UNIVERSITÉ DE MONTRÉAL

HIGH-YIELD PRODUCTION PROCESS OF INFLUENZA VIRUS-LIKE PARTICLES IN HUMAN CELLS TOWARD LARGE-SCALE VACCINE MANUFACTURING

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DEDICATION

"To my mom! For all the love, strength and dedication"

"A mi amada mami! Por su amor incondicional y por creer siempre en mi"

"To my sister and Mahdi for all the love and support!"

"Just keep swimming"

Dory

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RÉSUMÉ

Le virus influenza a été la cause d'épidémies et de pandémies parmi les plus anciennes et meurtrières rapportées dans l'histoire de l'humanité. La vaccination est le moyen le plus efficace de prévenir les infections. Par contre, la fabrication actuelle des vaccins contre l'influenza se fait dans les œufs, avec des embryons de poulet, un procédé lent et laborieux qui limite la capacité de répondre efficacement en cas de pandémie ou suite à une forte demande pour la grippe saisonnière. De plus, ces vaccins induisent principalement une réponse humorale contre les antigènes dominants hémagglutinine (HA) et neuraminidase (NA), deux protéines du virus, causant un manque de protection croisée contre certaines des nouvelles souches. Leur efficacité est également réduite chez certains groupes plus vulnérables (par ex. personnes âgées et jeunes enfants). Par conséquent, l'industrie se tourne vers le développement d'une nouvelle gamme de vaccins plus immunogéniques et produits à partir de plateformes plus efficaces. Les particules pseudo-virales (*Virus-like particles* en anglais, VLPs) constituent une alternative intéressante comme vaccin. Les VLPs présentent une structure qui s'apparente à celle des virus de type sauvage, en permettant la présentation à leur surface des antigènes principaux, dans leur conformation native. De plus, les particules pseudo-virales sont non-infectieuses et incapables de se répliquer. Les cellules de mammifères offrent plusieurs avantages comme plateforme d'expression pour la synthèse de nombreux produits biopharmaceutiques; elles sont capables d'effectuer des modifications post-traductionnelles complexes, de croître à haute densité et de produire des VLPs en suspension et en bioréacteur. Jusqu'à maintenant, les études traitant des VLPs influenza (produites avec des cellules de mammifères) se sont concentrées principalement sur l'assemblage du virion et sur le mécanisme de bourgeonnement cellulaire, alors que seulement un nombre limité d'études porte sur leur production à grande échelle et leur emploi potentiel comme vaccin. Dans le cadre de cette thèse, un bioprocédé transposable à grande échelle pour produire des quantités importantes de VLPs chimériques Gag-influenza à partir de cellules HEK-293 (cellules de reins issues d'un embryon humain) a été développé.

En premier lieu, nous avons généré une lignée cellulaire HEK-293 exprimant de façon stable les protéines HA et NA (souche H1N1 du virus Influenza) sous le contrôle d'un système inductible au cumate. Ensuite, la formation des VLPs chimériques a été induite et dirigée par la transfection de plasmides codant la protéine Gag du virus de l'immunodéficience humaine ou la protéine M1,

une composante de la matrice du virus de l'influenza. La protéine Gag a été fusionnée à la protéine fluorescente verte pour faciliter le suivi de la production des VLPs. Les protéines antigéniques ont été produites 7 fois plus efficacement en présence de la protéine Gag, ce qui indique qu'il s'agit d'une meilleure protéine structurale que M1 dans ce contexte. Par conséquent, la production de VLPs contenant HA-NA et Gag (par transfection) a donc été transférée à l'échelle d'un bioréacteur de 3L avec agitation. Les VLPs ont été recueillies par ultracentrifugation sur un coussin de sucrose, puis concentrées par filtration à flux tangentiel en employant une membrane ayant des pores d'une taille limite de 1000 kDa. Plusieurs techniques ont été employées pour caractériser les VLPs produites: immunodiffusion radiale simple, essai d'hémagglutination, immunobuvardage de type Western et dot blot, ainsi que la microscopie électronique à transmission. Par ailleurs, lors d'essais sur des animaux, l'immunisation intranasale à partir de VLPs a permis d'induire une réponse immunitaire spécifique en plus de conférer une protection totale à toutes les souris soumises à l'épreuve (100% d'efficacité) avec une souche homologue de l'influenza.

Après avoir démontré l'efficacité des VLPs comme vaccin *in vivo* (démonstration de faisabilité), une nouvelle lignée cellulaire inductible a été développée, exprimant cette fois les trois protéines HA, NA et Gag fusionnée à la GFP. Le but de générer une telle lignée était de simplifier le procédé de production en éliminant l'étape de transfection transitoire, qui peut être laborieuse à conduire à grande échelle. Sans sacrifier la production spécifique, le procédé a été optimisé pour obtenir une plus grande production volumétrique en augmentant notamment la densité cellulaire au moment de l'induction et en employant un mode de perfusion. L'opération a été réalisée à l'aide d'un bioréacteur de 3L et d'une filtration à flux tangentiel subséquente permettant d'augmenter les rendements de VLPs de 60 fois ($3x10^{11}$ Gag-GFP événements fluorescents/L de culture mesurés par cytométrie de flux) en comparaison à une production sans perfusion ($5x10^{9}$ Gag-GFP événements fluorescents/L). Le procédé a été caractérisé en déterminant la cinétique de production des protéines d'intérêts présentes dans les VLPs (le procédé en amont) ainsi que le taux de récupération pour chaque opération unitaire de la purification (le procédé en aval). L'opération du bioréacteur, en mode de perfusion et avec la lignée stable 293-HA/NA/Gag-GFP, a permis d'obtenir 5 fois plus de protéines antigéniques HA dans les VLPs que le bioréacteur opéré sans perfusion où la transfection transitoire a été employée. Le nouveau procédé développé

a permis de générer des rendements supérieurs à ceux publiés jusqu'à ce jour pour des VLPs influenza produits à partir de cellules de mammifères.

Finalement, dans le but de répondre à certaines préoccupations de biosécurité associées à un usage potentiel de VLPs comme vaccins (parce que ce sont des particules enveloppées qui bourgeonnent d'une cellule hôte et qui renferment des protéines cellulaires, de l'ADN et de l'ARN), nous avons effectué une caractérisation du protéome des VLPs Gag-influenza produites par transfection transitoire et des vésicules extracellulaires produites par des cellules HEK-293 de type sauvage. Les fonctions des protéines identifiées dans les VLPs et dans les vésicules extracellulaires ont été discutées.

Le procédé développé dans le cadre de cette thèse devrait être efficace pour produire des VLPs exposant les protéines HA et NA issues de différentes souches d'influenza. Les VLPs produites pourraient être évaluées dans le cadre d'essais cliniques dans le but de conduire au développement d'un vaccin efficace et sécuritaire pour remplacer ou complémenter le procédé actuel de production dans les embryons poulets.

ABSTRACT

Of the fatal infections noted in human history, influenza epidemics and pandemics are among the most ancient. Vaccination remains the most effective tool to prevent infection. However, the current production of influenza vaccines in embryonated chicken eggs has limited capacity during pandemics or high demand seasons, and is both labor-intensive and time-consuming. Furthermore, the seasonal egg-produced vaccines mainly induce humoral response to the Hemagglutinin (HA)/Neuraminidase (NA) dominant antigens, which leads to a lack of cross-protection against other non-matching novel strains. In addition, the vaccines provide low protection in high risk groups (e.g., elderly and young children). Consequently, the industry is moving toward the development of novel, more immunogenic influenza vaccines as well as more efficient production platforms. Virus-like particles (VLPs) constitute a promising alternative as influenza vaccine. They mimic the particulate structure of wild-type viruses while they are non-infectious, non-replicative particles, and the main antigens repetitively displayed on their surface maintain the native conformation. Mammalian cell culture offers several advantages for the production of biopharmaceuticals such as their ability to perform complex post-translational modifications and the high cell densities and productivities reached in suspension culture bioreactors. Up to now, the production of influenza VLPs from mammalian cells has been mostly addressed to study influenza assembly and budding mechanisms but little attention has been paid to its potential use for large-scale manufacturing of VLPs as influenza vaccine candidate. The aim of this thesis was to develop a scalable process to produce large quantities of chimeric influenza Gag-VLPs from stable human embryonic kidney HEK-293 cells in suspension culture.

First, a HEK-293 cell line stably expressing HA and NA proteins of influenza (subtype H1N1) under the regulation of the inducible cumate system was established. Then, the formation of VLPs was mediated by transient transfection of plasmids encoding human immunodeficiency virus (HIV) Gag or M1 influenza matrix protein. Gag protein was fused to the green fluorescent protein (GFP) to facilitate the monitoring of VLPs production. VLP antigenic proteins were produced seven times more efficiently in the presence of Gag, indicating that Gag is a better scaffolding protein than M1 in this context. Subsequently, the production of HA-NA containing VLPs after transient transfection of Gag as scaffold protein was successfully implemented in a 3-L controlled stirred tank bioreactor. VLPs were recovered by ultracentrifugation on a sucrose

cushion followed by concentration through tangential flow filtration (TFF) using a 1000 KDa cut-off membrane. Different techniques were employed to characterize the produced VLPs: Single radial immunodiffusion (SRID), hemagglutination assay, dot-blot, western-blot, and transmission electron microscopy (TEM). Of great significance, intranasal immunization of VLPs induced specific immunogenic response and provided complete protection in mice challenged with the homologous influenza strain.

Once the proof of concept of VLPs as an efficacious influenza vaccine was demonstrated in *vivo*, we developed a new inducible cell line expressing the three proteins HA, NA and the Gag fused to GFP. This was performed in an effort to streamline the production process by eliminating the transient transfection step that can be cumbersome at large scale. The process was optimized to reach a high volumetric yield of VLPs by increasing the cell density at the time of induction without sacrificing the cell specific productivity. By operating a 3L-bioreactor in perfusion mode followed by TFF, the yields of VLPs were improved by 60-fold ($3x10^{11}$ Gag-GFP fluorescent events/L of culture measured by flow cytometry) compared to a standard batch culture ($5x10^{9}$ Gag-GFP fluorescent events/L). The process was characterized for the upstream kinetics of production of VLP proteins and recovery rates for each downstream step. The production of a single bioreactor, operated in perfusion mode, with the stable cell line 293-HA/NA/Gag-GFP yielded 5-fold more total VLP antigenic HA proteins than what was produced with the 3L-batch bioreactor using transient transfection. Our process provided unprecedented yields of influenza VLPs produced from mammalian cells.

Finally, because VLPs are enveloped particles that bud from a host cell potentially enclosing host cell proteins, DNA and RNA, which could pose a safety concern, we performed a proteomic characterization of the influenza Gag-VLPs produced by transient transfection and also extracellular vesicles (EVs) produced from wild-type HEK-293 cells. The functions of all proteins identified within VLPs and EVs were critically discussed.

The process developed in this thesis could support the production of VLPs harboring HA and NA of different strains for clinical trials and could potentially result in a better vaccine candidate with higher efficacy and safety to replace the current labor-intensive egg-produced influenza vaccines.

TABLE OF CONTENTS

DEDICATION III

ACKNOWLEDGEMENTS IV

RÉSUMÉ VI

ABSTRACT IX

TABLE OF CONTENTS XI

LIST OF TABLES XVI

LIST OF FIGURES XVII

LIST OF SYMBOLS AND ABBREVIATIONS XVIII

CHAPTER 1 INTRODUCTION 1

1.1 Research problem 1

1.2 Hypothesis 2

1.3 Research objectives 2

1.4 Thesis organization 3

CHAPTER 2 LITERATURE REVIEW 4

2.1 Influenza 4

2.1.1 Classification and virus structure 4

2.1.2 Infective replication cycle 9

2.1.3 Influenza pathogenesis 12

2.2 Influenza vaccines 12

2.2.1 Immune response to current influenza vaccines 14

2.2.2 Limitations of current vaccine manufacturing process 15

2.2.3 Recombinant influenza vaccines 16

2.2.4 Influenza virus-like particles as vaccines 17

2.2.5 Gag as a core protein for VLPs 20

2.3 Mammalian cells as expression system 21

2.3.1 Mammalian cells expression regulation systems 23

2.3.2 Cell line development 25

2.4 Challenges associated to the large-scale manufacturing of VLPs for vaccine 27

2.4.1 VLPs design 27

2.4.2 Upstream process 29

2.4.3 Downstream process 30

CHAPTER 3 ARTICLE 1: HEMAGGLUTININ AND NEURAMINIDASE CONTAINING VIRUS-LIKE PARTICLES PRODUCED IN HEK-293 SUSPENSION CULTURE: AN EFFECTIVE INFLUENZA VACCINE CANDIDATE 33

3.1 Abstract 33

3.2 Introduction 34

3.3 Materials and Methods 36

3.3.1 Cells, plasmids and antibodies 36

3.3.2 Generation of 293CymR-rcTA cell line 37

3.3.3 Generation of 293HA-NA stable cells 37

3.3.4 Immunofluorescence assay 37

3.3.5 3L-Bioreactor 38

3.3.6 Ultracentrifugation and concentration of VLPs by sucrose cushion 25% 38

3.3.7 Tangential Flow Filtration 38

3.3.8 Cell lysate using RIPA buffer 38

3.3.9 Dot blot 39

3.3.10 Single Radial Immunodiffusion 39

3.3.11 Hemagglutination assay 39

3.3.12 Determination of host cell proteins and host cell DNA 39

3.3.13 Fluorescence intensity measurement and p24 quantification................................39

3.3.14 Transmission electron microscopy (TEM)..40

3.3.15 Immunization and viral challenge 40

3.3.16 Enzyme-linked immunosorbent assay (ELISA) for HA-specific antibodies detection……… 40

3.4 Results and Discussion...41

3.4.1 Development of 293HA-NA stable cells 41

3.4.2 Comparison of influenza matrix M1 and Gag proteins effect on influenza VLPs production...43

3.4.3 Influenza Gag-VLPs production in shake flasks and 3L-Bioreactor 44

3.4.4 Tangential Flow Filtration (TFF) of influenza Gag-VLPs...46

3.4.5 Quantification, characterization and yield of influenza Gag-VLPs 49

3.4.6 Immunization and mice challenge protection study..52

3.5 Acknowledgements 54

3.6 References 54

CHAPTER 4 ARTICLE 2: PROCESS INTENSIFICATION FOR HIGH YIELD EXPRESSION OF INFLUENZA H1N1 GAG VIRUS-LIKE PARTICLES USING AN INDUCIBLE HEK-293 PRODUCING CELL LINE 59

4.1 Abstract 59

4.2 Introduction 60

4.3 Materials and Methods 62

4.3.1 Cells, plasmids and culture conditions 62

4.3.2 Generation of 293-HA/NA/Gag-GFP stable cell line 62

4.3.3 Flow cytometry 63

4.3.3.1 Cells....63

4.3.3.2 Gag-GFP events count by flow cytometry 64

4.3.4 Perfusion Bioreactor....64

4.3.5 VLP proteins quantification and characterization....64

4.3.6 Clarification and Tangential flow filtration (TFF) 65

4.4 Results and Discussion....65

4.4.1 Stable cell line development 65

4.4.2 Media evaluation at small scale 66

4.4.3 Medium replacement at small scale 68

4.4.4 Perfusion Bioreactor....71

4.4.5 Tangential Flow Filtration (TFF) 73

4.5 Declaration of interest 76

4.6 Acknowledgements 76

4.7 References 76

CHAPTER 5 PROTEOMIC CHARACTERIZATION OF INFLUENZA H1N1 GAG VIRUS-LIKE PARTICLES AND EXTRACELLULAR VESICLES PRODUCED IN HEK-293SF…………… 79

5.1 Introduction 80

5.2 Methodology 81

5.2.1 nLC-MS/MS of Tryptic Digests....81

5.2.2 Production of VLPs and EVs 82

5.3 Results and Discussion....83

5.3.1 Proteins identified in VLPs 83

5.3.2 Extracellular vesicles unique proteins 96

5.4 Conclusions 99

CHAPTER 6 GENERAL DISCUSSION 101

CHAPTER 7 CONCLUSION AND RECOMENDATIONS 108

7.1 Conclusions 108

7.2 Recommendations for future studies 110

BIBLIOGRAPHY 112

LIST OF TABLES

Table 2-1 Influenza A virus strain A/PR/8/34 genome and proteins 7

Table 2-2 Virus-like particles in development phase and/or licensed for use as human vaccine. .19

Table 3-1 Summary of HA, NA and Gag quantification 45

Table 3-2 Recovery of the purification by TFF based on anti-HA Dot-Blot results 49

Table 4-1 Specific productivity, specific growth rate and productivity improvement factor from batch to perfusion 71

Table 4-2 Recovery of VLPs during the downstream process of the perfusion bioreactor harvest in terms of Gag-GFP events/ml and HA concentration...75

Table 5-1 Proteins identified by n-LC-MS/MS in VLPs sample 92

Table 5-2 Proteins uniquely identified in extracellular vesicles produced in HEK-293 cells 97

Table 6-1 Summary of the production performances of the two processes employed to produce VLPs..106

LIST OF FIGURES

Figure 2-1 Schematic representation of influenza virions.. 6

Figure 2-2 General steps in the infection cycle of influenza virus...11

Figure 2-3. Structure of Gag protein of HIV-1. 21

Figure 2-4 General method followed to create a stable cell line for large-scale production of recombinant protein...26

Figure 3-1 Development of the 293HA-NA stable cell clone and analysis of VLPs production efficiency.. 42

Figure 3-2 Gag-VLPs production in shake flasks and 3 L-bioreactor. 46

Figure 3-3 Tangential flow filtration (TFF) of influenza Gag-VLPs. 48

Figure 3-4 Quantification of HA and NA on influenza Gag-VLPs before and after TFF.. 51

Figure 3-5 Immunity of influenza Gag-VLPs and mice challenge.. 53

Figure 4-1 Development of stable cell line 293-HA/NA/Gag-GFP.. 66

Figure 4-2 Media evaluation at small scale.. 68

Figure 4-3 Production of VLPs in cultures with medium replacement (MR) at small scale. 70

Figure 4-4 Production of VLPs in a perfusion mode 3L-bioreactor.. 72

Figure 4-5 Tangential Flow Filtration (TFF) of VLPs produced in the 3-L perfusion bioreactor..75

Figure 5-1 Differential and common proteins identified by nano LC-MS/MS in Gag-VLPs and extracellular vesicles (EVs)...85

LIST OF SYMBOLS AND ABBREVIATIONS

ALIX	apoptosis-linked gene 2-interacting protein X
ATP	adenosine triphosphate
CCLs	continuous cell lines
CMV	cytomegalovirus
CR5	cumate responsive promoter
DNA	deoxyribonucleic acid
ESCRT	endosomal sorting complexes required for transport
EVs	extracellular vesicles
FDA	Food and Drug Administration
Gag	core structural protein of retrovirus
GFP	green fluorescent protein
GOI	gen of interest
HA	hemagglutinin
HCPs	host cell proteins
HEK-293SF	human embryonic kidney cells adapted to grow in serum-free medium
HIV-1	human immunodeficiency virus type 1
hpt	hours post-transfection
HSP	heat shock protein
IgG	immunoglobulin
ILVs	intraluminal vesicles
LAIVs	live attenuated influenza vaccine
LMH	flux in $L/m^2/hour$
M1	matrix protein of influenza virus

MDCK	Madin-Darby canine kidney cells
MHC	major histocompatibility complex
MR	medium replacement
MVBs	multi-vesicular bodies
NA	neuraminidase
NADPH	nicotinamide adenine dinucleotide phosphate
nLC-MS/MS	nanoscale liquid chromatography tandem mass spectrometry
p-Cym	operon of *Pseudomonas putida*
PEI	polyethyleneimine
pfu	plaque-forming units
PR	Puerto Rico
RBC	red blood cells
rcTA	reverse transactivator
RNA	Ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SRID	single radial immunodiffusion
TCA	Tricarboxylic acid
TEM	transmission electron microscopy
TFF	tangential flow filtration
TIVs	trivalent influenza vaccine
Vero	African green monkey kidney cells
VLPs	virus-like particles
vRNPs	virus ribonucleoprotein
WHO	World Health Organization

293CymR-rcTA HEK-293SF containing the cumate switch system

293HA-NA HEK-293SF stably expressing HA and NA (H1N1 A/PR/8/34)

293HA/NA/Gag-GFP HEK-293SF stably expressing HA, NA (H1N1 A/PR/8/34) and Gag-GFP

CHAPTER 1 INTRODUCTION

1.1 Research problem

Influenza is still a major threat to human health causing high morbidity and mortality in humans Influenza outbreaks are among the most ancient and lethal infections in human history. The reason for the persistence of this disease is because the causative agent is a virus with high and fast mutation rates. The mutations occur in the dominant antigenic proteins exposed in the viral envelope, hemagglutinin and neuraminidase. Thus far, there exist 18 types of HA and 11 NA that conform different strain subtypes. The new mutant strains cannot be recognized by the host immune system and therefore can cause severe infections. Vaccination remains the most effective way to combat the disease. Influenza vaccines have been produced in eggs for more than 70 years, but this system carries major drawbacks such as limited production capacity in case of high demand seasons or pandemics, egg allergies in some patients and low adaptability of some strains to eggs, which can result in mismatch with the circulating strains. Furthermore, the seasonal vaccines are highly efficacious in healthy adults but poorly immunogenic in high-risk groups (elderly, young children and pregnant women). With the great progress made in the development of cell culture process to produce biopharmaceuticals, many researches are now focused on switching the manufacturing of influenza vaccines to cell based production. Mammalian cells offer several advantages for the production of complex biomolecules such as superior post-translational modifications and easy adaptability to grow in serum-free suspension cultures. Among mammalian cells, human cell lines provide a glycosylation pattern homologous to endogenous human proteins reducing the risk of reactogenicity of biopharmaceuticals for human use. On the other hand, there is a need for new generation of influenza vaccines that mitigate the limitations of current seasonal vaccines and induce cross-protective immune response. Virus-like particles constitute an attractive platform to present the dominant antigens HA and NA in their native conformational structure on a non-infectious, non-replicative particulate structure similar to wild-type virus. The repetitive high-density arrays of antigenic proteins elicit a strong humoral response while the particulate structure induces the uptake by professional antigen-presenting cells stimulating cellular immune response. The immunogenicity and efficacy of VLPs as vaccine candidates have been extensively proven by *in vivo* experiments. However, there are still

numerous challenges for the large-scale manufacturing of these nanoparticles and their approval as vaccines. There is lack of robust and scalable processes to produce sufficient amount of VLPs from mammalian cells. This fact has limited the development of efficient methods to purify and characterize influenza VLPs for use in clinical trials. As enveloped particles, VLPs bud from the host cell membrane taking up not only the viral antigenic proteins but also host cell proteins, DNA and RNA. In mammalian cells, VLPs are co-produced with extracellular vesicles (EVs) also containing host cell contaminants and having a similar size to the VLPs, which makes difficult the development of an efficient purification process.

