscillatory cyclic-like profile of virus and HA titers would be expected, as this is the typical outcome of continuous two-stage production using lytic systems (Tapia et al., 2019) resulting from an equal oscillatory profile of DIPs accumulating along process (Frensing et al., 2013). A peak of high DIPs concentration induces a

peak of low infectious virus MOI, which is then followed by a new round of prevalence of infectious viruses and consequently a significant increase in the MOI, completing the first oscillatory cycle. Similar to the propagation of other viruses, the formation of DIPs in IC-BEVS (Kool et al., 1991) is a direct consequence of passage effect (Krell, 1996; Zwart et al., 2013).

As the formation of DIPs arises from random and/or prevalent mutations, specific genomic regions including those coding for the gene of interest (Pijlman et al., 2003) can be lost, which can be overcome through rBAC vector design (Giri et al., 2012; Pijlman et al., 2020, 2004). This is particularly concerning in bacmid-origin baculovirus as a consequence of a pivotal role of non-homologous regions of DNA replication on the generation of defective baculoviruses (Pijlman et al., 2002). This evidence may explain the high rate of loss of the genes of interest (HA and M1) from the baculovirus particles generated throughout this continuous process. Thus, it is postulated that the overall inefficiency of this continuous process in producing influenza HA-VLPs may derive from a combination of DIPs overaccumulation and specific elimination of the regions coding for the genes of interest from the particles being generated. The use of other methods of baculovirus stock generation (e.g. flashBAC) can potentially alleviate passage effect.

Insect High Five cells are usually preferred for recombinant protein production whereas insect *Sf*-9 cells are more suited for baculovirus amplification (Wilde et al., 2014). Since neutral pH adapted cells arise from High Five cells, passage effect may have been more prominent thus limiting generation of infectious baculoviruses throughout this continuous process (when comparing to as if *Sf*-9 cells had been used), which ultimately compromises HA-VLPs production in the long-term. For the same reason, *Sf*-9 cells may not be the most suited host to estimate infectious baculovirus titer as herein devised. Thus, estimating

baculovirus infectious titer using neutral pH adapted cells may allow to draw better conclusions regarding virus kinetics profile during this and upcoming continuous operations.

HA and M1 proteins were identified by western blot, and particles resembling influenza HA-VLPs characterized by HA molecules (spikes) uniformly displayed on VLPs surface and multi-component organization (McCraw et al., 2018) were identified by TEM at the end of the experiment. These observations confirm that high quality VLPs were produced even after approx. 2 weeks of continuous operation. Further assessment of particle concentration (e.g. using nanoparticle tracking analysis in Nanosight equipment) could prove helpful (in this and forthcoming studies) to assess the amount of particles being produced towards facilitated integration with a continuous downstream process.

The results here presented pave the way for further development of this two-stage bioreactor continuous production process using IC-BEVS. Performing multiple infections at specific time-points of the continuous process with the same rBAC construct herein used could be a way to provide the culture with infectious and non-mutated viruses, therefore promoting the production of high quality baculovirus and, as a result, influenza HA-VLPs. Alternatively, a new rBAC construct of non-bacmid origin could allow to perform this process without re-infections.