1.2 Hypothesis

To address the issues mentioned above, in this thesis we have made the following hypothesis.

The design and development of a VLP producer HEK-293SF stable cell line adapted to suspension growth will allow to develop and characterize a scalable process to produce, and further characterize high yields of influenza VLPs that are immunogenic and protective.

1.3 Research objectives

To demonstrate the veracity of the hypothesis, the following objectives were defined:

- To develop a HEK-293SF inducible stable cell line expressing the main influenza antigenic proteins HA and NA and to compare the VLPs production yields after transient co-expression of M1 influenza matrix protein and Gag structural core protein of HIV-1.
- To produce VLPs in a laboratory scale bioreactor and partially purify them to assess their immunogenicity and efficacy by *in vivo* experiments.
- To develop and apply process intensification strategies to achieve high yields of VLPs aiming at large scale manufacturing.
- To characterize the protein composition of VLPs and extracellular vesicles by LC-MS/MS.

1.4 Thesis organization

Following this introduction, Chapter 2 provides a detailed literature review on the state-of-the-art of influenza vaccines, mammalian cells platform and virus-like particles; Chapter 3 presents the production process of VLPs in a 3L-bioreactor after transient transfection of a gag-containing plasmid and the results of the immunization with VLPs in an *in vivo* experiment. The methodology and results of this chapter have been published in the ***Vaccine*** journal and presented as a poster at the *Cell Culture Engineering XV, Vaccine Technology VI and Virus-like particle & nanoparticle vaccines* conferences.

- Venereo-Sanchez A, Gilbert R, Simoneau M, Caron A, Chahal P, Chen W, et al. (2016). "Hemagglutinin and neuraminidase containing virus-like particles produced in HEK-293 suspension culture: An effective influenza vaccine candidate". *Vaccine*, 34:3371-80.

Chapter 4 presents the process intensification strategies performed to increase the yields of VLPs produced from the stable cell line 293-HA/NA/Gag-GFP including a 3L perfusion bioreactor and the concentration of 9.5L harvest by tangential flow filtration. These results are presented in a manuscript entitled ***"Process Intensification for High Yield Expression of Influenza H1N1 Gag Virus-Like Particles using an Inducible HEK-293 Producing Cell Line"*** which has been submitted to the ***Vaccine*** journal.

The proteomic composition of VLPs and EVs as well as a critical discussion of the function of each host cell protein identified in both nanoparticles species are presented in Chapter 5. This chapter is entitled "***Proteomic characterization of influenza H1N1 Gag virus-like particles and extracellular vesicles produced in HEK-293SF".***

Chapter 6 provides a general discussion of all the results obtained in each chapter; and, finally, Chapter 7 presents the main conclusions and recommendations.

CHAPTER 2 LITERATURE REVIEW

2.1 Influenza

The name *Influenza* arose in the fifteenth-century in Italy, from an epidemic attributed to the "*influence* of the stars" (Barberis, Myles *et al.*, 2016). In the years 1918-1920, influenza A virus caused one of the worst worldwide human pandemics in history. The so-called "Spanish flu" (H1N1) led to the death of at least 20 million people (Taubenberger, Reid *et al.*, 1997). The "Asian flu" (H2N2) in 1957 (70,000 deaths in North America) (Dunn, 1958; Neumann, Noda *et al.*, 2009; Trotter, Dunn *et al.*, 1959) and the "Hong Kong flu" (H3N2) in 1967 (Cockburn, Delon *et al.*, 1969) caused the second and third most important influenza A pandemics. Recently, the "swine flu" pandemic of 2009–2010 provoked over 16,000 deaths worldwide (Garten, Davis *et al.*, 2009). Most people call "flu" the "common cold" which is usually caused by some upper respiratory viruses such as rhinoviruses and coronaviruses, not by influenza viruses. Influenza is a severe acute disease that can occur in all age groups with symptoms such as sore throat, cough, high fever, headache, and muscular pains (Dermody & Chappell, 2011; Osterholm, Kelley *et al.*, 2012). The virus spreads quite rapidly through the air by droplets released from infected person sneezes or cough, and also by mucosal contact (eyes, mouth) with contaminated hands. The World Health Organization (WHO) reports approximately 3-5 million cases of severe illness worldwide and up to 500,000 deaths annually (http://www.who.int/mediacentre/factsheets/fs211/en/). Vaccination remains the best method to control and prevent seasonal and pandemic influenza.

2.1.1 Classification and virus structure

Influenza viruses have a genome of approximately 13.5 kb and belong to the *Orthomyxoviridae* family. The viruses are classified into three types: A, B and C (Milián & Kamen, 2015; Noda, 2011; Subbarao & Matsuoka, 2013; Zambon, 2014). Recently, a novel genus termed influenza virus type D has been isolated from cattle in the United States (Collin, Sheng *et al.*, 2015) and in Europe (Ducatez, Pelletier *et al.*, 2015). The main differences between influenza types A, B and C lie in the number of RNA segments, 8 in the case of type A and B and 7 segments for influenza C; unlike influenza A and B, influenza C only contains one envelope protein, the HEF

(hemagglutinin-esterase fusion) that binds to 9-O-acetylneuraminic acid receptors instead of the N-acetylneuraminic acid receptors recognized by influenza A and B (Gao, Brydon *et al.*, 2008; Muraki & Hongo, 2010). Influenza type A viruses are the best-studied orthomyxoviruses causing the most serious infections in humans, and are also widespread in many avian and mammalian species (Steel, 2011). Influenza type B is limited to humans, although occasional infections to other mammalian species such as seals have been documented (Bodewes, Morick *et al.*, 2013; Chen & Holmes, 2008). Two lineages of influenza B have been identified referred to as B/Yamagata and B/Victoria (Shaw, Xu *et al.*, 2002). The influenza type C causes mild infections in both pigs and humans, thereby having less importance for public health (Zambon, 2014).

Influenza A viruses are categorized into different subtypes based on the hemagglutinin (HA) or neuraminidase (NA). Recently, novel influenza viruses have been isolated from bats in Central America giving rise to a total of 18 different types of HA (H1-H18) (Tong, Li *et al.*, 2012; Tong, Zhu *et al.*, 2013) and 11 NA (N1-N11) (Jagadesh, Salam *et al.*, 2016) identified to date. Combination of these proteins gives rise to several influenza virus subtypes with different antigenicity. The virus classification refers to the surface antigens exposed (e.g. H3N2, H5N1, H1N1). The nomenclature used to describe each isolated influenza viral strain is descriptive and includes: the type of virus (A, B, C), the original host (this parameter is not indicated in human isolations), the geographic site of origin, the registered strain number and the year of isolation. For influenza A viruses, the corresponding identified HA and NA subtype must appear (e.g. A/H1N1/PuertoRico/8/1934, A/Chicken/Pennsylvania/1370/83 (H5N2) (Horimoto & Kawaoka, 2001).

The influenza virions are enveloped with a negative-sense, single-stranded, segmented RNA genome. Influenza viruses type A contain eight RNA genome segments encoding at least 12 viral proteins. Each one of the first 6 segments mostly gives rise to one protein: PB2, PB1, PA, HA, NP, NA. The PB2 (basic polymerase 2), (PB1 basic polymerase 1), and PA (acidic polymerase) proteins form the RNA polymerase complex (Kawaoka & Neumann, 2012). Hemagglutinin (HA) is a type I transmembrane protein and is the most represented envelope glycoprotein. The nucleocapsid protein (NP) tightly wraps the genomic viral RNA. Neuraminidase (NA), type II transmembrane protein, is the second most represented envelope glycoprotein. There are some exceptions where some of these first 6 segments code for more than one protein. In some influenza A strains, the RNA segment that codes PB1 has a second open reading frame for the

protein PB1-F2 which have been involved in the degree of virulence of the virions (Conenello, Zamarin *et al.*, 2007). Meanwhile, the 6th segment of influenza B virus also encodes a protein called NB that is not essential for viral replication in vitro (Hatta & Kawaoka, 2003) and structurally resembles the M2 protein of influenza A (Bouvier & Palese, 2008). The segments 7 and 8 are spliced into two messenger RNA (mRNA) encoding two different viral proteins; segment 7 codes for the matrix protein (M1) and the third enveloped protein (M2), whereas segment 8 codes for two nonstructural proteins (NS1 and NS2). The highly conserved 5' and 3' ends of the viral RNA segments form a helical hairpin at which is bound the trimeric PB2, PB1, and PA RNA polymerase complex. Arginine-rich nucleoproteins with positive net charge coat the rest of the negative-charged RNA phosphate backbone, approximately one NP per 24 nucleotides. These structures called the viral ribonucleoprotein (vRNP) are exported from the host cell nucleus and packaged at the plasma membrane to form the virion (Noda, Sugita *et al.*, 2012) (Figure 2-1). The shape of the virions can be either roughly spherical or filamentous with an approximated size of 80-120 nm in diameter, and a length of up to 20 µm in the case of filamentous particles. The morphology of the virions appears to be related to the source of isolation. Mostly filamentous structure virions are observed from clinical isolations and more spherical/rounded viruses are found during *in vitro* productions (eggs passage or cell culture) (Noda, 2011). The Table 2-1 summarizes the main functions of the viral proteins.

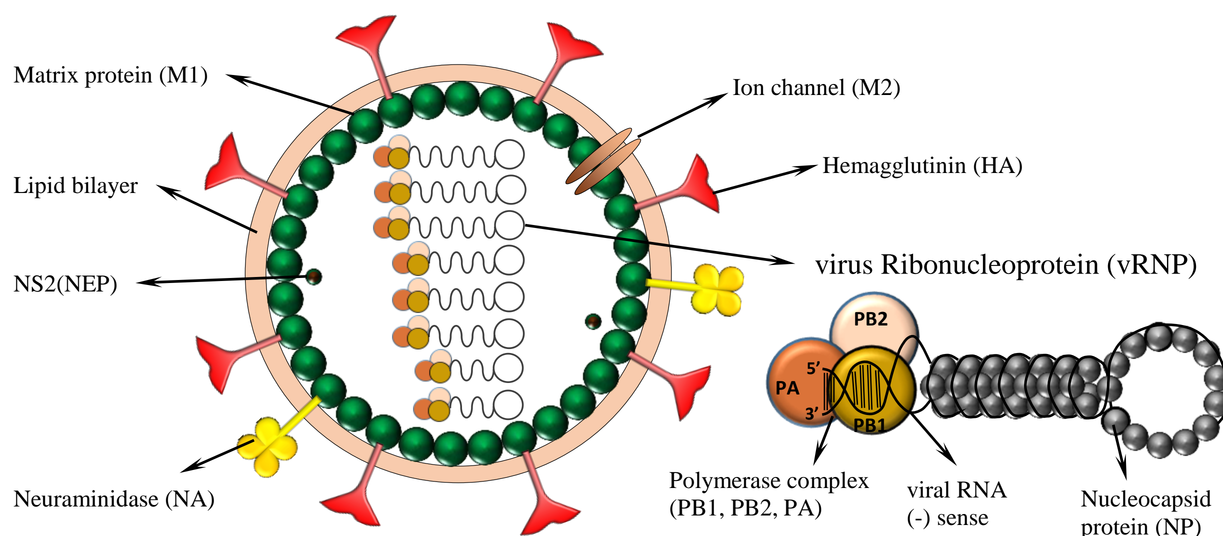

Figure 2-1 Schematic representation of influenza virions. Hemagglutinin, Neuraminidase and M2 ion channel are transmembrane proteins exposed in the viral envelope. The M1 matrix protein is found underneath the viral membrane. Inside of the enclosed lipid bilayer are the eight RNA

individual segments associated to the nucleocapsid protein and the RNA polymerase complex (PB1, PB2 and PA) forming the 8 helical vRNPs (adapted from (Dermody & Chappell, 2011)).

Table 2-1 Influenza A virus strain A/PR/8/34 genome and proteins

RNA segment	Length (nucleotide)	Encoded Proteins	Protein size (aa)	Protein function
1 2 3	2341 2341 2233	PB2 PB1 PA	759 757 716	These three proteins form the heterotrimeric RNA-dependent RNA polymerase complex. The PA endonuclease generates the primers; the PB2 binds the capped RNA and PB1 subunit performs the RNA synthesis (Stevaert & Naesens, 2016).
4	1778	HA	566	The hemagglutinin is a homotrimeric transmembrane glycoprotein and the main antigenic determinant; it is crucial for the binding to sialic acid receptors at cell virus entry and membrane fusion at low pH in the endosome.
5	1565	NP	498	The nucleoprotein encapsidates the viral RNA, control functions during RNA synthesis and is also involved in the virion morphology, probably through interaction with M1 (Bialas, Bussey *et al.*, 2014).

6	1413	NA	454	The neuraminidase is a tetrameric surface glycoprotein and the second antigenic determinant. It cleaves terminal N-acetyl neuraminic acid (sialic acid) mediating the release of the new virions progeny. It also facilitates hemagglutinin-mediated fusion and viral spread in the respiratory epithelium (Jagadesh *et al.*, 2016). Mostly, the HA:NA ratio in wild type virus is approximately 1:4.
7	1027	M1	252	The matrix protein (M1) interacts with the cytoplasmic tails of HA, NA, M2 (Chen, Leser *et al.*, 2007) and also with the nucleocapsids in the vRNPs, and NS2 (Noda, 2011). M1 is involved in the virion assembly, budding and morphology as well as the nuclear export of the vRNPs.
		M2	97	Integral membrane protein forming an ion channel crucial in the un-coating of virions inside the endosome and scission membrane for viral budding. The HA:M2 ratio is around 10 to 100 M2 per HA molecule (Bouvier & Palese, 2008).
8	890	NS1	230	The nonstructural protein 1 is an interferon-antagonist that down-regulates host cell mRNA processing; reduces apoptosis of infected cells.
		NS2(NEP)	121	The nonstructural protein 2 mediates the export of viral nucleocapsids from the nucleus; interacts with M1.

2.1.2 Infective replication cycle

Influenza infection begins with the binding of hemagglutinin glycoprotein to the N-acetylneuraminic (sialic) acid-containing receptors exposed on the host cell membrane. The term hemagglutinin arise from the protein's ability to agglutinate red blood cells. HA is synthesized as a homotrimeric molecule (HA0) that is sensitive to cleavage from cellular serine-proteases. After cleavage, polypeptide HA0 is excised in two subunits: the immunodominant globular head HA1 subunit that is responsible for the binding to sialic acid and the stalk domain HA2 that contains the fusion peptide involved in membrane fusion inside the endosome (Carr & Kim, 1993; Lorieau, Louis *et al.*, 2013). The virus enters the cell via clathrin-mediated endocytosis. The low endosomal pH causes an important conformational change in the HA protein which is comparable to the opening of a coiled spring (Baker & Agard, 1994; Dermody & Chappell, 2011; Lorieau *et al.*, 2013). The fusion peptide hidden within a hydrophobic region of HA moves out toward the membrane of the endosome causing the fusion of the virus envelope with the endosomal membrane (Ivanovic, Choi *et al.*, 2013). After membrane fusion, the next step includes the release of the helical nucleocapsids from the virion to the cytoplasm mediated by the M2 ion channel present in the virus envelope. The hydronium protons enter into the virion through the M2 channel protein and this acidic environment in the inner virion causes weakening of the interaction between M1 and the helical nucleocapsids, provoking their release across the previously fused membrane to the host cell cytoplasm (Bouvier & Palese, 2008).

Unlike most other RNA-containing viruses, influenza virus replication occurs in the nucleus (Pohl, Lanz *et al.*, 2016). The RNA segments of infecting virions liberated to the cytoplasmic lumen are transported into the nucleus via the classical importin-α– importin-β1 (IMPα–IMPβ1)-dependent nuclear import pathway (Eisfeld, Neumann *et al.*, 2015). This is facilitated by nuclear localization signals present in the vRNPs. Once the viral genome segments get into the nucleus the negative-sense viral RNAs are transcribed to messenger RNAs. The subunit PB2 of the vRNP complex binds to cellular capped pre-messenger RNA while PA subunit cleaves them to generate primers for RNA synthesis. The PB1 polymerase transcribes the RNA terminating at 5' poly U sequence found at the end of the RNA. These mature mRNAs are exported to the cytoplasm for translation in cellular ribosomes (Fodor, 2012). The polymerase also replicates the vRNA to a complementary RNA (cRNA) which serves as template for *de novo* vRNAs synthesis. Because of

their nuclear localization signals, NP and the trimetric polymerase are redirected again to the nucleus for the assembly of the ribonucleocapsids. Hence, the new progeny of vRNAs are assembled with NP and the RNA polymerase complex proteins newly synthesized inside the nucleus and then transported to the cytoplasm. This process involves the interaction with M1 and NEP. The transportation of mature vRNPs to the budding site is mediated by Rab11 vesicles (Eisfeld *et al.*, 2015).

The proteins of the viral envelope (hemagglutinin, neuraminidase and M2) take the secretory pathway through the endoplasmic reticulum (ER) and Golgi apparatus to the cell surface facilitated by the protein-trafficking machinery. HA and NA head towards cellular membrane zones enriched in cholesterol, named lipid rafts, whereas M2 is not associated to these domains (Rossman & Lamb, 2011). M1 is one of the most abundant proteins in the virion and it is located throughout the inner side of the viral envelope. M1 interacts directly with the cytoplasmic tails of HA, NA and M2 and also binds to virus nucleocapsids giving it an important structural role in maintaining the morphology of the virus (Chen *et al.*, 2007; Thompson, Petiot *et al.*, 2015). The exact mechanism for viral budding initiation remains unknown. Studies on the formation of influenza virus-like particles revealed that the accumulation of HA at the lipid-rafts patches is sufficient to mediate membrane curvature and release of HA-containing vesicles (Chen *et al.*, 2007; Rossman & Lamb, 2011). Viral budding occurs on the apical membrane previously filled up with HA, NA, and M1 associated to the vRNP segments and M2 could mediate the membrane scission and viral release. The review of Rossman, and Lamb (2011) proposed that, considering the inconsistencies encountered in the viral budding studies done with VLPs, there is not one single protein totally responsible of the influenza viral budding but rather it is a reduntant cooperation among them. Figure 2-2 shows the major steps in the infection cycle of influenza virus.

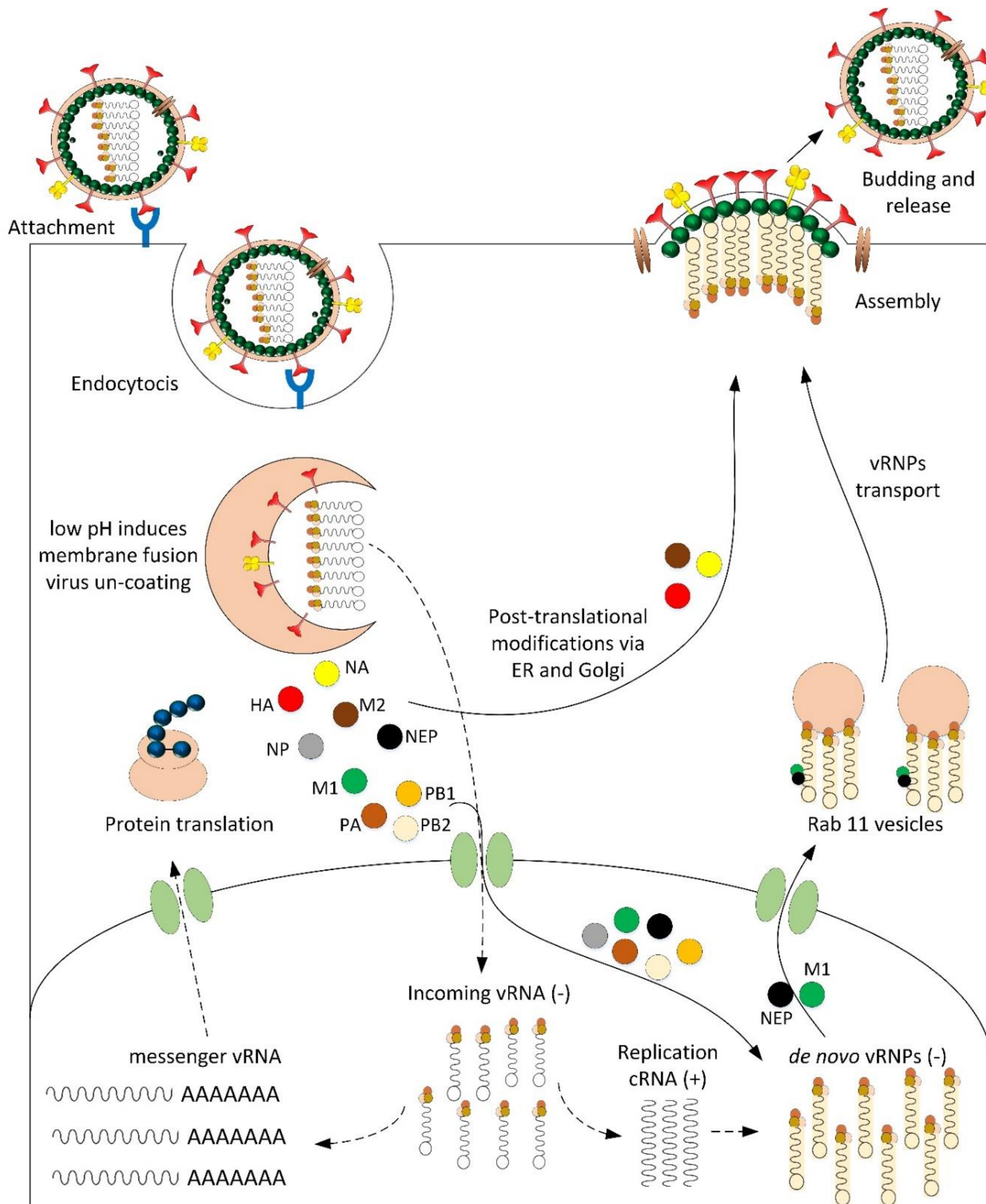

Figure 2-2 General steps in the infection cycle of influenza virus. First, the binding of virions to the cellular membrane and internalization in endosomes take place. Subsequently, there is release of the RNA genomes through the lipid bilayer after virus and endosome membrane fusion. Nucleocapsids enter into the nucleus for transcription and replication of the RNA segments. Finally, translation of the viral proteins, virus ribonucleoprotein assembly in the nucleus and viral budding at the host plasma membrane take place.

2.1.3 Influenza pathogenesis

The pathogenicity of influenza is determined by both viral and host factors (Behrens, Stoll *et al.*, 2006). The amount of host cell receptors, the abundancy of enzymes responsible of virus entry and replication (e.g. availability of protease mediating the cleavage of HA0), and the immunocompetency of the individual are among the factors related to the host individual. Viral factors are determined by the affinity and ability of the virus strain subtype to bind to the host cell receptors, the susceptibility to antigenic mutation and reassortment with other co-infecting viral strains, and the capability to attenuate the host immune response, etc.

In humans, the influenza virus infects and replicates in epithelial cells of both upper and lower respiratory tract (Taubenberger & Morens, 2008). Infected persons usually show asymptomatic to mild illness. The symptoms persist for 7-10 days and include fever, inflammation of the respiratory tract, cough, prostration, headache, etc. An experiment in healthy volunteers showed that a peak of influenza virus replication occurred 48 hours post-inoculation in the nasopharynx and little to no virus shedding was observed after 6 days (Barnard, 2009; Carrat, Vergu *et al.*, 2008). However, these results cannot be generalized since children or older persons were not included in the experiment, in whom the illness evolves differently. Since influenza virus infects epithelial cells, complications of the respiratory tract including tracheitis, bronquitis, bronchiolitis, otitis media, and sinusitis are commonly developed. Pneumonia and acute respiratory distress syndrome (ARDS) are the most severe complications of influenza infection (Li & Cao, 2016). The major reason of the high mortality during influenza pandemics is due to secondary bacterial infections mainly caused by *Streptococcus pneumonia* (Stegemann, Dahlberg *et al.*, 2009). A strong overactivated immune response due to an uncontrolled production of cytokines have been reported in severe influenza infections causing lung damage (Li & Cao, 2016).

2.2 Influenza vaccines

The source of variation in influenza viruses arises from two phenomena: 1) the accumulation of point mutations in the coding RNA segments, which is known as antigenic drift and 2) the reassortment between RNA segments of two or more different virus subtypes co-infecting the same host cell, which is termed antigenic shift. The major variations introduced due to the

antigenic drift occur in the main antigenic determinants, the globular domain of HA (HA1) and NA. Consequently, the new mutant virions progeny cannot be recognized by the host immune system causing severe infections. The antigenic shift occurs occasionally but can be extremely dangerous. By genome segments interchange, strains originally infecting one species may pass to another one appearing new to the immune system making the infection potentially highly fatal (Dermody & Chappell, 2011).

These viral strategies used to escape the host immune system have made it very difficult for researchers to find an efficient vaccine to totally eradicate the disease. The first human influenza virus was isolated after propagation in ferrets in 1933 (Hannoun, 2013). The discovery of the causative agent of influenza triggered several investigations to develop an effective vaccine. The progress in the development of methods allowing large-scale cultivation in embryonated chicken eggs and methods for virus quantification by hemagglutination assay, made possible the production of the first inactivated vaccines in 1942, which showed protective efficacy against influenza A and B after evaluation in trials among US military recruits (Francis, 1953). Currently, after more than 70 years, the basic technology and principles of influenza vaccine production remain much the same (Sambhara & Stephenson, 2009).

Thus far, influenza vaccines have to be annually updated to be effective against new emerging strains. Every year, through a surveillance system, the World Health Organization (WHO) identifies newly evolved antigenic strains most likely to predominate in the following influenza season. Twice a year, a committee assembled by WHO provides guidance to vaccine manufacturers on the viruses that will be included in the trivalent or quadrivalent influenza vaccines for the next seasons in the northern and southern hemispheres (Barr, 2014). Currently, vaccine companies have 6 months to produce their vaccines and provide them to public health systems. Two types of trivalent vaccines are approved for use: trivalent inactivated vaccine (TIV) and live attenuated virus vaccine (LAIV). These vaccines are composed of the 3 currently circulating seasonal influenza virus strains: two subtypes of influenza A virus (H3N2 and H1N1) and one strain of influenza B. The vaccine update is carried out by genetic reassortment between the field strains chosen by WHO surveillance and the backbone of A/PR8/34 master strain that has been shown to grow to high titers in embryonated eggs (HA and NA genes are exchanged). For influenza B, the field isolates are the seed strains for vaccine production because there is no evidence thus far of any master influenza B with high growth in the egg-based production

system. Once the seed strains are confirmed to be the reference strains by identity testing and sequence analysis, vaccine production begins. The TIVs can be prepared as whole inactivated virus- and split virus or subunit vaccines. Viruses are grown in the allantoic cavity of embryonated eggs, harvested, chemically inactivated by formalin or β-propriolactone and purified by ultra-centrifugation (whole inactivated virus vaccine), then, viruses can be split using a nonionic detergent and the HA and NA are purified (split virus or subunit vaccines) (Gerdil, 2003; Milián & Kamen, 2015). The LAIVs were produced in the 1960s by serial passage of the virus in eggs under suboptimal conditions at low temperature, selecting for variants that could grow in the cool nasopharynx but could not grow in the lung. Master donor strains were established for vaccine production (Shaw, 2012; Wong & Webby, 2013). Recently, quadrivalent inactivated vaccines have been approved by the Food and Drug Administration (FDA) including another influenza B-lineage leading to superior immunogenicity against the added B strain (Greenberg, Robertson *et al.*, 2013; Tinoco, Pavia-Ruz *et al.*, 2014).

2.2.1 Immune response to current influenza vaccines

The immunogenic efficacy of the licensed seasonal influenza vaccines is associated with the patient age, displaying less protection in young children and elderly. Current conventional TIVs stimulate strong serum antibody responses against the major envelope glycoproteins HA and NA but elicit poorly mucosal IgA influenza-specific antibody and cellular immunity (Cox, Brokstad *et al.*, 2004). Due to the antigenic drift that takes place in influenza A viruses, the main surface proteins mutate almost every year leading to new strains that cannot be neutralized by the host immune system. Thus, the immunity induced by seasonal TIVs vaccines is subtype-specific providing short-time protection.

The primary immune response to LAIV vaccines consists in serum IgA and IgM that peak two weeks post-vaccination while the IgG serum can last for at least 1 year post-vaccination. The nasal fluid IgA elicited is significantly higher than those elicited with TIVs. The LAIVs induce secretion of interferon-γ, proliferation of T lymphocytes including cytotoxic T-cells. Both vaccines have shown high rate of protection in human, but LAIVs can be more immunogenic in young children since a prior natural infection is needed for eliciting a potent humoral response against TIVs (Cox *et al.*, 2004). Both vaccines offer low protection in the elderly.

Considering that cytotoxic T-cell immunity is responsive to more conserved internal proteins which could be efficient for mediating cross-protection and fast recovery from the disease, vaccine candidates stimulating influenza A virus-specific CD8+ T cells are very promising (Grant, Quiñones-Parra *et al.*, 2016; Johansson & Brett, 2007; Kang, Yoo *et al.*, 2009; Trollfors, 2006).

2.2.2 Limitations of current vaccine manufacturing process

The egg-embryonated technology that supports production of seasonal influenza vaccines carries some drawbacks that are pushing the scientific community to find novel and more efficient vaccine production platforms. Disadvantages associated with the egg-based production platform include: 1) adaptation of virus to eggs can introduce mutations that alter the composition of the hemagglutinin and negatively affect its antigenicity, 2) some virus strains cannot be efficiently grown in eggs which leads to a time-consuming process with the risk of developing undesired reassortants 3) the supply of eggs in the cases of pandemics or high demand seasons can be a limiting factor (one or two high quality eggs are needed per dose of vaccine). Also, diseases affecting chickens such as avian influenza virus outbreak can disrupt the supply eggs for vaccine production; 4) local or systemic allergic reactions to egg-derived vaccine components in vaccinated persons and 5) the requirement of biosafety level 3 to manipulate highly pathogenic viral strains or non-human influenza viruses (Genzel, Behrendt *et al.*, 2013; Quan, Steinhauer *et al.*, 2008; Tree, Richardson *et al.*, 2001).

Therefore, there is a need to develop a new generation of influenza vaccines and a robust production system that takes advantage of recent progress in bioengineering, biotechnology, molecular and cellular biology. Most of the available expression systems have already been explored for the production of influenza vaccines: mammalian cell culture (Chu, Lugovtsev *et al.*, 2010; Le Ru, Jacob *et al.*, 2010; Paillet, Forno *et al.*, 2011); insect cells (Cox & Hollister, 2009; Pushko, Tumpey *et al.*, 2005); plants (Landry, Ward *et al.*, 2010); bacteria (Rudolph & Ben-yedidia, 2011). An excellent review from Milián, and Kamen (2015) provides a very complete state-of-the-art on current influenza vaccines that have been approved by the FDA along with the expression system used. For example, the Flucelvax influenza vaccine, originally developed by Novartis and recently acquired by Seqirus™ (a CSL company), is produced in Madin Darby Canine Kidney (MDCK) suspension cells and the Flublok, manufactured by Protein Sciences

Corp., is produced in insect cells and both vaccines have been licensed by FDA, demonstrating the potential of cell culture technology as a modern and robust influenza vaccine production system.

2.2.3 Recombinant influenza vaccines

Influenza vaccines design is a key issue for achieving broader humoral and cellular immunity combined with improved safety. Different influenza vaccine strategies have been examined so far mainly looking for a universal vaccine that can prevent infections from new strains, and also for vaccine presentation formats and adjuvants to increase immunogenicity; and induce a long-lasting response. The subunit vaccines mainly based on the engineering of the HA antigen have been extensively approached (He, Leyrer *et al.*, 2016; Song, 2016). Toward a universal vaccine based on HA, chimeric globular HA domain containing the main conserved epitopes from different clades/subtypes based on computationally optimized broadly reactive antigens (COBRA) (Ducatez, Bahl *et al.*, 2011; Giles, Bissel *et al.*, 2012; He, Prabakaran *et al.*, 2014) and headless HA vaccine candidates containing conserved epitopes of the stalk domain able to elicit broadly protective antibodies (Krammer & Palese, 2013; Neu, Dunand *et al.*, 2016) constitute promising approaches. In the same direction of finding universal vaccines targeted to conserved epitopes of viral proteins, the highly conserved extracellular domain of the ion channel M2 protein (M2e) has been studied (Deng, Cho *et al.*, 2015). Considering the role of M2 during the viral infection, the antibodies raised against this protein could inhibit the virus growth. However, since this protein is the least represented in the viral envelope, little M2e-specific humoral response have been detected by natural infection. In general, vaccine candidates based on M2e do not induce neutralizing antibodies; they have to be enhanced with strong adjuvants and/or advanced antigen presentation formats to be efficacious (Krammer & Palese, 2015; Shaw, 2012).

As an alternative approach, DNA- and viral vector-based vaccines have shown to preserve the native structure of the antigens efficiently (Chen, Cheng *et al.*, 2008; Laddy, Yan *et al.*, 2008; Smith, Wloch *et al.*, 2010; Van Kampen, Shi *et al.*, 2005). Several other studies have focused on peptide-based vaccines by combining different conserved epitopes as synthetic peptides to enhance T-cells response (Alexander, Bilsel *et al.*, 2010). Finally, virus-like particle vaccines are becoming a promising way to safely elicit both humoral and cellular response in the host immune system.

2.2.4 Influenza virus-like particles as vaccines

Influenza virus-like particles (VLPs) are usually formed by recombinant expression of the major viral proteins, e.g., HA, NA, M1, and M2. These proteins mediate the budding of particles that resemble native influenza virus as observed by electron microscopy. The VLPs do not contain viral genetic material; they are non-replicating and non-infectious particles displaying HA and NA antigens in the lipid envelope. The displayed viral glycoproteins keep their native conformational structure and intact biochemical functions (Haynes, 2009). VLPs are more immunogenic than subunit vaccines and induce an immune response similar to the wild-type virus, without the associated safety concerns. The repetitive display of the main antigens on VLPs and their particulate structure enhance phagocytosis such that they are easily taken up by antigen-presenting cells, especially dendritic cells. The antigenic epitopes are cross-presented through MHC I molecules resulting in the activation of cytotoxic T lymphocytes. Furthermore, VLPs can induce strong activation of B cell response in a T cell-independent mode. The high-density of epitopes on the VLPs envelope induces the crosslinking of the B-cell receptor promoting the B cells proliferation, T-helper activation by MHC II presentation, immunoglobulins secretion and generation of long-lasting memory B cells (Chen & Lai, 2013; Rynda-Apple, Patterson *et al.*, 2014).

The viral proteins required for the formation of VLPs have varied in many studies. VLPs have been assembled from the co-expression of HA/NA/M1/M2 (Pan, Wei *et al.*, 2010), HA/NA/M1 (Pushko *et al.*, 2005), HA/M1 (Song, Hossain *et al.*, 2010), or by the single expression of M1 (Gómez-Puertas, Albo *et al.*, 2000), M2 (Rossman, Jing *et al.*, 2010), NA (Lai, Chan *et al.*, 2010), and HA (Chen *et al.*, 2007; D'Aoust, Couture *et al.*, 2010). These inconsistencies observed in the production of VLPs have brought to light that the mechanisms of viral budding of influenza viruses are not completely understood. As mentioned previously, it appears that there is not a single protein responsible for the viral budding but rather there is a certain redundancy among them (Rossman & Lamb, 2011). It is worth mentioning that most of the studies done to elucidate viral budding mechanism have been performed in different expression systems and using different vectors to deliver the recombinant genes. The expression system may influence the budding mechanism, since the host cell provides the cellular machinery for the virus replication and budding.

The baculovirus/insect cell-expression system is the most widely used to produce influenza VLPs (Vicente, Roldão *et al.*, 2011). Several studies producing influenza VLPs in insect cells have shown the efficacy of the VLPs to induce protective immunity against influenza virus challenges (Bright, Carter *et al.*, 2007; Galarza, Latham *et al.*, 2005; Kapczynski, Tumpey *et al.*, 2016; Pushko *et al.*, 2005; Pushko, Tumpey *et al.*, 2007; Quan, Huang *et al.*, 2007; Smith, Flyer *et al.*, 2013). Moreover, infuenza VLPs have been generated in *Nicotiana benthamiana* (a close relative to the tobacco plant) providing cross-protection in ferrets and promising immunogenicity in humans (Landry *et al.*, 2010). Generation of influenza VLPs has also been achieved in different types of mammalian cells, including: HEK-293T (HEK-293 containing the SV40 large T-antigen), HeLa (human cervical cancer derived cells), Cos-1 (monkey kidney cells), and Vero cells (derived from the kidney of an African green monkey) resulting in efficacious protection in *in vivo* challenge experiments (Carter, Darby *et al.*, 2016; Tang, Lu *et al.*, 2011; Wu, Yeh *et al.*, 2010). Various strategies have been followed to deliver influenza genes in mammalian cells to generate VLPs. Tang *et al.* (2011) carried out the production of VLPs using a unique BacMam containing individual expression cassettes of HA, NA, M1 under the control of the CMV promoter. In this work, very basic optimization of the transduction conditions was performed. The MOI, incubation time, and the addition of sodium butyrate (an additive used for enhancing gene expression in baculovirus transduced cells) were established. Following sucrose gradient purification, baculoviral DNA was detected but there was no detectable baculovirus in the purified VLPs. A positive hemagglutination assay and protective immunity in animals showed the efficacy of the produced VLPs. Wu *et al.* (2010) obtained VLPs from a stable Vero cell line expressing M1, M2, NA and HA using plasmids with the inducible system based on tetracycline operon. The work was focused on the characterization of VLPs recovered by ultracentrifugation. A detailed analysis of the VLP proteomic composition, glycosylation profile and demonstration of VLPs efficacy in animals was presented. These studies demonstrated the immune-protective efficacy of influenza VLPs derived from mammalian cells. However, more efforts are needed in order to develop and optimize a process to scale-up the influenza VLP production in mammalian cells with the ultimate goal that this approach can become seriously considered for large-scale vaccine manufacturing.

Table 2-2 shows different types of VLPs, including influenza-like, that have been licensed and/or are in the process of acceptation as vaccines.

Table 2-2 Virus-like particles in development phase and/or licensed for use as human vaccine (Effio & Hubbuch, 2015). The expression system used is specified for influenza VLPs.

Development stage	Virus/pathogen/disease
Preclinical	Chikungunyavirus (Metz, Martina *et al.*, 2013), Coxsackievirus B3 (Koho, Koivunen *et al.*, 2014), Cytomegalovirus (Vicente, Burri *et al.*, 2014), Dengue virus (Schmitz, Roehrig *et al.*, 2011), Enterovirus 71 (Chung, Chen *et al.*, 2010; Ku, Liu *et al.*, 2014), Group A Streptococcus (Chuan, Wibowo *et al.*, 2014), Human B19 parvovirus (Chandramouli, Medina-Selby *et al.*, 2013), Human immunodeficiency virus (Buonaguro, Tagliamonte *et al.*, 2012; Negrete, Pai *et al.*, 2014), Human papillomavirus (Gissmann, 2009), Rotavirus (Li, Lin *et al.*, 2014)
Phase 1	Allergenic rhinitis/Asthma (anti IgE Qβ-VLP), Chikungunyavirus (Chang, Dowd *et al.*, 2014), Ebola virus (Mohammadi, 2014), Influenza H1N1 (Low, Lee *et al.*, 2014; Ward, Landry *et al.*, 2014) (produced in bacteria and plant *Nicotiana benthamiana,* respectively), Influenza H7N9 (Fries, Smith *et al.*, 2013) (produced in insect cells (HA,NA and M1), Respiratory syncytial virus (Glenn, Smith *et al.*, 2013)
Phase 2	Alzheimer's disease (amyloid Qβ-VLP) (Bachmann & Whitehead, 2013; Caputo, Graf *et al.*, 2014), Human B19 parvovirus (Bernstein, Sahly *et al.*, 2011), Influenza H1N1 (López-Macías, Ferat-Osorio *et al.*, 2011) (produced in insect cells HA, NA and M1), Influenza H5N1 (Ward et al., 2014) (produced in plant *Nicotiana benthamiana*) , Norovirus (Sundararajan, Sangster *et al.*, 2015)
Phase 3	Human papillomavirus (Erickson, Landers *et al.*, 2014; Hildesheim, Wacholder *et al.*, 2014), Malaria P. falciparum (RTS,S) (Umeh, Oguche *et al.*, 2014)
Licensed	Human papillomavirus (Gardasil®, Cervarix®) (Dochez, Bogers *et al.*, 2014), Hepatitis B virus (Recombivax HB®, Engerix-B®) (Lacson, Teng *et al.*, 2005), Hepatitis E virus (Hecolin®) (Zhang, Wei *et al.*, 2014)

2.2.5 Gag as a core protein for VLPs

Gag is the major structural protein of HIV-1 and comprises about 50% of viral particle mass. This protein is sufficient to produce non-infectious virus-like particles in the absence of other viral proteins. The major structural components of HIV-1 are derived from Gag precursor polyprotein and consist in three polypeptides: matrix layer (MA), conical capsid or p24 (CA) and the nucleocapsid (NC) and spacer proteins SP, SP2 and p6 (Briggs, Simon *et al.*, 2004) (Figure 2-3). The gag gene is translated by the ribosomes, followed by post-translational myristoylation and it is translocated throughout the cytoplasm to the plasma membrane where Gag molecules assemble and bud as immature particles (Ganser-Pornillos, Yeager *et al.*, 2008) . There is a preference of Gag-based particles to bud from lipid rafts domain facilitated by interaction with host cell proteins (Spearman & Freed, 2009). For example, the association with caveoline-1 has been related to this fact (Yu, Beer *et al.*, 2006). This feature is very significant for the use of Gag protein as a budding trigger of pseudo-type VLPs, like influenza, where the main envelope proteins are accumulated in lipid rafts regions of the membrane. Concurrent with the assembly and budding, the protease mediate the cleavage of Gag polyprotein in 6 proteins that get rearranged inside the virion to form the mature particles. The Gag core protein of retroviruses has been employed to produce influenza-pseudotype Gag VLPs in insect cells (Haynes, Dokken *et al.*, 2009). The co-expression of Gag and the envelope proteins of influenza gave rise to abundant quantities of homogeneous sized particles with the antigens correctly displayed in the envelope. The immunization of mice and ferrets with these VLPs elicited robust immunogenicity and protection.