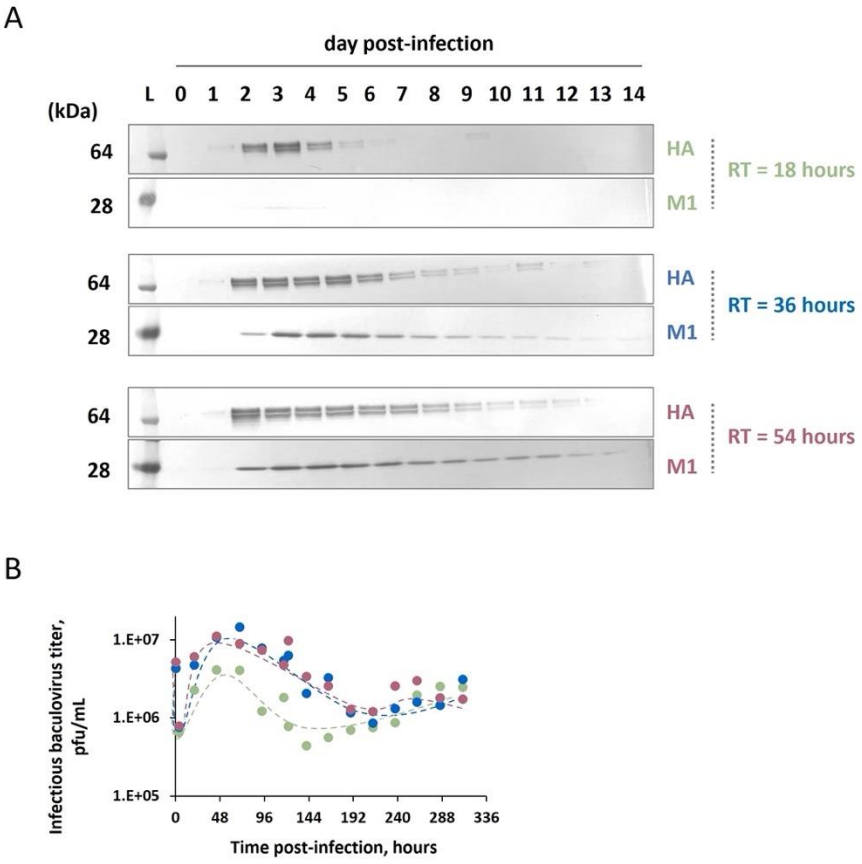
5. Conclusion

In this study, we implemented a continuous two-stage bioreactor production setup using IC-BEVS to produce influenza HA-VLPs, which successfully operated for 14 days. The RT of the production bioreactor was shown to have significant impact on the efficiency of the continuous production as different RT resulted

in different profiles of cell concentration and viability, infectious and total baculovirus titer, and HA titer. Using higher RT was shown to be the beneficial. However, expression of HA-VLPs decreased throughout continuous operation in all conditions tested, possibly due a passage effect on baculovirus which resulted in loss of the genes of interest. Nonetheless, VLPs produced showed correct morphology and size after 14 days of continuous operation.

Despite not being yet operating close to its full potential, the strategy here implemented allowed to gather useful data for further optimization. Re-infection of the production bioreactors may allow to restore quality of the viruses in culture and achieve a cyclic-like profile of HA-VLPs expression. The successful implementation of this continuous process could potentiate the further development of a fully integrated continuous process of HA-VLPs production.

6. Supplementary Material



Supplementary Figure 1 Continuous production of influenza HA-VLPs in two-stage bioreactors. (A) identification of HA and M1 proteins by Western blot in in-process samples during continuous production. Samples loaded are clarified supernatants, non-diluted; same volume was loaded in the gel for all samples. L: Pre-stained protein standard SeeBlue® Plus2; (B) infectious baculovirus titer (pfu/ML) assessed by MTT assay.

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Chapter 6

Discussion

Author contribution

Ricardo Correia wrote the chapter.

TABLE OF CONTENTS

1. Discussion

The emergence of new viruses and/or virus strains demands continued development of improved platforms for vaccine production. The previous chapters described improvements achieved in different stages of the vaccine manufacturing process using insect cells, namely the upstream processing and storage.

The use of atypical culture conditions was assessed to improve production of two potential vaccine candidates, specifically an influenza VLP-based vaccine candidate and a malaria vaccine candidate based on the blood-stage antigen protein PfRipr5. The outcome of these studies displays the efficiency in manipulating two different culture parameters (pH and temperature) through two different approaches (time-point shift in culture parameter and adaptive laboratory evolution), thus demonstrating the versatility of using non-standard culture parameters. Being broadly used for the development of microbial strains with industrial relevance, evolutionary approaches have been devalued for improving performance of insect cells.