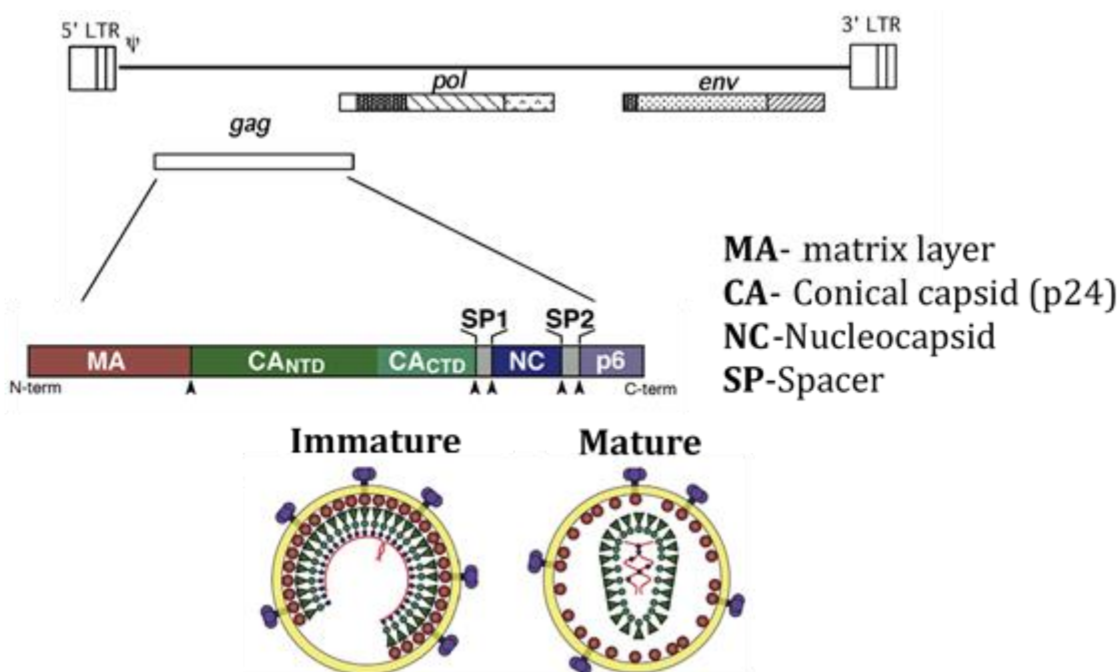

Figure 2-3. Structure of Gag protein of HIV-1 (adapted from (Ganser-Pornillos et al., 2008).

2.3 Mammalian cells as expression system

Mammalian cell culture has become an efficient technology to sustain high yield production of therapeutic proteins or vaccines in the biopharmaceutical industry (Le Ru *et al.*, 2010). One of the major advantages associated with this expression system is that mammalian cells can perform complex post-transcriptional and post-translational modifications which are important for protein stability, ligand binding, functionality and immunogenic responses (Liste-Calleja, Lecina *et al.*, 2013). Thus, the development of the platform arises as a need to efficiently produce high-quality biopharmaceuticals that are less immunogenic for human use than those obtained from traditional methods, e.g., protein expression in bacteria, yeast or production of inactivated viral vaccine by passages in animals or in embryonated eggs as in the case of influenza (Barrett, Mundt *et al.*, 2009). Since the approval in 1980s of insulin and the licensure of Inactivated Poliovirus Vaccine produced in cell culture many investigations have moved toward this direction. From 2003 to 2010 more than half of approved biopharmaceuticals were produced in mammalian cell cultures (Liste-Calleja *et al.*, 2013).

In general, there are three types of mammalian cell cultures that have been used so far. The first culture to be used was primary cell culture, in which the cells were directly taken from a human

or animal tissue. The inconsistency of the starting material (it may be composed of mixtures of cell types), very short lifespan and concerns about contamination with a number of potential adventitious agents limited their use (Barrett *et al.*, 2009). The second type of cell culture employed was diploid cell lines. They are defined as "having a finite *in vitro* lifespan in which the chromosomes are paired (euploid) and are structurally identical to those of the species from which they were derived" (Barrett *et al.*, 2009). These cells are restricted to a limited number of passages before senescence, which is not suitable for large-scale production. Diploid cell lines need a more demanding growth medium and are not easy adapted to serum-free conditions. The third type of cell culture involves the use of continuous cell lines (CCLs) that are defined as immortalized cell lines with an infinite lifespan. They are either derived from tumorigenic cells or from a normal population with potential tumorigenicity. Due to the tumorigenic origin of CCLs, for many years, they were not considered suitable for the production of biopharmaceuticals. However, taking advantage of the development of new techniques in immunology, virology and molecular biology; WHO established requirements for using CCLs to produce biological products. For a long time, the CCLs were grown in adherent culture and required the constant supplementation of animal serum (leading to increased production costs and lot-to-lot variability), which has hindered their use for large-scale production. Moreover, the serum is an animal derived product that is poorly characterized and can be contaminated by adventitious agents. The successful adaptation of some CCLs to serum-free media allowed to reach higher cell densities, increased yields of biological products and reduced manufacturing costs (Werner, Walz *et al.*, 1992).

Currently, many CCLs are well characterized and have been widely used either for recombinant protein expression or viral production. Specifically for the production of influenza viral vaccines, Madin Darbin Canine Kidney (MDCK) cells (Voeten, Brands *et al.*, 1999), human embryonic retinal cells (PER.C6) (Pau, Ophorst *et al.*, 2001) or Vero cells (Paillet *et al.*, 2011) are among the most widely used. Influenza VLPs have been produced in Vero cells (Wu *et al.*, 2010) and HEK-293T (Chen *et al.*, 2007; Tang *et al.*, 2011). MDCK and Vero cells are the preferred host for influenza virus production because MDCK cells can produce infectious particles at levels comparable with eggs and Vero cells are recommended by WHO for meeting the established requirements to produce viral vaccines (Barrett *et al.*, 2009; Belsey, Lima *et al.*, 2006). However,

these cell lines must be grown adherently in serum-containing medium which can be cumbersome for large scale production (cells are grown attached to microcarriers).

The HEK-293 cell line has been widely used in the last decades for the production of viral vectors for gene therapy (Dumont, Euwart *et al.*, 2015; Le Ru *et al.*, 2010). Several studies about metabolism, scalability, and viral transfection have validated the use of HEK-293 cells as an efficient and well-documented platform for the large scale production of recombinant proteins and viral vectors (Ansorge, Transfiguracion *et al.*, 2009; Durocher, Pham *et al.*, 2007; Henry, Dormond *et al.*, 2004; Henry, Jolicoeur *et al.*, 2011; Petiot, Jacob *et al.*, 2011; Shen & Kamen, 2012). HEK-293 cells grown in suspension culture (batch mode) with serum-free medium (HEK-293SF) can reach cell densities up to 10^7 cells/mL (Côté, Garnier *et al.*, 1998). When HEK-293SF cells were infected with influenza virus, titers of infectious influenza viruses as high as those obtained with the MDCK reference cell line, up to 10^9 infectious virus particles (IVP)/ml, were achieved (Le Ru *et al.*, 2010). Recently, after baculovirus transduction in several cell lines (including Vero, HeLa, MDCK and HEK-293T) to produce influenza VLPs, it was demonstrated that 293T was the most susceptible cell line and yielded the highest protein expression (Tang *et al.*, 2011).The major concern about the use of HEK-293 cells has been associated to their potential tumorigenicity but a previous study clearly established a relationship between tumorigenicity and the viable passage number. The study concluded that using low-passages cultures (<52) should be acceptable to regulatory authorities (Shen, Gu *et al.*, 2008). There are now various biotherapeutic products produced in HEK-293 cell that have been approved by the FDA or the European Medicine Agency, showing the possibility of fast regulatory clearance and good safety profile of these cells for manufacturing biopharmaceuticals (Dumont *et al.*, 2015).

2.3.1 Mammalian cells expression regulation systems

Living organisms have the ability to adapt to the environment by internal changes allowing their continued survival on our planet. These internal changes known as regulation mechanisms have been identified for a long time, but it was not until 1961 that the first mechanism was described at the molecular level (Jacob & Monod, 1961). The lactose regulation system in *Escherichia coli* became a paradigm for understanding the general concept of gene regulation. It represents a recurring molecular principle for adaptation of life to changing environmental conditions, even for gene regulation systems of higher eukaryotes (Fussenegger, 2001). *E. coli* can redirect its

metabolism for utilization of lactose as carbon source by expressing a set of genes encoded by the lactose (Lac) operon. The mechanism works depending on the absence or presence of lactose as the only source of carbon.

Analogous mechanisms to this system are found in mammalian cells and are able to integrate metabolic, hormonal and environmental signals and orchestrate vital regulatory networks such as the cell-cycle, programmed cell death (apoptosis) and development (Fussenegger & Bailey, 1998). In general, inducible regulation systems have been widely used in biotechnology and gene therapy since their discovery. The potential applications of these systems are huge, e.g. the possibility to express a protein or set of proteins in a regulated manner helps the study of metabolic pathways and the understanding of specific functions of proteins. Additionally, it provides the possibility to regulate both the level and duration of the expression of some proteins that are toxic by constitutive expression (Mullick, Xu *et al.*, 2006). Furthermore, for large-scale production of proteins, even for nontoxic proteins, inducible regulation systems seem better because of the facility to express the recombinant protein at the desired cell density. The constitutive overexpression of a foreign protein in the culture could reduce cell growth and decrease cell viability (Spitzer, Landthaler *et al.*, 2012). Several studies to develop a very efficient and tightly controlled inducible system to regulate the expression of proteins in mammalian cells have been performed. The ideal inducible system should have a very low basal protein expression ("off" state) as well as reversible and very high expression ("on" state) after induction (ability to switch efficiently from on to off state, and vice versa). Furthermore, the inducer should not have a pleiotropic effect on host cells and the ability to induce protein synthesis rapidly (Russell, 1999).

Currently, the most widely used mammalian regulation systems are ecdysone (insect hormone) system (Melito, Qiu *et al.*, 2008) and (Tet) tetracycline resistance operon from *E. coli* modified to function in mammalian cells (several different versions of Tet system have been developed so far). However, some limitations are still found in these systems, which has motivated the need to develop other inducible systems to extend their use to other specific biological applications. Mullick *et al.* (2006) have developed an inducible transcription system (cumate switch), derived from the p-cym operon of *Pseudomonas putida,* for expression in mammalian cells. This work demonstrated that the cumate gene switch can tightly control gene regulation for both transient and stable expression. Later on, researchers have used the stable cell line with this inducible

transcription system to generate stable lentivirus (LVs) from a packaging cell line (293SF-PacLV) with the capacity to produce high-titers (up to 3.4×10^7 transducing units (TU)/ml) of LVs in suspension culture and in serum-free medium (Broussau, Jabbour *et al.*, 2008). This last work confirmed the feasibility of harnessing the regulatory elements of the p-cym operon from *P. putida* to regulate gene expression in mammalian cells.

2.3.2 Cell line development

The growing demand for biopharmaceuticals produced in mammalian cells has compelled researchers to improve media composition, optimize culture conditions and develop sophisticated manufacturing technology to sustain higher yields of biological products in a cost-effective manner. Substantial progress has been made in the development of mammalian cell lines for stable expression of recombinant proteins (Noh, Sathyamurthy *et al.*, 2013). Once it is established, the cell line can be optimized for large-scale production without the variability typically associated with the type of method or the delivery vectors involved in transient transfection (Browne & Al-Rubeai, 2007). The general method used to create a stable cell line for protein expression consists of various steps (Figure 2-4). First, the gene of interest (GOI) should be cloned in an appropriate plasmid containing the selection marker cassette. Also, two independent plasmids can be used, one containing the selection marker and the other one the GOI. Subsequently, the plasmid (or plasmids) is transiently transfected in the target cell line and the expression as well as the correct folding of the protein are verified before continuing to the next steps. Once the GOI expression is validated, the cells are cultivated under selective pressure in medium containing a selective agent (e.g. antibiotic). Only those cells that have integrated the plasmid can survive. To generate clonal cells, it is necessary to obtain single and isolated high-producer cells because large variability between the transfectants is observed. This phenomenon occurs due to random integration of the gene copies in the chromosomes giving rise to different phenotypes. The gene can be inserted in a genomic region disrupting endogenous genes and affecting the normal growth of the cells (Noh *et al.*, 2013). The expression levels of the recombinant protein are variable between the clones, probably depending on the number of inserted gene copies or the site of insertion. However, it has been shown that this variability appears in both transfected cells that produce a recombinant protein and the untransfected

parental cell line, indicating that variability is an inherent characteristic of the cells and does not just arise as a result of the gene insertion (Browne & Al-Rubeai, 2007).

Since stable cell lines usually take time to be generated, a lot of effort has been made to find more efficient, cost-effective and high throughput methods for the selection of high-producer clonal cells (Browne & Al-Rubeai, 2007). Traditional methods include limiting dilution cloning in 96 wells plates. This method is still widely used owing to its relative simplicity and low cost but is labor intensive, time-consuming, has a low-throughput and requires exhaustive downstream analysis of product levels. New methods allow an increase in the number of screened samples and a reduction of the time required to select cells with suitable characteristics. It is worth to mention, flow cytometry and cell sorting, as well as automated systems, such as, ClonePix (Genetix), and CellCelector^{TM} (Aviso), which permit to identify and pick higher producer clonal cells as early as seven days after seeding by pre-programmed software. Finally, after selection of the best clone, various passages are made with the selected clone to confirm insertion stability. Then, the production from the stable cell line is scaled up and optimized for production.

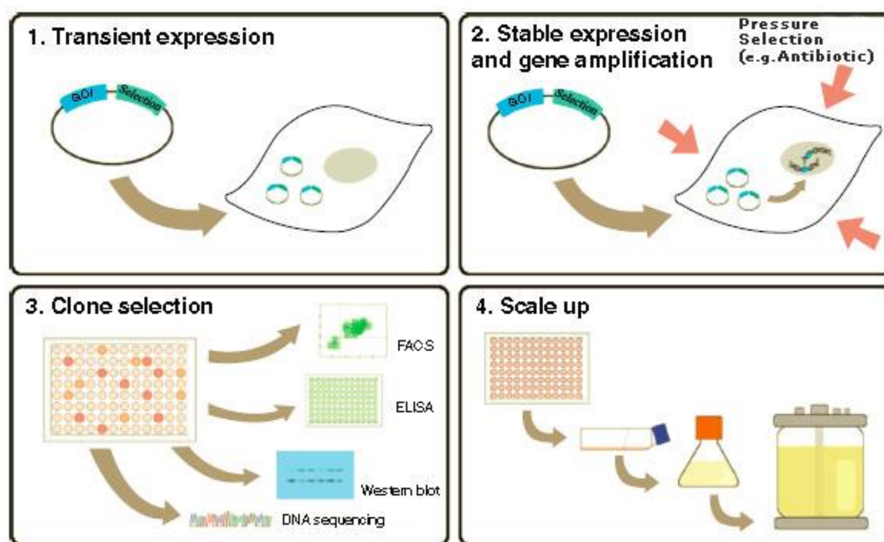

Figure 2-4 General method followed to create a stable cell line for large-scale production of recombinant protein. 1) To make the plasmid construction with the gene of interest-selection marker and pursues transient transfection in the host cell for checking functionality of the expression cassette. 2) To stress transformed cells under selective pressure with the antibiotic. 3) Traditional method of clone selection by limiting dilution cloning in 96 wells plates and downstream analysis to check the expression by different techniques. 4) Scale up to large-scale suspension (Noh *et al.*, 2013).

2.4 Challenges associated to the large-scale manufacturing of VLPs for vaccine

As mentioned before VLPs constitute a promising influenza vaccine candidate. However, to finally validate these particles as vaccines, more work should be done to improve VLPs design, and develop the upstream and downstream processing. Due to the versatility of VLP functions and structures, many innovative and creative designs can be implemented toward more immunogenicity and cross protection. Also, the upstream process should be further developed and optimized to reach high VLP titers in a cost-effective manner. The downstream process of enveloped VLPs needs to be developed as well. Harvesting high-yields of VLPs-containing broths from the upstream process will allow to test several purification strategies in order to obtain highly purified VLPs for a better characterization of the final product. The process development should be supported by robust qualitative and quantitative analytical methods for production monitoring and characterization of polished VLPs (Thompson, Petiot *et al.*, 2013; Vicente *et al.*, 2011).

2.4.1 VLPs design

VLPs can be classified as enveloped or non-enveloped depending on the presence or not of the lipid bilayer. The intrinsic nature of the viral proteins involved in the VLP self-assembly will dictate the structure of the particle. Non-enveloped VLPs are classified as: single-protein (e.g. the Human Papillomavirus (HPV) licensed vaccine) and multi-protein (e.g. VLPs derived from poliovirus (Bräutigam, Snezhkov *et al.*, 1993) and infectious bursal disease virus-like particles (Kibenge, Qian *et al.*, 1999)). Single protein non-enveloped VLPs are quite simple in structure while multi-protein VLPs are far more complex forming two or three capsid layers as in the case of rotavirus VLPs (Crawford, Labbe *et al.*, 1994). In enveloped VLPs, such as influenza and HIV-VLPs, the matrix proteins are enclosed with the host cell lipid membrane at the budding site while glycoproteins are embedded and exposed in the membrane. The presence of the lipid envelope makes these VLPs less structurally homogeneous than their non-enveloped counterpart (Donaldson, Al-Barwani *et al.*, 2015; Lua, Connors *et al.*, 2014).

The structural and functional versatility of VLPs give room for innovative designs. One single VLP can harbor different antigens to combat different diseases and/or disease mutants. Also,

even though VLPs possess self-adjuvanting properties, the combination of VLPs with molecular adjuvants can further enhance their immunogenicity. VLPs can be used to deliver proteins, DNA or RNA to specific cell types which have application for gene therapy (Garcea & Gissmann, 2004; Kaczmarczyk, Sitaraman *et al.*, 2011; Robert, Lytvyn *et al.*, 2016). Several studies from Medigen, USA have reported the expression of multiples HA subtypes within a VLP (triple and quadri-subtyped VLPs) which conferred protection in ferrets against the corresponding strains (Pushko, Pearce *et al.*, 2011; Pushko, Sun *et al.*, 2016; Tretyakova, Hidajat *et al.*, 2016; Tretyakova, Pearce *et al.*, 2013). Also, multiple studies have investigated chimeric VLPs, where foreign proteins or antigens from other viruses, together with a core/capsid protein are co-expressed within the same cell through genetic engineering. In the last decades, the recombinant hepatitis B virus core (HBc) has been widely used as carrier of foreign epitopes from many viruses such as influenza A, papillomavirus, human and simian immunodeficiency virus, cytomegalovirus and also bacterial protein epitopes (López-Sagaseta, Malito *et al.*, 2016; Pumpens & Grens, 2001). In fact, in the malaria vaccine candidates Malarivax (Nardin, Oliveira *et al.*, 2004) and Mosquirix (Cohen, Nussenzweig *et al.*, 2010), the epitopes from circumsporozoite protein (CSP) of the *Plasmodium falciparum* malaria parasite are presented on chimeric nanoparticles using as capsid core the HBcAg or HBsAg (hepatitis B surface antigen), respectively.

VLPs can serve as a robust presentation platform for proteins that are naturally less immunogenic. An interesting work done by Kim, Song *et al.* (2013) showed that the incorporation of a tamden repeat of M2e domain together with HA on VLPs elicited a significant higher immunogenicity and cross-protection in absence of adjuvant compared with the wild-type M2 VLPs, in mice. Also, the immunogenicity of VLPs has been enhanced by incorporation of flagellin and granulocyte-macrophage colony-stimulating factor (Liu, Liu *et al.*, 2016). The immunization with these modified VLPs promoted stronger Th1 and Th2 responses along with an enhanced B cells proliferation. Original and efficient VLP designs will hopefully speed up the path toward universal vaccines and vaccines of new generation. The rational design of VLPs should take advantages of computational tools. More and more databases are available going from full sequences of isolated viruses genome (NCBI) to databases like Immune Epitope Database Analysis Resource (IEDB) where a compilation of reported immune T- and B-cell epitopes and MHC molecules are available (Wong & Ross, 2016).

2.4.2 Upstream process

The demonstration that VLPs are immunogenic and efficacious in small *in vivo* experiments can be easily supported with laboratory scale productions. However, in order to produce sufficient amounts of VLPs to accumulate stronger pre-clinical data and perform clinical trials, the production process should be carefully developed to be scalable, robust, reproducible, cost-effective and rapid. Several aspects can be looked up and improved during VLPs production in cell culture processes. If working with stable cell lines, the screening process should be implemented with high throughput screening techniques that allow to select clones with optimal VLPs protein expression and growth. Both, protein expression and cell growth rate and/or metabolism can be differently affected in each clone depending on the integration site of the gene of interest. Hence, a combination of high cell specific productivity and cell specific growth rate will provide a more suitable clone for large scale manufacturing. In the case of enveloped VLPs, like influenza, where more than one protein is involved in their formation, it appears more reasonable to employ an inducible system to reduce the extra metabolic burden during growth phase due to the expression of the recombinant proteins.

Most of the VLP productions in mammalian cells found in literature have been commonly mediated through transient transfection of at least one of the genes of interest (Cervera, Gutiérrez-Granados *et al.*, 2013; Chen *et al.*, 2007; Robert *et al.*, 2016; Wu *et al.*, 2010). Transient transfection of recombinant genes is very efficient for fast expression of the corresponding heterologous proteins (Durocher, Perret *et al.*, 2002). In the case of influenza VLPs, transient transfection has been efficiently used to elucidate budding mechanisms (Chen *et al.*, 2007) and also for small laboratory scale productions to demonstrate immunogenicity and efficacy of VLPs in animal experiments (Wu *et al.*, 2010). However, for large-scale manufacturing, having a transient transfection step in the production process requires the use of an extra closed vessel connected to the bioreactor to mix DNA with the transfection agent. Moreover, some transfection agents are toxic for the cells (Ansorge *et al.*, 2009) and stocks of plasmids should be maintained. For a process based on transient transfection, the DNA:transfection agent ratio and cell density at the time of transfection has to be optimized (Cervera *et al.*, 2013). In order to achieve high production yields, different media should be also evaluated at small scale (Vicente *et al.*, 2011). Metabolic flux studies can help to implement rational feeding strategies based on the culture limitations (Henry, Perrier *et al.*, 2005). Certainly,

the metabolic state of the cells may vary throughout the culture time, so the bioreactor parameters should be carefully controlled and methods for online monitoring of crucial cell physiological aspects and product formation should be implemented for an efficient process performance (Ansorge, Lanthier *et al.*, 2011; Henry, Kamen *et al.*, 2007; Liu, Wang *et al.*, 2016).

Fed-batch and continuous perfusion processes are the most common strategies employed to increase cell density and product yields in mammalian cell cultures. In the fed-batch operation mode, cell cultures are supplemented with nutrients that are being consumed by the cells, preventing their depletion in the culture medium. However, the accumulation of metabolic by-products might also inhibit cell growth (Hiller, Ovalle *et al.*, 2017). Finding an optimum feed requires a meticulous study of the cell line metabolism to identify the limiting factors and best timing to overcome these limitations (Henry *et al.*, 2004; Petiot *et al.*, 2011). The use of a perfusion process can alleviate some of the limitations of fed-batch cultures; by continuous removal and addition of conditioned and fresh medium, respectively, the growth inhibitors are eliminated and favorable culture conditions can be maintained (Tapia, Vázquez-Ramírez *et al.*, 2016). This mode of operation is particularly suited to products that are unstable, because they are sensitive to temperature and/or degradation by proteases (Ansorge *et al.*, 2009). To our knowledge, there are no reports applying this process intensification strategy to influenza VLPs produced from mammalian cells.

2.4.3 Downstream process

The use of influenza VPLs as vaccine is not only limited by the upstream stages of the production process, but also due to the lack of efficient purification and characterization methods. With the increased interest on these nanoparticles, more efforts have been dedicated to determine and solve the challenges associated with their downstream processing.

The contaminants present in the harvest broth collected from the upstream process can be classified as product and/or process-related contaminants. The process-related contaminants refer to host cell proteins (HCPs), DNA, RNA, endotoxins, extracellular vesicles, etc. while product-related contaminants are aggregates, misfolding structures and unassembled VLPs (Effio & Hubbuch, 2015). The purification process of VLPs involves different steps: clarification, concentration, diafiltration, and finally chromatography for intermediate purification and polishing.

The clarification process consists in the separation of cells and cell debris from supernatant. For enveloped VLPs, cell separation has been mostly done by centrifugation. However, other approaches have been implemented to make the process scalable. Negrete *et al.* (2014) succeeded to remove cells from harvest broth containing insect cells-produced Gag-VLPs by microfiltration at low shear forces using hollow fiber membranes of 0.45 μm. Maintaining low shear forces during clarification is critical for cells integrity and avoid increasing contaminants due to cell lysis. Also, low shear forces are favorable for envelope VLPs since these particles are highly sensitive to shear stress. Depth-filtration methodology has also been employed for cell removal of non-enveloped VLPs such as rotavirus-like particles (Peixoto, Sousa *et al.*, 2007). Digestion with benzonase endonuclease is a popular method used early at the clarification step to remove DNA. Stabilizers or protease inhibitors can also be added at this step. The cell culture platform, upstream process strategy and VLP type will all determine the downstream process strategy. For example, the harvest broths collected from perfusion processes are almost free of cells due to the cell retention device, but greater volumes need to be processed. Harvest broths collected from production process in insect cells cultures contain not only VLPs, but also high amount of baculovirus.

Once the harvest broth is clarified and is free of cells, the second step in VLPs downstream processing is the concentration and diafiltration of the clarified material. Originally, the concentration and purification of VLPs were mainly performed through ultracentrifugation. However, this technique is difficult to scale up, laborious, time-consuming and also VLPs are not recovered completely pure due to separation inefficiency (Effio & Hubbuch, 2015; Morenweiser, 2005). Ultracentrifugation is still used for bench scale research, but it is not practical for large-scale manufacturing. Nowadays, the most used technique to concentrate VLPs is tangential flow filtration using membranes of different cut-off depending on the VLP size. This ultra- and diafiltration (UF/DF) step allows to remove small extracellular vesicles and cell debris reducing HCPs and DNA contaminants. HIV-Gag VLPs have been efficiently concentrated using membranes with cut-off of 300 KDa (Cruz, Peixoto *et al.*, 2000) and 500 KDa (Negrete *et al.*, 2014). Precipitation by polyethylene glycol (PEG) can be an alternative method for VLPs concentration, however the technique has some drawbacks such as irreversible aggregation and co-precipitation with other contaminants like nucleic acids (Effio & Hubbuch, 2015).

Recovering highly pure enveloped VLPs containing only the heterologous viral proteins has become challenging for the downstream processing of VLPs. These large multimeric protein complexes surrounded by lipid membrane contain (inside and/or attached) HCPs, DNA and RNA that cannot be removed even with the most refined polishing methods. However, chromatographic methods could be efficient for the removal of extracellular vesicles (EVs) that are co-produced with VLPs in mammalian cell cultures. The removal of EVs will increase purity since EVs are nanoparticles involved in cell-cell communication and they also contain HCPs, DNA and microRNA. Affinity chromatography methods could be the most rational approach to separate both nanoparticles species. Most likely, different types of proteins are expressed on the surface of each nanoparticle, which, once identified, can be used as potential targets in affinity chromatography. The protein identification can be done by LC-MS/MS. Other kind of chromatography like size exclusion may not be very efficient in this case, because VLPs and EVs are similar in size. Anion-exchange chromatography has been often used to remove HCPs and DNA (Negrete *et al.*, 2014; Pincus, Boddapati *et al.*, 2010).

Analytical methods play a crucial role in the development of a robust manufacturing process. So far, most of the studies employed for VLP quantification rely on analytical methods used for influenza virus such as hemagglutination assay and single radial immunodiffusion. These methods are not high throughput and are not suitable for online monitoring of influenza VLPs during upstream and downstream processing (Thompson *et al.*, 2013). The fusion of reporter proteins like GFP will facilitate the monitoring of VLPs production from in-process samples (Gutiérrez-Granados, Cervera *et al.*, 2013).

CHAPTER 3 ARTICLE 1: HEMAGGLUTININ AND NEURAMINIDASE CONTAINING VIRUS-LIKE PARTICLES PRODUCED IN HEK-293 SUSPENSION CULTURE: AN EFFECTIVE INFLUENZA VACCINE CANDIDATE

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3.1 Abstract

Virus-like particles (VLPs) constitute a promising alternative as influenza vaccine. They are non-replicative particles that mimic the morphology of native viruses, which make them more immunogenic than classical subunit vaccines. In this study, we propose HEK-293 cells in suspension culture in serum-free medium as an efficient platform to produce large quantities of VLPs. For this purpose, a stable cell line expressing the main influenza viral antigens hemagglutinin (HA) and neuraminidase (NA) (subtype H1N1) under the regulation of a cumate inducible promoter was developed (293HA-NA cells). The production of VLPs was evaluated by transient transfection of plasmids encoding human immunodeficiency virus (HIV) Gag or M1 influenza matrix protein. To facilitate the monitoring of VLPs production, Gag was fused to the green fluorescence protein (GFP). The transient transfection of the gag containing plasmid in

293HA-NA cells increased the release of HA and NA seven times more than its counterpart transfected with the M1 encoding plasmid. Consequently, the production of HA-NA containing VLPs using Gag as scaffold was evaluated in a 3-L controlled stirred tank bioreactor. The VLPs secreted in the culture medium were recovered by ultracentrifugation on a sucrose cushion and ultrafiltered by tangential flow filtration. Transmission electron micrographs of final sample revealed the presence of particles with the average typical size (150-200 nm) and morphology of HIV-1 immature particles. The concentration of the influenza glycoproteins on the Gag-VLPs was estimated by single radial immunodiffusion and hemagglutination assay for HA and by Dot-Blot for HA and NA. More significantly, intranasal immunization of mice with influenza Gag-VLPs induced strong antigen-specific mucosal and systemic antibody responses and provided full protection against a lethal intranasal challenge with the homologous virus strain. These data suggest that, with further optimization and characterization the process could support mass production of safer and better-controlled VLPs-based influenza vaccine candidate.

Keywords: Influenza vaccine; Virus-like particles; TFF; Stable cell line; Quantification; HEK-293

3.2 Introduction

Influenza is an illness that causes high morbidity and mortality to human population worldwide (Wurm, 2004). The antigenic “drift” and “shift” phenomena are the origin of the appearance of new strains that cannot be recognized by the host immune system causing severe infections (Herzog, Hartmann *et al.*, 2009; Rossman & Lamb, 2011). Thus, every year, seasonal influenza vaccines are produced in embryonated chicken eggs depending on the circulating strains (Effio & Hubbuch, 2015). The production in hen’s eggs carries major drawbacks that have been described elsewhere (Genzel *et al.*, 2013; Le Ru *et al.*, 2010). Additionally, the egg produced seasonal vaccines do not provide full protection in all age groups (Hessel, Schwendinger *et al.*, 2010; Pica & Palese, 2013). These facts are driving the scientific community to urgently develop a new generation of influenza vaccines that is supported by a robust production platform taking advantages of recent progress in the fields of immunology, molecular and cellular biology, and bioprocessing sciences.

Mammalian cells possess several attractive attributes as a robust production platform candidate due to their ability to perform complex post-translational modifications and the high cell densities reached in suspension cultures in bioreactors. This fact has allowed to increase the platform yields and produce bioproducts of very high quality (Genzel *et al.*, 2013). Additionally, the required-time to develop stable cell lines has decreased considerably in the past decade. Inducible promotors have been developed in mammalian cells as well, mainly to deal with the overexpression of toxic proteins (Mullick *et al.*, 2006). The use of inducible promotors also allows to separate the growth phase from the production phase, thereby reducing the metabolic burden during biomass growth (Aucoin, McMurray-Beaulieu *et al.*, 2006; Pattenden, Middelberg *et al.*, 2005).

From the perspective of new generation influenza vaccines, different approaches have been investigated to overcome the disadvantages associated to egg produced vaccines (Jegerlehner, Schmitz *et al.*, 2004; Kim, Lee *et al.*, 2014; Lin, Huang *et al.*, 2011; Nadeau & Kamen, 2003; Sedova, Shcherbinin *et al.*, 2012; Shaw, 2012; Smith *et al.*, 2010; Van Kampen *et al.*, 2005). Virus-like particle (VLPs) constitute a promising alternative to safely elicit an effective immune response since they mimic native virus (Wurm, 2004). Influenza VLPs have been mostly produced in insect cells. However, this expression platform has the inconvenience of baculovirus contamination in final samples (Krammer, Schinko *et al.*, 2010). With the aim of avoiding these drawbacks and exploit the advantages of a superior platform, previous works have explored the production of influenza VLPs in mammalian cells. Most of these studies have been focused in elucidating the virus budding mechanisms (Chen *et al.*, 2007; Dunn, 1958; Gómez-Puertas *et al.*, 2000; Lai *et al.*, 2010) or testing the protective immunogenicity of VLPs in animals by directly using the sucrose cushion preparations (Cockburn *et al.*, 1969; Tang *et al.*, 2011). From a bioprocessing perspective, influenza VLPs have been produced in human embryonic kidney cells (HEK-293) cells but the production levels were significantly lower in comparison with insect cells, and contamination with extracellular vesicles was observed in final samples (Thompson *et al.*, 2015). In this work, we have developed and characterized an efficient procedure to produce influenza VLPs from a cell clone stably expressing the hemagglutinin (HA) and neuraminidase (NA) of influenza subtype H1N1 (293HA-NA cells). It is demonstrated that HA and NA containing VLPs can be efficiently produced following transfection of the 293HA-NA cells with a plasmid encoding the gag gene of human immunodeficiency virus (HIV) whose product acted

as scaffold. The extracellular vesicles were efficiently removed from final preparation by tangential flow filtration (TFF). The Gag-made nanoparticles assembled from 293HA-NA cells showed the typical morphology and expected size for immature HIV-1 particles. An extensive characterization and quantification of the influenza VLPs produced was performed by using different analytical techniques. The immunogenicity and protective efficacy of the VLPs was demonstrated in mice.

3.3 Materials and Methods

3.3.1 Cells, plasmids and antibodies

The cells were cultured in Hyclone SFM4Transfx-293 supplemented with 4-6 mM of glutamine in a humidified incubator at 37 ºC with 5% CO2, at an agitation rate of 100-110 rpm. The gene for M1 (CY033578.1) was codon optimized to human cells and the restriction sites for Hind III were added at both ends of the gene (GenScript) for subsequent cloning in pKCR5 plasmid. The plasmids pUC-HA and pUC-NA H1N1 A/Puerto Rico/8/1934 described in (Thompson *et al.*, 2015) were used as template for PCR to introduce the Kozak sequence and enzymatic sites. A NheI site was introduced toward the 5' end (forward primers 5'-ACTAGCTAGCGCCACCATGAAGGCAAACCTACTGGTCCTG-3'HA and 5'-ACTAGCTAGCGCCACCATGAATCCAAATCAGAAAATAATAACC-3' NA) and HindIII site at the 3' end (reverse primers 5'-ACCCAAGCTTAGATCTTCAGATGCATATTCTGCAC-3' HA and 5'-ACCCAAGCTTAGATCTCTACTTGTCAATGCTGAATG-3' NA). The plasmid pKCR5 contains the CR5 promoter that has been described in (Mullick *et al.*, 2006). The HIV-1 (HBX2) gag DNA with the Kozak consensus sequence was synthetized by TOPGene Technologies (Canada) and cloned PmeI/NheI in pAdCMV5-GFPq (Garten *et al.*, 2009) to give rise the plasmid pAdCMV5-gagGFP. The primary antibodies used for the immunofluorescense assay were: Anti-HA LS-C140660 (LifeSpan BioSiences, USA) (I) and Anti-NA ref: 04-230 (NIBSC, UK) (II); for Dot-Blot assay: anti-HA monoclonal produce in-house (III), universal rabbit anti-NA HCA-2 (IV) ; for SRID: anti-HA ref: 03/242 (NIBSC, UK) (V); for western blots: (III), (II), Anti-M1 antibody [GA2B] (ab22396) (Abcam, UK) (VI), and anti-GAG monoclonal 2p24 Biotinylated (Hybridoma 31-90-25, ATCC) (VII). The secondary antibodies for the Immunofluorescense assay were #115-495-003 (Jackson Immunoresearch, USA) (VIII) and

Alexa Fluor® #594 A11005 (ThermoFisher Scientific, USA) (IX); for Dot-Blot: the infrared-conjugated secondary antibodies IRDye (LI-COR Bioscience, USA); for western blots anti-mouse and anti-sheep IgG-HRP (Jackson Immunoresearch, USA).

3.3.2 Generation of 293CymR-rcTA cell line

The 293CymR-rcTA cell line was constructed at the National Research Council of Canada (NRC) by stably transfecting the plasmid pMPG-CMV5-CymROpt and pKCMV5-CuO-rcTA in the 293SF-3F6 cell line adapted to serum-free culture (Côté *et al.*, 1998). CymR is the repressor and rcTA is the reverse transactivator of the cumate regulation system (Mullick *et al.*, 2006). The addition of cumate in 293CymR-rcTA cells triggers the synthesis of rcTA and its binding, resulting in the activation of the cumate responsive promoter (CR5).

3.3.3 Generation of 293HA-NA stable cells

293HA-NA cells were generated by transfecting $1x10^6$ cells/ml of 293CymR-rcTA cells with 8µg total DNA of XbaI-linearized plasmids pKCR5-HA, pKCR5-NA and pcDNA6/his-Blasticidin (amount of 3:3:2 µg) using PEIpro (Polyplus transfection, France). The selection with Blasticidin S HCl (Enzo Life Sciences, USA) was applied 48 hours post transfection (hpt). Cells were split in 96-well plates with 8-10 µg/ml of Blasticidin. After 3 weeks under selection, several clones were isolated, induced with cumate and screened by Western Blot.

The induction of protein expression for all productions in 293HA-NA cells was done at a cell density of $1x10^6$ cells/m using 100 µg/ml cumate (stock of 100 mg/ml in Ethanol 70%) (Ark Pharm, Inc., USA). The cultures were stopped at 48-72 h post-transfection.

3.3.4 Immunofluorescence assay

To perform this assay, 293CymR-rcTA and 293HA-NA cells were seeded at $0.8x10^6$ cells/ml in 6-well plates. At 48 h post-induction the culture was centrifuged to discard the supernatant. Cell pellet was washed with 1% ice-cold BSA/PBS (bovine serum albumin/phosphate buffer saline) and incubated in 750 µl of this solution for 30 min. Then, 1.5 µL of antibody (I) at 3,7 mg/ml and 2 µL of antibody (II) were added to the cells and incubated for 1h. The cells were washed twice in 1%BSA/PBS. 2.5 µL of VIII and IX secondary antibodies were diluted in 500 µl 1% BSA/PBS and added to the cells for 1h incubation in the dark at 4ºC. Three washes with

1%BSA/PBS were done. The cells were re-suspended in 200 µl 1% ice-cold BSA/PBS and transferred to a glass bottom 4-chamber dish for confocal analysis using a Fluoview FV10i, Olympus microscope.

3.3.5 3L-Bioreactor

A 3-L Chemap type SG bioreactor (Mannedorf, Switzerland) was employed to produce the VLPs under controlled and monitored conditions. The bioreactor features have been previously published (Le Ru *et al.*, 2010).

3.3.6 Ultracentrifugation and concentration of VLPs by sucrose cushion 25%

VLPs were concentrated following a protocol that has been previously described elsewhere (Thompson *et al.*, 2015). All the sucrose cushion samples presented in this work had a concentration factor of 25X.

3.3.7 Tangential Flow Filtration

The Minimate™ TFF System (PALL Corporation) was used for the ultrafiltration of the influenza Gag-VLPs from the sucrose cushion sample. A Biomax cartridge (1000 kDa 0.005 m^2 PXB01MC50 | Pellicon XL Ultrafiltration Module) was employed in the purification of influenza Gag-VLPs. Before start the purification, the cartridge was sanitized with 0.5 M NaOH overnight. Subsequently, the system was neutralized with SuperQ water. The tubing size used was 16 SI and the retention volume of tubing was measured as 8 ml. Hollow fiber cartridges were used to concentrate control-VLPs and influenza Gag-VLPs recovered from TFF at 1000 KDa cut-off (ref: D02-E300-05-N, D02-E750-05-N, Spectrum Laboratories, USA).

3.3.8 Cell lysate using RIPA buffer

The cell culture was centrifuged at 1000 rpm for 5 min. The cell pellet was washed twice with 1 ml of PBS1X. The washed pellet was incubated 30 min on ice with 100 µl of RIPA buffer (50 mM Tris-HCl at pH8, 150 mM NaCl 0,1 % SDS, 1% NP-40, 0,25% sodium deoxycholate). Then, the mix was centrifuged at 12000 rpm for 5 min. Samples were analyzed by SDS-PAGE and Western Blot, as previously described (Thompson *et al.*, 2015).

3.3.9 Dot blot

The protocol followed in this study has been previously published (Li, Jaentschke *et al.*, 2010). Briefly, the standards anti-HA and anti-NA employed were hemagglutinin H1N1/A/Puerto Rico/8/34, (Protein Science, USA) and neuraminidase H1N1/A/USSR/90/77, (Sino Biological Inc., China). The detection system used was Odyssey CLx imaging, (LI-COR Bioscience, USA).

3.3.10 Single Radial Immunodiffusion

The previously published protocol (Thompson *et al.*, 2015) was used and the standard curve was constructed with the same material used for Dot-Blot.

3.3.11 Hemagglutination assay

The protocol for hemagglutination assay (Thompson *et al.*, 2015) was used. The quantity of VLPs/ml was calculated with the equation:

Viral particle/ml= $[RBC] * 10^{(logHAtiter)}$; where RBC is red blood cells per well ~$2x10^6$ cells/well

The number of RBCs are proportional to the number of viral particles at the end point dilution. 1 virus=1 RBC (Donald & Isaacs, 1954; Thompson *et al.*, 2013).

3.3.12 Determination of host cell proteins and host cell DNA

The HEK-293 host cell proteins (HCPs) ELISA kit (Cygnus Technologies, USA) was employed to determine the concentration of HCPs in the samples. The host cell DNA was estimated using the Quant-iT™ PicoGreen® dsDNA Assay Kit (ThermoFisher Scientific, USA).

3.3.13 Fluorescence intensity measurement and p24 quantification

The supernatant from daily sampling was recovered by centrifugation at 1000 rpm for 5 min. The fluorescence intensity was measured using the Spectrophotometer Synergy H1 BioteK microplate reader set as follows: λ_{em}=485nm and λ_{ex}=528nm. The values graphed were calculated by subtracting the fluorescence intensity values obtained for non-transfected 293CmR-rcTA cells used as negative control. The concentration of p24 was determined using the HIV-1 p24 ELISA assay cat# XB-1000 (XpressBio, USA).

3.3.14 Transmission electron microscopy (TEM)

Transmission electron micrographs were obtained using a Hitachi H-7500 TEM, operating in high contrast mode at an acceleration voltage of 80kV and at a magnification between 30,000x and 200,000x. Briefly, a 10µL of sample solution was adsorbed to a glow-discharged carbon-coated copper TEM grid (Cu-300HD, Pacific Grid-Tech, CA). Sample was then stained with 10 µL of freshly prepared and filtered 2% uranyl acetate which is applied directly on the grid. After 10 s, the stain was removed by touching the edge of the grid with a filter paper. The grid was dried at room temperature prior to the TEM observation.