Improving vaccine storage might be just as important as improving production. Maintaining the vaccine cold chain presents a significant economic burden and can be especially challenging in lower income countries due to lack of specialized equipment or particularly higher ambient temperature. Here, a water-free storage formulation based on natural deep eutectic solvents has shown to confer higher storage stability to influenza VLPs with potential for vaccine storage at room temperature. The development of improved storage formulations dismissing the need for refrigeration can significantly reduce costs and logistics associated with vaccine storage and transportation.

A multi-stage bioreactor set-up was implemented to crave the first steps for establishing a continuous production process of influenza VLPs using insect cells and baculovirus infection. Though not fully optimized, this work allowed to derive conclusions on the dynamics of virus and protein production, regarding quantity and quality. It represents a step forward towards the establishment of a fully continuous process for further integration with downstream processing, following the current trend in vaccine manufacturing.

1.1. The less evident advantages of using atypical culture conditions

In addition to enabling higher productivity as demonstrated throughout this thesis, the use of non-standard values of culture parameters can have impact (and potentially be advantageous) in other product features.

Protein conformation changes upon variations in their thermodynamic states as a result of alterations in solution's parameters such as temperature or pH (O'Brien et al., 2012). pH-dependent protein conformational changes derive from variations in the protonation state of side chains of ionizable amino acid residues, which largely influence protein structure, stability and solubility (Di Russo et al., 2012; Ozog and Bechet, 1995). Correct protein conformation and structure stability is necessary to maintain its efficient functionality, and pH alterations may induce denaturation (Guckeisen et al., 2021). As for antigenic proteins contained in vaccines, correct conformation and therefore biologic activity is essential for proper immunogenicity (Scheiblhofer et al., 2017). Thus, a shift in culture pH, besides its impact on cell performance, can be the key for harvesting an antigen with the desired conformation and biological function. Likewise, modulation of culture temperature may influence the quality

(structural stability and consequently activity) of the antigens being produced. Protein synthesis, folding, unfolding and turnover is mediated by chaperone and protease systems, ensuring that proteins are correctly folded and functional (Saibil, 2013). Chaperone activity can be promoted by manipulating temperature (Haslbeck, 1999). This parameter can even be used to switch functions of heat shock proteins between molecular chaperone and proteolytic activity (Spiess et al., 1999). In fact, activity of chaperones can be behind the dynamics of evolutionary events allowing cell adaptation (Çetinbaş and Shakhnovich, 2013). Therefore, manipulation of culture temperature during vaccine production can theoretically be influencing the function of the molecular entities responsible for proper protein folding, with direct consequence on the structure and activity of the vaccine candidates, and thus on vaccine efficacy.

The aforementioned examples suggest that the use of atypical culture conditions can impact on other factors than just productivity, namely on the quality of the vaccines being produced. The use of atypical culture conditions has allowed to produce recombinant proteins with better quality in addition to higher titer (McHugh et al., 2020; Zheng et al., 2018), which could in some cases be associated with improved glycosylation events (Alhuthali et al., 2021; Donaldson et al., 1999). Using non-standard culture conditions to improve vaccine quality, even if compromising production load, could be a valid possibility. Despite not being assessed throughout this thesis, the conformational stability of the proteins produced at non-standard culture parameters in Chapter 2 and Chapter 4, and consequently their functionality as vaccines, should be assessed and compared with those produced at standard culture conditions. Most common methods for assessment of protein structure include circular dichroism and x-ray crystallography (Guckeisen et al.,

2021; Manta et al., 2011; Papageorgiou and Mattsson, 2014), whereas the immunogenicity of these vaccine candidates could be evaluated through immunization/challenge studies.

Using atypical culture conditions can also allow to reduce costs. For example, cultivation at reduced (≈ room) temperature as described in Chapter 4 can be cheaper at an industrial level than the standard procedure for not requiring heating for cell incubation. Moreover, the medium formulation used in Chapter 2 for culture at higher pH was obtained by diluting a commercially available insect cell medium, therefore being a cheaper alternative at least at laboratory scale.

1.2. Shift in culture parameter vs evolutionary engineering – what to choose?

Manipulation of culture conditions to attain higher productivity may be achieved either by performing a time-point shift in a culture parameter (Chapter 4) or by following an adaptive laboratory evolution approach, i.e. adaptation of cells to grow continuously under the influence of the non-standard culture parameter (Chapter 2). There is no universal formula for using non-standard culture conditions to improve productivity, both in terms of approach and parameter being manipulated; the same procedure can lead to opposite outcomes even in closely related products (Mason et al., 2014).

Most studies addressing changes in culture parameters focus on time-point shifts rather than evolutionary engineering. Lowering culture temperature is one of the main approaches for improving recombinant protein production using mammalian cells (Kaufmann et al., 1999; Vergara et al., 2014). Adaptive laboratory evolution has been mostly used to develop bacteria and yeast

strains with improved fitness (cell growth, nutrient utilization, byproduct tolerance) for industrial applications (Dragosits and Mattanovich, 2013). Both strategies are far from being used to their full extent in insect cells.

Time-point shift in culture parameters is theoretically facilitated when manipulating parameters which are conferred by external factors, such as temperature and oxygenation (DO, CO2). When manipulating parameters associated with the composition of the culture medium, such as pH (Correia et al., 2020), osmolarity (Olejnik et al., 2003), or the presence/absence of specific nutrients or byproducts towards personalized nutrient utilization (Seong et al., 2020), an evolutionary approach could suit better the process. Time-point shift in these parameters may potentially require renewal of the culture medium to provide the new conditions, thus being less practical. Despite parameters such as osmolarity or pH being adjustable on site by adding salts or base, abrupt fluctuations in these parameters can have a detrimental effect on cells (Krampe and Al-Rubeai, 2010), which can be especially concerning if the target value to be tested is considerably different from the standard value. For that reason, evolutionary approaches may require a step-wise adaptation approach, to move from the standard to adapted conditions (Correia et al., 2020; Fernandes et al., 2020).

Another important aspect to take into consideration is the timespan of the production process, and how the use of non-standard culture conditions influences it. For instance, within Chapter 4, we have also assessed the use of insect cells adapted to low culture temperature via ALE (adapted as described in (Fernandes et al., 2020)) (data not shown). However, the titers achieved with adapted cells were similar to those obtained when performing the temperature shift to the same lower temperature (22 °C) as reported in Chapter 4. Thus, for requiring an adaptation process (which can account for up

to 2-3 months) and for having a slower growth rate than cells cultured at 27 °C (Fernandes et al., 2020) thus prolonging cell expansion, the use of cells adapted to lower culture temperature was not an attractive option when comparing to a shift in this parameter at the time of infection.

1.3. Continuous production using IC-BEVS: the burden of a lytic system

Continuous production of vaccines using IC-BEVS is challenging due to the fact that this is a lytic system, which in principle implies cell lysis and end of culture at some point. Engineering approaches using cascades of bioreactors have been used with great success for the production of live viruses (Frensing et al., 2013; Tapia et al., 2016, 2017, 2019a) in other lytic systems. The use of these multi-stage setups for continuous recombinant protein production using insect cells and baculovirus infection has however, to the best of the author's knowledge, not been assessed in almost 20 years (Kompier et al., 1988; Pijlman et al., 2004; van Lier et al., 1996, 1994, 1992, 1990).

The results achieved in Chapter 5 demonstrate the challenges with implementing such processes. The quality of the baculovirus being generated over time is highly compromised by passage effect (Krell, 1996). This outcome might be a consequence of the formation of defective interfering particles (Kool et al., 1991) amplified by the bacmid origin of the recombinant baculovirus used in this work (Pijlman et al., 2003). The use of another non-bacmid related method to generate the baculovirus stock (Schaly et al., 2021), such as the FlashBAC (used in Chapter 4), could mitigate this problem. Alternatively, different bioreactor configurations such as tubular plug-flow

bioreactors could avoid the oscillations in titers resulting of the periodic accumulation of defective interfering particles (Tapia et al., 2019b).