3.3.15 Immunization and viral challenge

Six to eight-week-old female BALB/c mice were purchased from Charles Rivers Laboratories (St. Constant, Quebec). All immunizations were done intranasally at day 0, 14, 21 under lightly anesthesia with isofluorane and using as adjuvant 1 µg cholera toxin (CT, Sigma-Aldrich Canada Ltd., Canada). A group of mice (n=10) was immunized with 50 µl of influenza Gag-VLPs containing 2 µg of HA based on concentration determined by Dot-Blot. Two groups of 5 mice each were immunized with 2 µg of recombinant influenza H1N1 HA (rHA) (A/Puerto Rico/8/34, Sino Biological Inc., USA) or unimmunized (naïve). A group of 7 mice was immunized with control-VLPs containing the same Gag concentration of influenza Gag-VLPs dose. At day 42, five influenza Gag-VLPs immunized mice and 2 control-VLPs immunized mice were sacrificed for blood and nasal lavage fluid collection whereas non-terminal blood samples were collected in 5 rHA-immunized and 5 naïve mice as described elsewhere (Collin *et al.*, 2015). All samples were stored at -20ºC until assay. Five weeks after the last immunization, mice were intranasally challenged with 10^3 plaque-forming units (pfu) of the mouse-adapted influenza H1N1 virus (A/Puerto Rico/8/34) in 50 µl PBS. Challenged mice were observed daily for 12 days to monitor body weight and surviving rates.

3.3.16 Enzyme-linked immunosorbent assay (ELISA) for HA-specific antibodies detection

The 96-wells Immunolon 2R microplates (Thermo Electron Corporation, USA) were coated with 0.2 µg rHA/well in 50 µl of bicarbonate buffer (pH 9.6) at 4ºC overnight. All the subsequent incubations were carried out at room temperature. The plates were blocked with 5% bovine

serum in PBS for 1 h, and washed three times with PBS-0.05% Tween 20. Duplicates of 100 µl pre-diluted samples (1:10 for nasal IgA, 1:100 for serum IgA, IgG1 and IgG2a) were added to the wells. After 3 h incubation, alkaline phosphatase-conjugated goat antibodies specific for mouse IgA, IgG1 and IgG2a (Caltag Laboratories, UK) were added and incubated for 1 h. Color reactions were developed by the addition of p-nitrophenyl phosphate (pNPP) substrate (KPL, Inc., USA), and optical density was measured at 405 nm with an automated ELISA plate reader (Synergy H1, Bio-Tek Instruments Inc, USA). Pooled samples collected from mice that had been intranasally immunized with the rHA+CT or from the naïve mice were used as positive or negative controls for the assays, respectively.

3.4 Results and Discussion

3.4.1 Development of 293HA-NA stable cells

To generate the 293HA-NA cells the HA, NA and Blasticidin containing plasmids were transfected into the parental cell line 293CymR-rcTA (Figure 3-1A). At 48h post-transfection (hpt) cells were maintained under blasticidin selection. Several cell clones were isolated, induced with cumate and the cell lysates were analyzed by Western Blot (data not shown). The clone exhibiting the highest expression levels of HA and NA was selected as our stable 293HA-NA cells. The expression of HA and NA proteins at the surface of the 293HA-NA cells was also confirmed by confocal fluorescence microscopy. The two secondary antibodies employed in the immunoassay were conjugated with two different fluorophore signals: green (NA) and red (HA), respectively (Figure 3-1B). The membrane of the cells expressing both proteins turned orange because of the two-color mixed effect. Some cells that are expressing only HA (red membrane) or NA (green membrane) can be observed as well. The non-transfected 293CymR-rcTA cells were treated in the same way and no evidence of non-specific antibody binding was detected (Figure 3-1B).

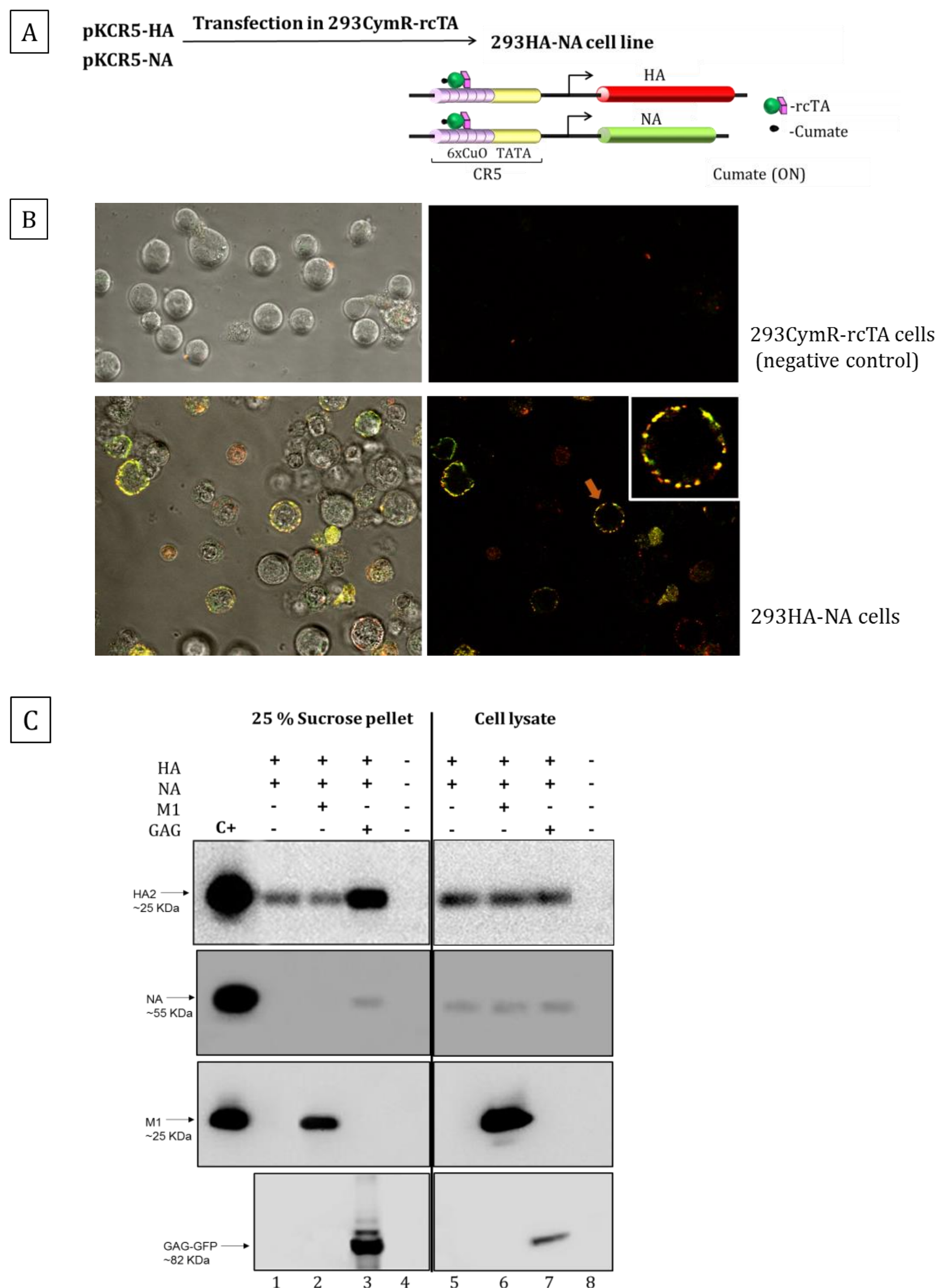

Figure 3-1 Development of the 293HA-NA stable cell clone and analysis of VLPs production efficiency. (A) Schematic representation of plasmids pKCR5-HA and NA transfection in the cell line 293CymR-rcTA to create the stable cells 293HA-NA under the cumate regulation system (Mullick *et al.*, 2006) (B) 72 h post-induction with cumate, live cells (no fixation) were observed by confocal microscopy. The arrow points to cell membrane with an orange tone due to

simultaneous expression of the two proteins HA and NA. (C) The influence of M1 and Gag proteins on VLPs production from 293HA-NA cells. Lanes (1 and 5) non-transfected 293HA-NA stable cells; (2 and 6) 293HA-NA cells transfected with pKCR5-M1; (3 and 7) 293HA-NA cells transfected with pAdCMV5-GagGFP; (4 and 8) non-transfected parental cell line 293CymR-rcTA as negative control; (C+) sucrose cushion of influenza virus H1N1 A/PR/8/34 produced in HEK-293 cells. The culture supernatants were 25× concentrated through a 25% sucrose cushion and the cells were lysed with RIPA buffer. The same total protein quantity was loaded in each lane, 6 µg for sucrose cushion pellets and 50 µg for cell lysates.

3.4.2 Comparison of influenza matrix M1 and Gag proteins effect on influenza VLPs production

The effect of two different proteins in triggering the release of VLPs from 293HA-NA cells was studied. There are discrepancies in the literature regarding the role of M1 protein as the driving force for virus budding and assembly (Chen *et al.*, 2007; Gómez-Puertas *et al.*, 2000). It appears that for influenza virus, the budding process is not driven by a single protein but rather is due to a redundant cooperation among them (Rossman & Lamb, 2011). For that reason, we evaluated in parallel the effect of HIV-1 Gag structural polyprotein, which is a well-known protein to promote viral budding of HIV and other viruses including influenza (Guo, Lu *et al.*, 2003; Haynes, 2009; Haynes *et al.*, 2009; Shaw *et al.*, 2002). To facilitate the monitoring of Gag expression, the protein was fused with the green fluorescent protein.

Thus, three different protein combinations were examined. Co-expression of HA-NA (non-transfected 293HA-NA cells), co-expression of HA-NA-M1 (293HA-NA cells transfected with pKCR5-M1), and co-expression of HA-NA-Gag (293HA-NA cells transfected with pAdCMV5-gagGFP). Since it is expected that VLPs will be released from the cells, a Western Blot analysis was performed to provide first evidence of VLPs presence in the supernatant. The cell lysate was also analyzed. The results showed that co-expression of HA-NA from non-transfected cells released HA in the supernatant while NA expression could not be detected (Figure 3-1C). With the combined expression of HA-NA-M1, the detection level of HA in the sucrose cushion was similar to that observed for HA-NA co-expression suggesting no positive impact on protein expression after transfection with M1. The matrix protein M1 was released from the cells, but a larger amount remained trapped within the cells. Our observations support the results obtained by (Chen *et al.*, 2007) that M1 by itself is not the driving force of influenza virus budding. The

presence of M1 in the influenza VLPs seems to influence the functionality and morphology of the VLPs rather than its production (Chen *et al.*, 2007; Thompson *et al.*, 2015). Most likely the current discrepancies in the literature are due to the fact that different expression systems and/or delivery vectors have been employed, which makes it difficult to compare the results (Chen *et al.*, 2007; Gómez-Puertas *et al.*, 2000; Li, Jaentschke*, et al.*, 2010). The expression system employed may influence the budding mechanism since the host cell provides the cellular machinery for the viral budding, and many host proteins are found in the VLPs (Cockburn *et al.*, 1969).

In contrast with M1, transfection with a gag encoding plasmid was highly efficient to mediate the release of both NA and HA from the cells. This is supported by previous work in insect cells (Haynes *et al.*, 2009). The expression of HA after HA-NA-Gag co-expression was 7-fold greater than in all previous combinations (HA-NA and HA-NA-M1). This experiment was performed in triplicate and the concentrations of HA and NA after sucrose cushion were estimated by Dot-Blot assay as shown in Table 3-1.

3.4.3 Influenza Gag-VLPs production in shake flasks and 3L-Bioreactor

The results obtained in Figure 3-1C and Table 3-1 provided evidences of VLPs production when HA, NA and Gag were co-expressed in the cells. With the goal to assess process scalability, influenza Gag-VLPs production was characterized and compared in shake flasks (50 mL working volume) and fully instrumented bioreactor (3L scale). The timeline for the production process is shown in Figure 3-2A. The experiment in shake flasks showed an increase in the fluorescence of supernatant and accumulation of HA protein following transfection/induction (Figure 3-2B). The corresponding results obtained in bioreactor are shown in Figure 3-2C. While culture performances were overall very similar at both scales, the fluorescence intensity and HA expression in the supernatant was slightly greater in the bioreactor due to better control of cell culture parameters. The transfection efficiency was found to be 81% in shake flasks and 86 % in bioreactor, as measured by flow cytometry analysis of GFP expressing cells 24 hpt (data not shown). At both scales it was observed a reduction in cell growth rate and viability after transfection/induction, probably due to a toxic effect of PEI (Ansorge *et al.*, 2009) and/or Gag protein.

Table 3-1 Summary of HA, NA and Gag quantification

Samples	Dot-Blot (µg/ml)		SRID (µg/ml)	HA assay (HAU/ml)	ELISA (ng/ml)	Based on HA assay
	HA	NA	HA	HA	p24	VLPs/ml
HA-NA	1.18 ± 0.14	0.05 ± 0.01	-	-	N/A	-
HA-NA-M1	1.19 ± 0.38	0.08 ± 0.00	-	-	N/A	-
HA-NA-Gag	7.59 ± 0.21	0.40 ± 0.02	-	-	-	-
TFF Feed	8.41	0.4	9.2	12589	28.8	2.52×10^{10}
Purified Gag-VLPs	6.05	0.16	3.4	8913	25.7	1.78×10^{10}
Purified Gag-VLPs conc.	42	1.86	30.2	-	178.4	-
Control-VLPs	N/A	N/A	N/A	N/A	100.8	N/A
H1N1 A/Puerto Rico/8/34	80	19.62	-	-	-	-

**(N/A) non-applicable*

**(-) not measured*

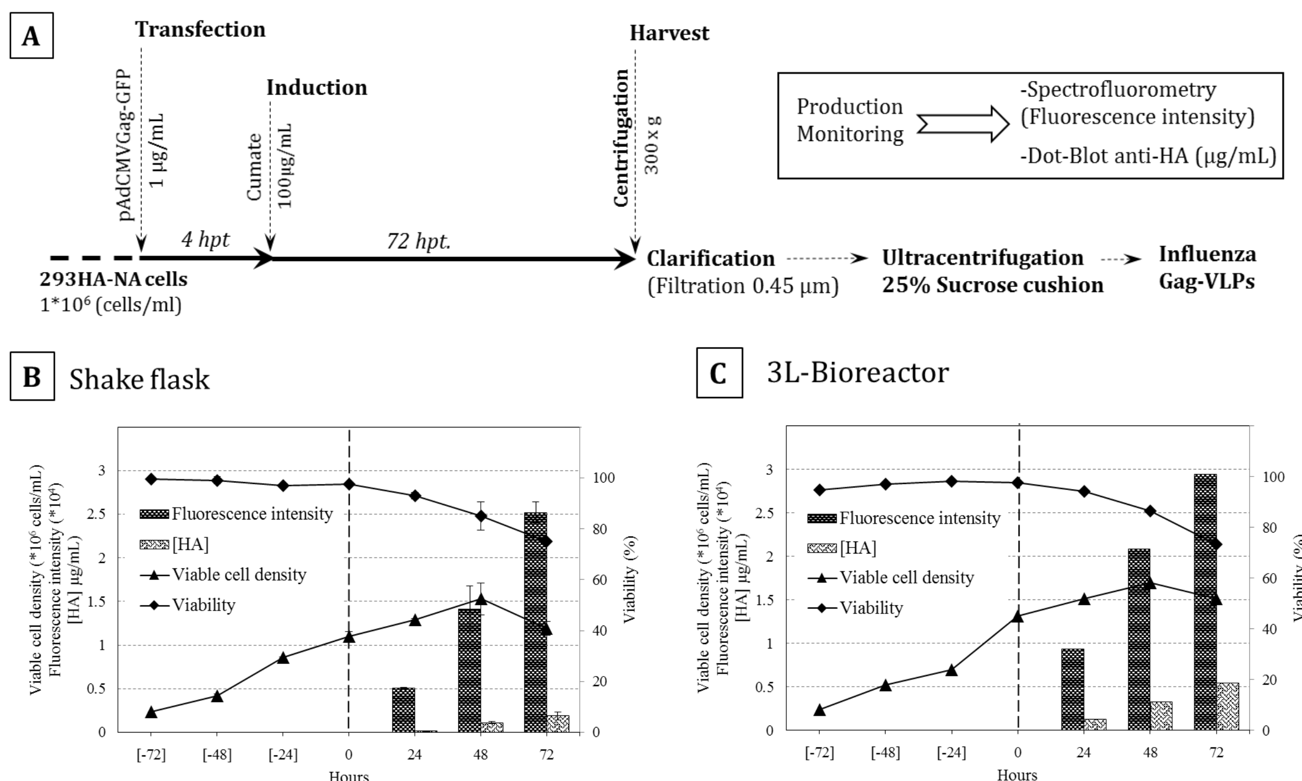

Figure 3-2 Gag-VLPs production in shake flasks and 3 L-bioreactor. (A) Diagram of the production process of influenza Gag-VLPs from 293HA-NA cells; (B) and (C) Time course of viable cell growth, cell viability, supernatant HA concentration, and GFP fluorescence intensity in shake flasks and 3 L-bioreactor, respectively. The production in shake flask was repeated twice and errors bars shown are standard deviations. The time of transfection was set as 0 h. The culture was stopped at 72 h post-transfection.

3.4.4 Tangential Flow Filtration (TFF) of influenza Gag-VLPs

The production of VLPs from mammalian cells is accompanied by extracellular vesicles that contain host cell proteins, DNA and RNA, which are undesired material in a vaccine (Barberis *et al.*, 2016; Thompson *et al.*, 2015). Therefore, purification of influenza VLPs is a critical issue to address (Vicente *et al.*, 2011). In this work, we have evaluated TFF as an important step to remove small extracellular vesicles still present in sucrose cushion sample of Gag-VLPs produced in 293HA-NA cells.

The influenza Gag-VLPs sucrose cushion from the 3L-bioreactor (Feed) was passed through an ultrafiltration cassette with 1000 KDa cut-off. The TFF feed employed was the sucrose cushion since after bioreactor production we aimed testing different purification methods while keeping

the aliquots of VLPs well-stored (data not shown). Better process scalability and higher recoveries could be obtained in the future by avoiding centrifugation steps (Morenweiser, 2005). Thus, the influenza Gag-VLPs sucrose cushion (32 ml) were diafiltered by 4 volume exchanges with a trans-membrane pressure (TMP) kept constant at 8psi. The three proteins HA, NA and Gag were clearly present in the retentate (Figure 3-3A). Interestingly, proteins with very low molecular weight were also observed in the TFF retentate suggesting that these small proteins are either part of the influenza Gag-VLPs or adsorbed to the influenza particles (Figure 3-3A SDS-PAGE).

The 25% sucrose cushion pellet from supernatant of non-transfected 293CymR-rcTA cells was observed by transmission electron microscopy (TEM) revealing the presence of very small vesicles (10-90 nm) (Figure 3-3B). This observation supports the need of more refined process to remove these contaminants from final samples. Figure 3-3C and D show the TEM images of influenza Gag-VLPs before and after TFF, respectively. The presence of vesicles clusters and/or cell membrane can be observed in the feed (Figure 3-3C). However, after ultrafiltration, the retentate recovered from TFF revealed more pure influenza Gag-VLPs by TEM (Figure 3-3D). The size of the influenza Gag-VLPs after TFF ranged from 50-220 nm. TFF using 1000 KDa cut-off membrane appears as a novel approach in the purification of HIV-Gag VLPs, since other studies have reported 300 KDa (Hatta & Kawaoka, 2003) and 500 KDa (Negrete *et al.*, 2014) cut-off.