Unlike other continuous processes, the product of interest in IC-BEVS is generally a by-product (recombinant protein) of the virus replication and not the virus itself. Thus, fine-tuning the residence time in the production bioreactor (Tapia et al., 2019a) to optimize the process might not be as straightforward as when viruses are the end target. Lower residence times (around 24 hours) might suit continuous virus production; however, higher residence time was necessary to observe a first significant increase in protein titer in this work, this being in line with the evidences of batch processes (Correia et al., 2020).

1.4. Using NADES for vaccine storage

Advancements in vaccine storage specifically aiming for less restrictive temperature control are needed to alleviate the burden posed by maintaining the cold chain. When used as storage medium, NADES formulations have the potential to bypass the inherent vulnerability of protein biologics to high temperature (Lee et al., 2018) and enable their storage at room temperature.

In Chapter 3, we have shown improved storage stability of influenza HA-VLPs formulated in a trehalose-glycerol (TGly) NADES. This specific formulation was suggested as cryoprotective agent and reduced the formation of ice crystals (upon freezing) when mixed at different proportions with water (Castro et al., 2018), suggesting that its use to formulate vaccines could prove beneficial during freeze-thaw cycles. The high viscosity of pure TGly may limit its direct applicability for product formulation in downstream processing. However, an appropriate dilution of NADES systems (inc. TGly) can significantly reduce their

viscosity while maintaining protective properties (Correia et al., 2021; Lee et al., 2018; Liu et al., 2018).

NADES could also be easily implemented for storage of other types of vaccines such as veterinary; in fact, the thermal stability conferred by NADES could be important for the development of oral vaccines that are made available to wildlife directly on the field (Maki et al., 2017), conferring better protection to adverse ambient conditions. The noticeable higher stability of influenza HA-VLPs conferred by TGly upon exposure to high temperature encourages to further investigate this type of formulations. Other NADES formulations composed of common vaccine excipients generally regarded as safe ("GRAS") including aminoacids (e.g. proline, arginine), sugars (e.g. sucrose, frutose), antioxidants (e.g. malic acid), among others, could be suitable alternatives (Cardoso et al., 2017; Dai et al., 2013). The fact that this was the first report of improving vaccine storage using a NADES formulation demonstrates that there is unquestionably room for improvement.

2. Conclusions and future perspectives

This thesis described improvements in the production of two vaccine candidates using atypical culture conditions, enhanced vaccine storage using a potential water-free formulation, and preliminary data regarding implementation of a continuous vaccine production process using insect cells and baculovirus infection. The following conclusions could be withdrawn from this work:

- Adaptation of insect High Five cells to grow at neutral pH (rather than the standard 6.2) allows to improve production of influenza virus-like particles;
- Adaptation to neutral pH was essential to achieve the outcome described above, since a time-point shift in culture pH was inefficient in improving VLP titers;
- The adaptation process had no influence on the morphological characteristics of the VLPs;
- A storage formulation based on a natural deep eutectic solvent system composed of trehalose and glycerol allowed to protect influenza VLPs (maintain HA titer and particle integrity) at a higher level than an aqueous standard formulation upon thermal stress;
- This formulation was promising in storing VLPs at room temperature, which could significantly impact the costs and logistics of vaccine storage;
- The production of the malaria vaccine candidate PfRipr5 could be improved by performing a temperature shift to mild hypothermic conditions, using insect High Five and *Sf*-9 cells;
- Temperature shift was shown to be a more suitable approach than adaptation to improve production of PfRipr5 (data not shown);
- Optimization of the design of the recombinant baculovirus vector, specifically the inclusion of an alanine amino acid residue adjacent to the signal peptide cleavage site and the inclusion of a glycine-serine spacer upstream of the purification tag, also resulted in improved PfRipr5 production. When combined with temperature shift, the optimization strategy allowed to improve production yield by over 5-fold;

- Assessing conformational stability and efficacy of the vaccine candidates here produced could be an added value, to confirm the null impact of using atypical culture conditions;
- In a multi-stage bioreactor production system using IC-BEVS, the residence time in the production bioreactor was shown to strongly affect the kinetics of infection (such as profiles of viable cell concentration, cell viability, virus titer, VLPs titer);
- The low quality of the recombinant baculovirus (i.e. decreasing ratio infectious/total particles and content of gene of interest) being produced over time of continuous operation suggests a prominent virus passage effect.

The work here developed showed improvements in the upstream and storage of vaccines, however these are merely two stages of the entire manufacturing process. Advancements in downstream processing of vaccines need to accompany the progresses achieved in the upstream, to avoid purification from being the limiting step on the process intensification route. As stated throughout this thesis, the expected natural route of progression of the vaccine manufacturing field is thought to steer towards process intensification and integration. With that in mind, this work converged to the preliminary development of a continuous production process of an influenza vaccine candidate. In addition to reduce the batch-to-batch variability, continuous processing allows to reduce equipment footprint since processes can be prolonged rather than spatially upscaled or replicated (as in batch or fed-batch). However, continuous upstream implies constant harvesting of potentially large volumes, hence the importance of revamping downstream as much as upstream. Continuous multi-column chromatography has arisen as an attractive technique for continuous culture bulk processing, whereas perfusion

processes using hollow fiber membranes not only support the production process but can also serve as continuous clarification process to feed downstream.

Process integration is therefore foreseen in a near future assuming that the continuous process here suggested can be optimized. If continuous downstream cannot support upstream, repetitive batch-like purification rounds could be a solution. However, the hold times of harvested culture should be carefully considered to avoid compromising product quality. In line with the process intensification direction and given the higher cell-specific production rate of the adapted cells described in this thesis, these could be employed in a perfusion process at high cell density to significantly increase the volumetric titers, since this strategy has proven efficient in overcoming cell density effect in prior studies.

The next steps of optimization of the continuous process will focus on trying to compensate or avoid the production of defective baculoviruses. A periodic multi-infection process could recover the quality of these viruses in the production bioreactor and promote a cyclic-like profile of virus and recombinant protein titer. However, the feasibility of this strategy could be compromised by a competition of cell infection between the new viruses being added and the defective ones generated in culture. Importantly, these new viruses could even serve as helper viruses for the replication and over-accumulation of the defective counterparts. Therefore, the use of baculoviruses with less propensity to periodic accumulation of defective particles could be a plausible solution, with the non-bacteria origin ones being a valid option, as previously discussed.

The mechanisms underlying higher performance of the adapted cells here established are, at this point, still unknown. It is not clear if the adaptation

process gave rise to beneficial mutation events or just filtered (as means of survival) cells with improved fitness to grow at the atypical conditions (due to differential metabolism, gene expression, or morphology). Further characterization of this cell population is foreseen hereafter. A multi-omics profiling of adapted cells is envisaged, with metabolomics (to assess different metabolic traits), membrane lipidomics (to assess cellular membrane content regarding components involved in budding of VLPs) and transcriptomics (RNA sequencing, to assess the signatures differentiating adapted cells regarding gene expression) being the upfront candidates for this analysis.

PfRipr5 produced by insect cells using standard culture conditions was used for immunization studies with animal models, and the serum collected revealed *in vitro* growth inhibitory capacity against a malaria parasite strain above the threshold defined within the scope of this project (data not shown). Similar work assessing PfRipr5 produced using the optimized strategy implemented in this thesis (i.e. shift to lower culture temperature) is envisaged and will allow to assess the quality of this vaccine candidate when produced at lower culture temperature. Likewise, immunogenicity of influenza HA-VLPs produced at neutral pH should be assessed and compared to that of HA-VLPs produced at standard conditions. Immunization and challenge studies could be performed in the future to assess whether the atypical culture conditions compromised immunogenicity.

Finally, the development of water-free formulations for vaccine storage could be improved by using natural deep eutectic solvent systems with lower viscosity, to facilitate the process of re-suspensions of the lyophilized vaccine. Noteworthy, an option with considerably lower viscosity could even be implemented directly at the end of the vaccine purification process, dismissing

the need of a lyophilization process. Such innovation could be foreseen in line with the intention to establish an integrated process.

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ITQB NOVA, Avenida da República, 2780-157 Oeiras, Portugal | Tel +351 214 469 100 | itqb.unl.pt