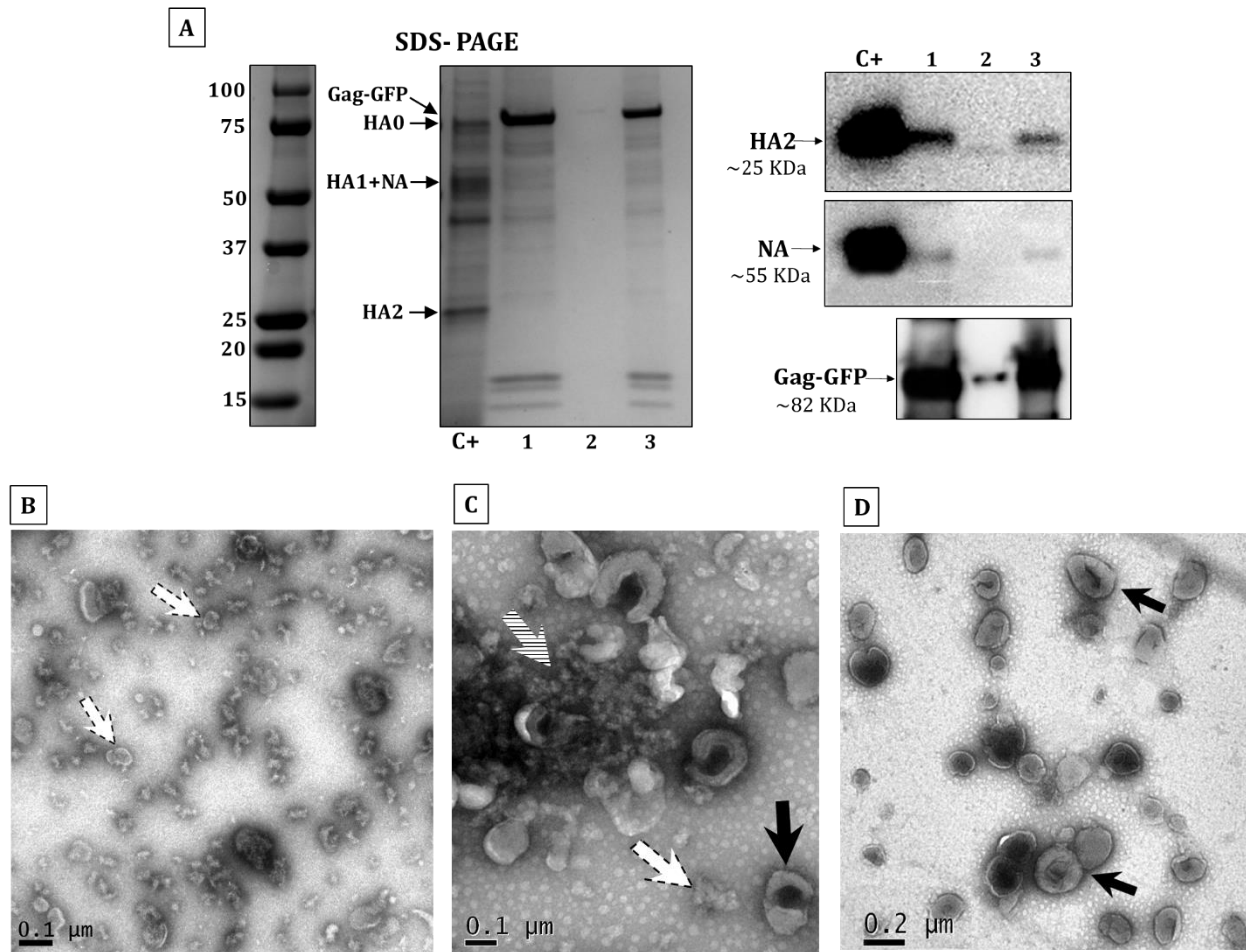

Figure 3-3 Tangential flow filtration (TFF) of influenza Gag-VLPs. (A) SDS-PAGE and Western Blots for HA, NA and Gag detection in the purification steps by TFF. Lanes are (C+) sucrose cushion Influenza virus H1N1 A/PR/8/34 produced in HEK-293 cells; (1) The feed which is sucrose cushion from supernatant of 3 L-bioreactor influenza Gag-VLPs production; (2) Permeate; (3) Retentate which is referred as “purified influenza Gag-VLPs”. The reducing agent used was DTT at 168 mM and samples were boiled for 5 min at 95◦C. The position of the relevant proteins is marked with an arrow according to their expected molecular weight and the detection by Western Blot (B) Transmission electron microscopy (TEM) Image 20,000× of 25% sucrose cushion from parental cell line 293CymR-rcTA supernatant. TEM Images 20,000× (C) Feed and (D) Purified influenza Gag-VLPs. Black arrows highlight the influenza Gag-VLPs, white arrows point to vesicles and striped arrow indicates vesicle clusters. The VLPs are identified as the particles showing the classical dense core structure and morphology of HIV, whereas the vesicles are those lacking of the dense core with more irregular shapes. A total of 10 different field images at different magnifications were taken of the purified influenza Gag-VLPs and none of them showed presence or traces of vesicles or vesicle clusters

Table 3-2 Recovery of the purification by TFF based on anti-HA Dot-Blot results

Samples	Volume (ml)	HA (µg/ml)	Total HA (µg)	Recovery (%)
Supernatant 3L-bioreactor (before clarification)	800	0.54	432	100
TFF Feed	32	8.41	269.12	62.3
Purified Gag-VLPs	32.4	6.05	196.02	45.4

3.4.5 Quantification, characterization and yield of influenza Gag-VLPs

Different methods have been described to quantify HA and NA, the main antigens of influenza virus (Thompson *et al.*, 2013). In this study, we have estimated the concentration of HA, NA and Gag proteins in our influenza Gag-VLPs before and after ultrafiltration. The concentration of HA in µg/ml was estimated by Dot-Blot, SRID and its biological activity in HAU/ml was assessed by Hemagglutination assay (Figure 3-4). NA and Gag concentrations were determined by Dot-Blot and HIV-1 p24 ELISA, respectively (Figure 3-4A). The amount of influenza Gag-VLPs/ml was estimated by correlation with the hemagglutination units. The results obtained from each quantification method are tabulated in Table 3-1. The slightly differences in concentration obtained by SRID and Dot-Blot could be simply due to intrinsic variability between the techniques or since SRID measures the antigenic conformation of HA (Li, Jaentschke*, et al.*, 2010), there might be less antigenic HA after TFF. The HA units values obtained by the HA assay (8913 HA units/ml) in our final VLPs preparation are greater compared with previous works producing influenza VLPs in mammalian and insect cells (Thompson *et al.*, 2013; Thompson *et al.*, 2015). The ratio of NA:HA on the purified Gag-VLPs estimated by Dot-Blot was approximately 1:40, while the ratio obtained for the sucrose cushion preparation of influenza virus H1N1 A/PR/8/34 produced in HEK-293 cells by using the same assay resulted in 1:4 (Table 3-1). Based on the results of HA concentration by Dot-Blot, the recovery after each step of the influenza Gag-VLPs downstream processing is shown in Table 3-2. A total HA recovery of 45% was obtained after TFF.

In order to assess the efficiency of the TFF step to remove host cell proteins (HCP) and host cell DNA, the concentration of these contaminants was measured before and after TFF. The results showed that the HCP concentration was 1.3 and 0.49 µg/ml whereas the host cell DNA was 3.7 and 2.46 µg/ml before and after TFF, respectively. These data show that 62.3% of HCP and

33.3% of DNA were removed by TFF.

Finally and with the aim to put our VLPs production process in context with other available recombinant vaccine approaches, the absolute yield and the overall timeline from the moment of receiving the sequence of the new circulating strain until the potential scaled up in a 100L bioreactor and purification has been predicted. Approximately 8 weeks would be required from receiving the DNA sequence until the final selection of the best expressing stable cell clone. Subsequently, it might take 5 weeks for cells amplification and VLPs production in a 100L bioreactor. The clarification, TFF and quantification analysis can be performed in one week. Thus, the production of the first bulk of VLPs might take approximately 14 weeks. Certainly, this time could be shortened by further optimizing the production process and by developing a better method to isolate clones (Tong *et al.*, 2013). The absolute yield of HA, by SRID, recovered by this process after TFF is 138 µg for 1L of culture volume.

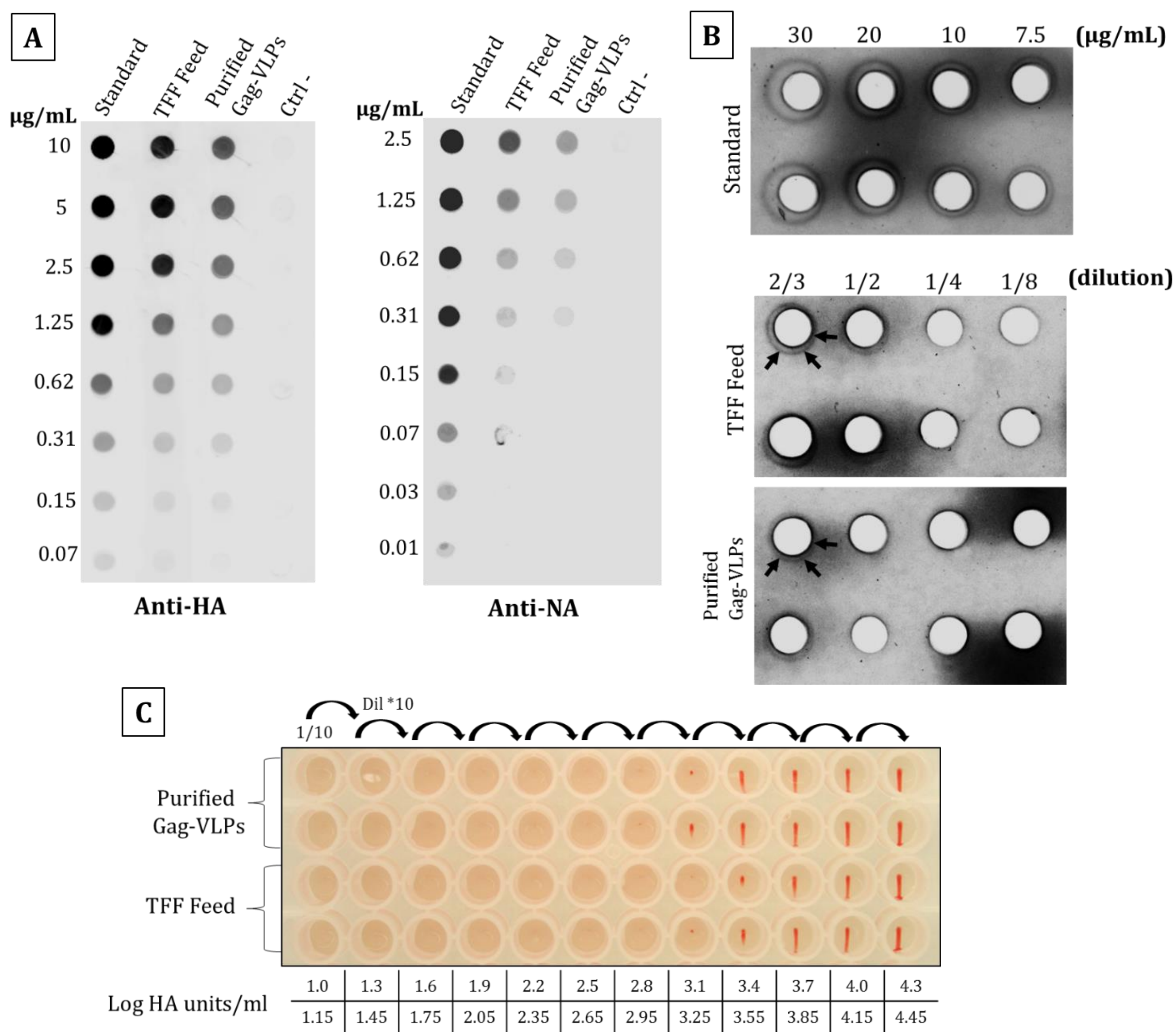

Figure 3-4 Quantification of HA and NA on influenza Gag-VLPs before and after TFF. (A) Dot-Blot using anti-HA and anti-NA antibodies. (Standard) Hemagglutinin recombinant protein from H1N1 A/Puerto Rico/8/34 (10 µg/ml top dot) and neuraminidase recombinant protein from H1N1 A/USSR/90/77 (2.5 µg/ml top dot); (Feed) sucrose cushion from supernatant of 3 L-bioreactor influenza Gag-VLPs production used as feed in the TFF, dilutions from 1/2 to 1/256 (top to bottom); (Purified Gag-VLPs) Retentate from TFF; (Ctrl −) sucrose cushion from 293CymR-rcTA cells supernatant, dilutions from 1/2 to 1/256 (vertical from top to bottom). (B) Single radial immunodiffusion (SRID) (Standard) same as for Dot-Blot anti-HA, horizontal dilutions from 30 to 7.5 µg/ml; samples horizontal dilutions from (2/3 to 1/8). The arrows are pointing to the periphery of the rings (C) Hemagglutination assay. Samples were diluted 1/10 in PBS, then sequentially diluted by 10.

3.4.6 Immunization and mice challenge protection study

The immunogenicity and protective efficacy of the influenza Gag-VLPs containing HA and NA (H1N1 subtype) were evaluated in mice. Before start the experiment, the VLPs recovered after ultrafiltration by TFF were 8X concentrated using a hollow fiber cartridge of 300 KDa pore size (purified Gag-VLPs conc.) due to volume restriction for intranasal immunization in animals. The control-VLPs were produced in shake flasks as described in Figure 3-2A but by transfecting the pAdCMV5-gagGFP in the parental cell line 293CymR-rcTA instead, such a way there is not HA and NA on the VLPs. The control-VLPs sucrose cushion sample was ultrafiltered and concentrated using 750 KDa and 300 KDa cut-off hollow fiber cartridges, respectively. The concentration of the corresponding proteins in concentrated samples was tabulated in Table 3-1.

Thus, mice were immunized following the schedule illustrated in Figure 3-5A. As shown in Figure 3-5B, HA-specific IgA were detected in the nasal lavage fluid of mice intranasally immunized with influenza Gag-VLPs while no or negligible amount of specific IgA was detected in the nasal lavage fluid of mice immunized with the control-VLPs. The fact that our VLPs induced mucosal IgA it is very significant since the respiratory tract is the natural route of influenza virus infection (Muraki & Hongo, 2010). The serum HA-specific IgA, IgG1 and IgG2a were only detected in mice immunized with influenza Gag-VLPs and the antibody levels were comparable to those in the mice immunized with rHA (Figure 3-5B). The detection of IgG1 is an indication of a T helper (Th) type 2 response which has been also observed after vaccination with rHA (Bright *et al.*, 2007). Interestingly, the vaccination with influenza Gag-VLPs induced higher titer of IgG2a than the rHA H1N1 vaccinated mice which is associated to a dominant Th1 response (Bialas *et al.*, 2014; Tong *et al.*, 2012). More importantly, the influenza Gag-VLPs immunized mice demonstrated impressive protection against a subsequent lethal intranasal challenge with the homologous virus strain in that the vaccinated mice showed little to no clinical signs or body weight loss throughout the course of infection and all the mice survived the challenge (Figure 3-5C). These results suggest that the influenza Gag-VLPs induce a potent protective immunity against lethal influenza virus challenge.

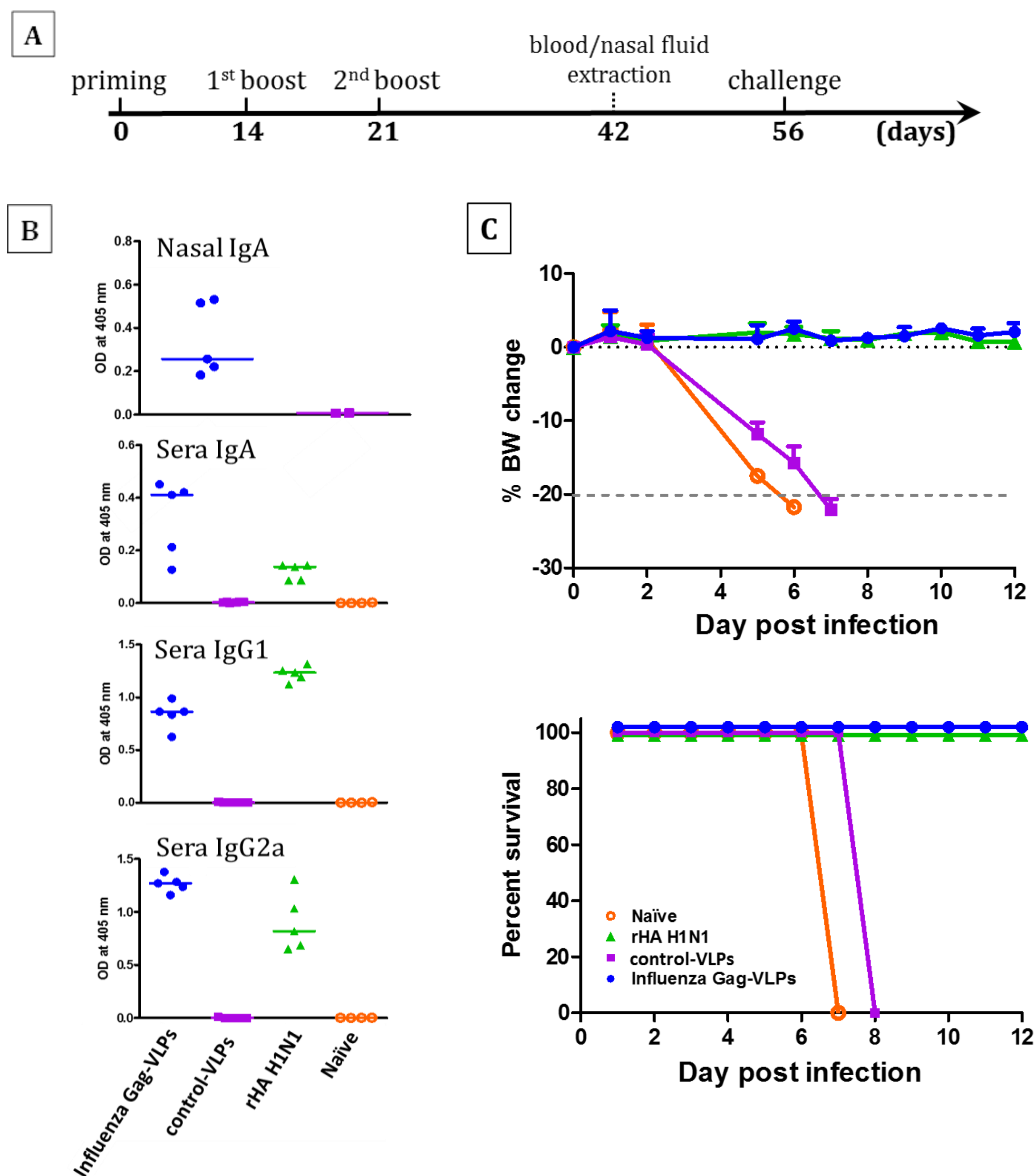

Figure 3-5 Immunity of influenza Gag-VLPs and mice challenge. (A) Vaccination schedule. (B) Sera IgG and/or nasal IgA specific to VLPs were evaluated by ELISA. At day 42, the nasal fluid and blood from sacrificed mice of groups immunized with (2 µg HA) influenza Gag-VLPs and control-VLPs were analyzed for nasal IgA and sera IgA, IgG1, and IgG2a response. Non-terminal blood samples from 2 µg rHA H1N1-immunized and naïve mice were assayed for HA-specific IgG sera antibodies. Each individual dot represents the value from a single mouse, and the horizontal line represents the median value for the group. The concentration of HA used per dose was based on the Dot-Blot assay results (C) Five weeks after last immunization, mice of all groups were intranasally challenged with 10^3 pfu of the mouse-adapted influenza H1N1 virus

(A/Puerto Rico/8/34). The percentages of body weight (BW) changes and survival were daily recorded for up to 12 days. Body weight data are the means for five mice per group ± SD.

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CHAPTER 4 ARTICLE 2: PROCESS INTENSIFICATION FOR HIGH YIELD EXPRESSION OF INFLUENZA H1N1 GAG VIRUS-LIKE PARTICLES USING AN INDUCIBLE HEK-293 PRODUCING CELL LINE

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4.1 Abstract

Influenza virus dominant antigens presentation using Virus like particle (VLP) platform is attractive for the development of new generation of influenza vaccines. Mammalian cell platform offers many advantages for VLPs production. However, limited attention has been paid to the processing of VLPs produced in mammalian cells. Better understanding of the production system could contribute to increasing the productivity and making large-scale VLPs vaccine manufacturing feasible. In a previous study, we have generated a human embryonic kidney HEK-293 inducible cell line expressing Hemagglutinin (HA) and Neuraminidase (NA), which was used to produce VLPs upon transient transfection with a plasmid containing HIV-1 Gag. In this work, to streamline the production process, we have developed a new inducible cell line expressing the three proteins HA, NA and the Gag fused to GFP for monitoring the VLPs production in suspension and serum-free medium cultures. The process was optimized to reach high volumetric yield of VLPs by increasing the cell density at the time of induction without sacrificing the cell specific productivity. A 5-fold improvement was achieved by doing media

evaluation at small scale. Furthermore, a 3-L perfusion bioreactor mirrored the performance of small-scale shake flask cultures with sequential medium replacement. The cell density was increased to $14x10^6$ cells/ml at the time of induction which augmented by 60-fold the volumetric yield to $1.54x10^{10}$ Gag-GFP fluorescent events/ml, as measured by flow cytometry. The 9.5-L harvest from the perfusion bioreactor was concentrated by tangential flow filtration at low shear forces. The electron micrographs revealed the presence of VLPs of 100-150 nm with the characteristic dense core of HIV-1 particles. The developed process shows the feasibility of producing high quantity of influenza VLPs from an inducible mammalian stable cell line aiming at large scale vaccine manufacturing.

Keywords: Stable cell line; Process development; Perfusion; TFF; Gag-VLPs; Influenza vaccine

4.2 Introduction

Well-assembled virus-like particles (VLPs) are highly attractive as vaccine candidates. The advantages of VLPs include: mimicry of the particulate structure of wild type viruses, absence of viral genome, and the repetitive display of major antigens on their surface while keeping the intact conformation (Haynes *et al.*, 2009; Roldão, Mellado *et al.*, 2010; Zeltins, 2013). An important step when proposing these nanoparticles as vaccine candidates is to demonstrate their immunogenicity and efficacy in preclinical *in vivo* experiments. Once the proof of concept is demonstrated, more efforts should be deployed to the establishment of a reproducible scalable production process that can support the vaccine candidate characterization for further preclinical and clinical studies (Effio & Hubbuch, 2015; Lua *et al.*, 2014; Pattenden *et al.*, 2005).

The production of VLPs from mammalian cells has been commonly achieved via transient transfection or stable expression of the corresponding recombinant gene/s (Carter *et al.*, 2016; Chen *et al.*, 2007; Robert *et al.*, 2016; Venereo-Sanchez, Gilbert *et al.*, 2016; Wu *et al.*, 2010). Developing a process based on transient transfection can be cumbersome for vaccine manufacturing at large scale. Transfection agents like PEI are toxic for the cells (Ansorge *et al.*, 2009), a complex bioreactor set-up is needed (one extra closed vessel for making the DNA:transfection agent polyplexes) and adequate plasmid stocks must be maintained. Meanwhile, stable cell line development has gained substantial progress with a significant reduction in the required time to obtain stable clones supported by robotic clone isolation (Caron,

Nicolas *et al.*, 2009) and powerful flow cytometry cell sorters (DeMaria, Cairns *et al.*, 2007). Once the cell line is established, it can be optimized for efficient protein expression, eliminating the drawbacks associated with methods relying on transient transfection.

A review of the state-of-the-art on the optimization of virus production reveals that driving the cultures to high cell densities at the time of infection has the potential to significantly improve the virus volumetric yield (Cortin, Thibault *et al.*, 2004; Ghani, Garnier *et al.*, 2006; Henry *et al.*, 2004; Petiot *et al.*, 2011). Similar scenario could be applied to stable cell lines producing VLPs under an inducible system in which the growth and production phases are separated. This approach of inducible expression seems very feasible for the production of enveloped VLPs, where more than one protein are often needed for the assembly. Accordingly, the cell biomass can be augmented to reach the targeted cell density at the time of induction without adding the extra metabolic burden imposed by heterologous protein expression. Perfusion culture process has been efficiently used to increase cell densities and productivities in mammalian cell cultures (Henry *et al.*, 2004; Petiot *et al.*, 2011). The perfusion mode allows to increase cell densities and extend culture times by continuously exchanging the culture medium with fresh one, and continuously removing by-products that might be toxic to the cells or inhibit the product formation. Perfusion process is very efficient in preserving quality and stability of products that may be negatively impacted if staying too long in the culture environment. However, in virus production, several studies have reported a decrease in the cell specific productivity at high cell densities (Maranga, Brazão *et al.*, 2003; Nadeau & Kamen, 2003; Tapia *et al.*, 2016).

The challenges associated to the bioprocess engineering of influenza VLPs production from mammalian cells have been far less studied. Wu *et al.* (2010) studied the production of influenza VLPs HA-NA-M1-M2 from Vero cells in a 3L-bioreactor using microcarriers while Thompson *et al.* (2015) reported the production of influenza VLPs from HEK-293 suspension cells transduced with a BacMan-HA-NA-M1 also in a 3L-bioreactor but no further process optimization was performed. Likewise, the production of HIV Gag-VLPs from HEK-293 cells was investigated using transient transfection. The productivity was increased by 2.4 fold using an optimized production protocol based on the use of a supplemented Freestyle medium and by performing medium exchange prior to transfection (Cervera *et al.*, 2013).

In previous work, we have characterized the production of influenza VLPs from a HEK-293HA-NA stable cell line after transient transfection with a Gag-containing plasmid (Venereo-Sanchez *et al.*, 2016). In the present study, to avoid the drawbacks related to transient transfection at large scale, we investigated the efficacy of a new HEK-293 stable cell line expressing the three main proteins involved in the formation of VLPs containing HA, NA and Gag-GFP, hereafter referred as "293-HA/NA/Gag-GFP". The production process from the stable cell line was developed to achieve higher yields. Media evaluation, sequential medium replacement at small scale and a perfusion strategy in bioreactor were implemented to drive the cultures to higher cell densities at the time of induction and improve the volumetric yield. The VLPs produced from the perfusion bioreactor were semi-purified and concentrated by Tangential Flow Filtration (TFF). The VLPs were quantified and characterized by transmission electron microscopy (TEM), single radial immunodiffusion (SRID), Dot-Blot, and hemagglutination assay. To our knowledge this is the first work describing the bioprocess development of influenza VLPs production in suspension culture of inducible mammalian cells.

4.3 Materials and Methods

4.3.1 Cells, plasmids and culture conditions

The cells were cultured in a humidified incubator at 37 ºC with 5% CO_2, at an agitation rate of 100–110 rpm. The medium was supplemented with 4–6 mM of glutamine during cell maintenance. For the construction of the plasmid pKCR5-Gag-GFP, the gag-GFP gene was enzymatically isolated from the plasmid pAdCMV5-gagGFP (Venereo-Sanchez *et al.*, 2016) by digestion with Pme I and BamH I. Then, the gag-GFP gene was blunted and inserted in the plasmid pKCR5-3CuO-GFP (Mullick *et al.*, 2006) previously digested with the restriction enzymes Age I and EcoR I.

4.3.2 Generation of 293-HA/NA/Gag-GFP stable cell line

The 293-HA/NA/Gag-GFP cells were generated by transfecting $1x10^6$ cells/ml of 293HA-NA cells (Venereo-Sanchez *et al.*, 2016) with the XbaI-linearized plasmid pKCR5-GagGFP and a plasmid encoding the resistance to G418, pMPG/tk-Neo/CMV-CymR linearized with AscI to remove the fragment containing the promoter and CymR. The transfection was performed in a

six-wells plate with a total DNA of 2.5µg/well, 1.9:0.6 µg respectively, using PEIpro (Polyplus transfection, France) at a DNA:PEI ratio of 1:1. The selection with G418 sulfate (geneticin selectin, ThermoFisher Scientific, USA) was applied 48 hours post transfection at 200µg/ml after passing the cells to two plates of 96-well seeded at $1x10^4$ cells/well. After 4-5 weeks under selection, 48 clones were isolated, induced with 100µg/ml of cumate and screened by flow cytometry for GFP and HA positive cells. The five pools showing the highest levels of HA and Gag-GFP expression were subjected to limiting dilution to isolate single clones. The cells were seeded in five 96-well plates at three different cell densities: 0.3, 1 and 3 cells/well. The plates were incubated until the clones reach 60-80 % of confluence. During this period 50 µL of medium was added twice. Three to four weeks later, subclones from plates seeded at 0.3 and 1 cell/well were transferred in 24-well plates and tested for GFP and HA positive cells. The best 8 subclones were expanded in 25 cm^2 flasks and then cultivated in 125 ml erlenmeyer flasks. The production of VLPs was assessed in sucrose cushion preparations by detecting HA, NA and Gag proteins by Western Blot.

4.3.3 Flow cytometry

4.3.3.1 Cells

The cells at 72 hours post-induction (hpi), including the corresponding positive and negative controls, were slowly re-suspended and transferred from a 96-flat bottom plate to a 96-V bottom plate. The cells were fixed by adding formaldehyde 10% to a final concentration of 2% under agitation. After 20 min incubation under agitation, the cells were washed with cold PBS and permeabilized with ethanol (70% final concentration); following two consecutive washes with cold PBS. The cells were incubated with blocking buffer for 15 min (4% Fetal Bovine Serum in PBS). The primary antibody anti-HA ref: 03/242 (NIBSC, UK) was added at a final dilution of 1:50 and incubated 30 min at room temperature. Two washes with cold PBS were done after each antibody incubation. Then, the secondary antibody Donkey anti-sheep Alexa Fluor 674 (ab150179, Abcam, UK) at 1:2000 dilution was added, followed by an incubation time of 15 min in the dark. The cells were resuspended in blocking buffer, transferred to a 96-filter plate (Multiscreen 60µm Nylon Mesh, EMD Millipore), and spun at 230 x g for 1 min at room temperature. The cells were transferred to 96-V bottom plates for the analysis by Fluorescence-

activated cell sorting (FACS) in the BD LSRFortessa-HTS, Biosciences (Becton-Dickinson, USA).

4.3.3.2 Gag-GFP events count by flow cytometry

The Gag-GFP fluorescent events per ml were quantified by detecting GFP green fluorescence and light scattering signals. The analysis of the samples was done using a BD LSR-Fortessa SORP flow cytometry system (Becton-Dickinson, USA) equipped with a 300mW 488nm laser and a Forward Scatter photomultiplier tube (PMT) detector. The volume passed in a given time was calculated using Becton-Dickinson Trucount^{TM} Tubes (Becton-Dickinson cat nb: 340334) of 52250 beads per tube. Based on 20 sec sample aspiration, the GFP fluorescent events counts were determined as the number of events per ml. Shero^{TM} Nano Fluorescent particles (Spherotech Inc.USA) from 0.13 to 0.88 µm were used to choose the adequate instrument settings.

4.3.4 Perfusion Bioreactor

A 3L Chemap CF-3000 bioreactor (Mannedorf, Switzerland) was set-up as previously published in (Henry *et al.*, 2004). The cells were inoculated in the bioreactor at around 0.3×10^6 cells/ml. The temperature, pH, dissolved oxygen and agitation were controlled at 37 ºC, 7.2, 40% and 90 rpm, respectively. The pH was regulated by addition of 7.5% w/v $NaHCO_3$ solution. Pure O_2 was sparged from day 4 of culture when surface aeration was not sufficient. A cooling system was installed to maintain the VLPs-containing harvest bottle at 4 ºC during the collecting time. Each day, the harvest bottle was replaced and kept at 4 ºC in the refrigerator for subsequent downstream processing.

4.3.5 VLP proteins quantification and characterization

The Dot-Blot, SRID, Hemagglutination assay, Western Blot, determination of host cell proteins, host cell DNA and TEM techniques were performed under the same conditions published in (Venereo-Sanchez *et al.*, 2016).

4.3.6 Clarification and Tangential flow filtration (TFF)

The VLPs-containing supernatant collected from the bioreactor was pre-filtered through a 78 mm fiberglass pre-filter and then filtered through a 0.45 µm, 1000 ml funnel filter (Corning Inc., USA). The TFF was set-up using two P2B01MC01 | Pellicon® 2 Mini Ultrafiltration Modules Biomax®- 1000 KDa 0.1 m^2 supported with the Pellicon 88cm^2 and 0.11m^2 cassette holder (EMD Millipore, USA). The ultrafiltration was performed by circulating the feed through the TFF membranes using a Masterflex I/P pump (Easy-Load model 77601-10) and a tubing Masterflex platinum-cured silicone 16 I/P. Two manometers (Anderson-Negele, Germany) were connected to the system to monitor the transmembrane pressure. The average flux across the membrane was estimated to be 310 L/m^2/hour (LMH).

The ultrafiltration and diafiltration of the supernatants collected from the shake flasks under medium replacement were performed using a hollow fiber cartridge (D02-E750-05-N, Spectrum Laboratories, USA).

4.4 Results and Discussion

4.4.1 Stable cell line development

To develop the 293-HA/NA/Gag-GFP cell line, that stably expresses HA, NA and Gag fused to the GFP, the gene Gag-GFP was cloned into a plasmid containing the cumate inducible CR5 promoter. The resulting plasmid (pKCR5-GagGFP) was co-transfected with a plasmid containing the resistance to neomycin in the cell line 293HA-NA (Venereo-Sanchez *et al.*, 2016). After selection, the neomycin resistant clones were screened by detecting Gag-GFP and HA by flow cytometry (Figure 4-1A). Different patterns were observed in the isolated clones; clones made up of cells expressing only Gag-GFP, only HA, or expressing both proteins. The eight subclones with the highest expression of both proteins were selected, cultivated in shake flasks (25 ml working volume) and induced with cumate for VLPs production. The subclone displaying the highest simultaneous expression of HA and Gag and with good cell growth characteristics (data not shown) was selected as our cell line 293-HA/NA/Gag-GFP. The presence of HA, NA and Gag-GFP in the chosen clone was confirmed by Western Blot analysis (Figure 4-1B).

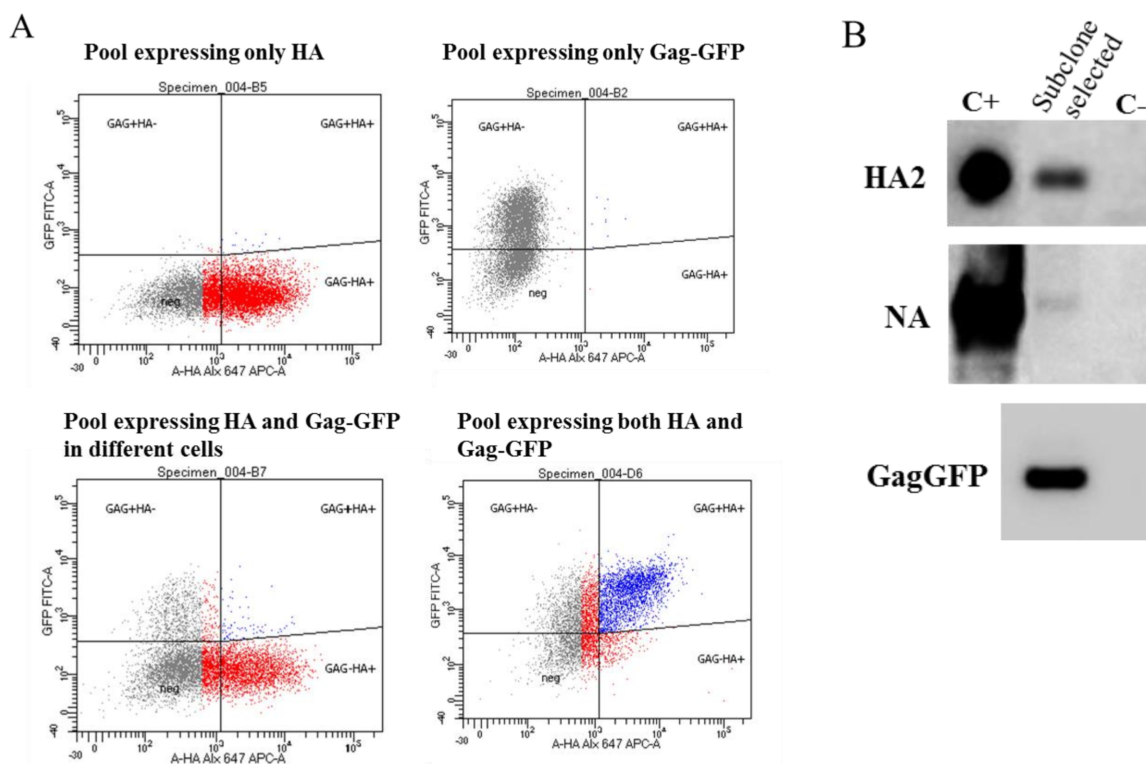

Figure 4-1 Development of stable cell line 293-HA/NA/Gag-GFP. A) Different expression patterns were observed in the clones. **B)** Western blot analysis. Each lane contains the sucrose cushion preparation of: (C+) influenza virus H1N1 A/PR/8/34 produced in HEK-293 cells; (Subclone selected) VLPs produced after induction of the highest producer stable clone 293-HA/NA/Gag and (C-) the supernatant of parental cell line 293CymR-rcTA.

4.4.2 Media evaluation at small scale

Once the highest-producer cell subclone was selected, process development efforts were undertaken with the aim to improve the volumetric yield of VLPs by increasing the cell density at the time of induction without sacrificing the cell specific productivity. Firstly, two different commercial media: SFM4TransFx-293 and HyCell^{TM}TransFx-H were evaluated in shake flasks (25 ml working volume) for VLPs production (Figure 4-2A). By using HyCell^{TM}TransFx-H medium it was possible to induce the cells 293-HA/NA/Gag-GFP at a higher cell density (~3 x 10^6 cells/ml) than when cells were cultured with SFM4-TransFx medium (~1 x 10^6 cells/ml). Cell viability was greater than 90% at the time of induction for both conditions.

The VLPs production was monitored by measuring the expression of HA via Dot-Blot and the quantity of Gag-GFP fluorescent events by flow cytometry. The results of production kinetics on supernatants revealed that the best production of Gag-GFP and HA was attained with HyCell-TransFx-H medium, $5x10^7$ Gag-GFP fluorescent events/ml and 0.3 µg/ml of HA (Figure 4-2B). It can be seen that after 48 hpi, the Gag-GFP fluorescent events count did not increase significantly. However, in the case of HA, the maximum quantity produced was at 72 hpi. The harvested supernatants from all conditions were 50X concentrated through a 25% sucrose cushion and the concentration values are shown in Figure 4-2C. The yields after concentration showed a production improvement of 5.7-fold in terms of Gag-GFP fluorescent events/ml and 4.7-fold for HA concentration compared to the culture with SFM4TransFx. For following experiments, we considered the batch production in SFM4Transfx as our reference control condition since our previous work (Venereo-Sanchez *et al.*, 2016) was run under the same conditions.

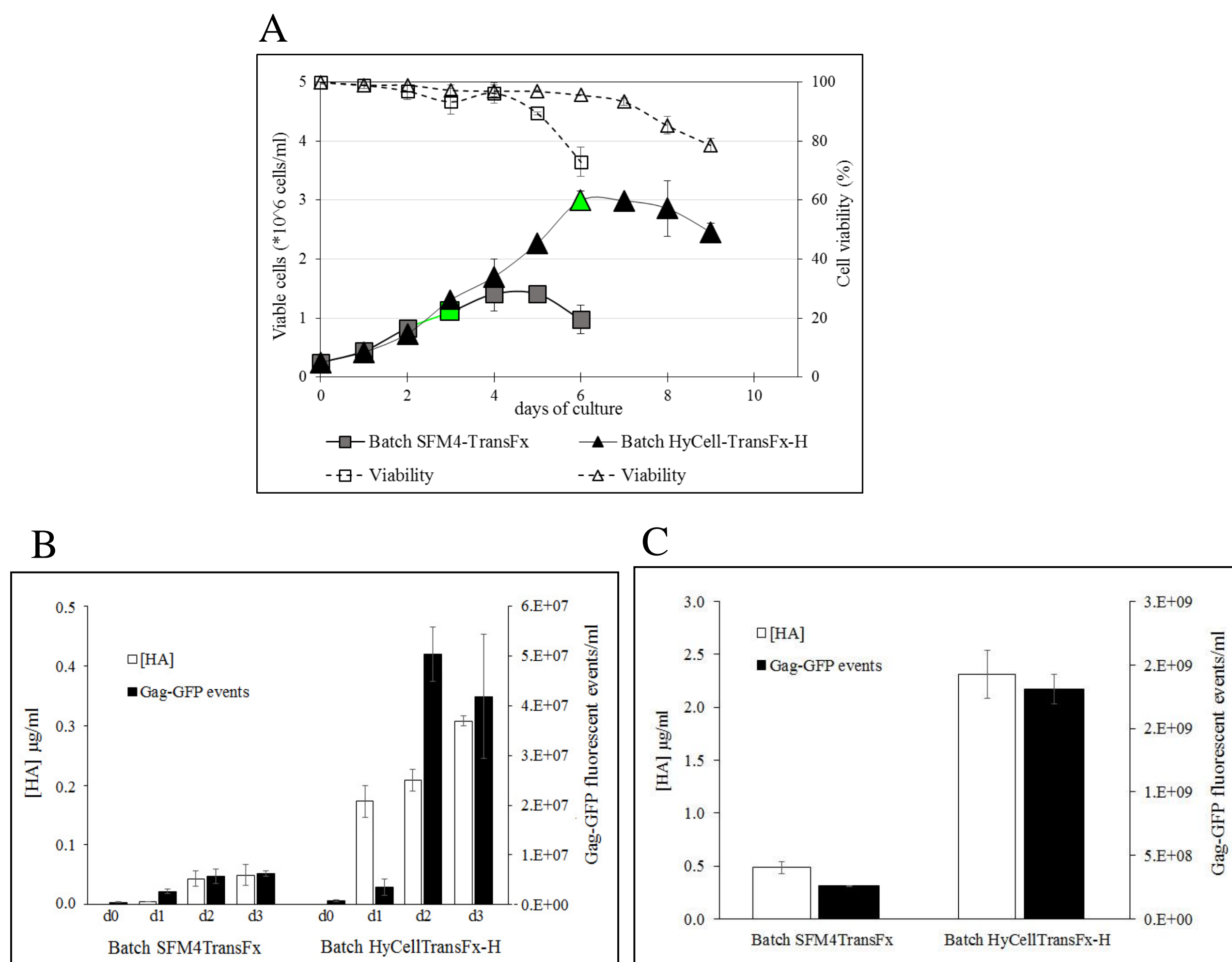

Figure 4-2 Media evaluation at small scale. A) Cell growth curves using 293-SFM4TransFx and HyCell-TransFx-H media. The production of VLPs was monitored in B) supernatants and C) 50X sucrose cushion concentrated harvests by the count of Gag-GFP fluorescent events/ml measured by flow cytometry and the concentration of HA (µg/ml) by Dot-Blot. The green marker indicates the day of induction. The error bars indicate the standard deviation for two replicates.

4.4.3 Medium replacement at small scale

As an attempt to increase further the volumetric yield of VLPs by increasing the cell density at the time of induction, a medium replacement (MR) strategy was explored at small scale. This constitutes a simple way to mimic the perfusion mode. Two experimental conditions were considered: 1) medium exchange was done on a daily basis during the growth phase but not after induction (no MR post-induction) and 2) medium replacement was performed throughout the

growth and production phases (MR post-induction). The cultures were performed in shake flasks (50 ml working volume) and consisted in exchanging 75 % of the culture volume with fresh medium every day. The growth curves corresponding to the two strategies are shown in Figure 4-3A. The cultures reached much higher cell densities with medium replacement (~14-18x10^6 cells/ml) in comparison with the corresponding batch culture (~3x10^6 cells/ml) (Figure 4-2A). It can be observed that, when medium exchange was maintained after induction, the cell growth and viability declined at 24 hpi while for the condition with no medium exchange post-induction, cells continued to grow and cell viability decreased more slowly. This is probably due to a shear stress effect on the cells resulting from the centrifugation steps, since all the culture parameters were identical (e.g. working volume, temperature, agitation rate, etc.).

The production in supernatant was monitored every 24 hpi (Figure 4-3B). The concentrations of HA and Gag-GFP fluorescent events in the supernatant are given as total cumulative values to consider the total volumetric yield per day. The Gag-GFP fluorescent events count increased after induction up to ~9.6x10^{10} at 96 hpi when medium replacement was continued during the production phase, compared to a maximum of 3.6x10^{10} at 72 hpi when feeding was restricted to the growth phase. Likely, the expression of HA continuously increased after induction for both conditions up to 93.2 and 25 µg total, respectively. Hence, the reduction in cell viability observed during the production phase does not seem to have a negative effect on productivity.

The VLPs-containing harvest collected from both conditions were 50X concentrated by TFF using a hollow fiber of 750KDa cut-off. The total amount of HA protein (µg) and Gag-GFP fluorescent events produced in “MR post-induction” strategy improved by 2.9-fold and 2.2-fold, respectively, in comparison with the “no MR post-induction” condition (Figure 4-3C). Compared to the control batch SFM4TransFx, a volumetric yield improvement of 128 fold (Gag-GFP events/L) and 17 fold for HA was achieved in the condition where medium exchange was maintained after induction (Table 4-1). The differences between the increasing fold factor for each gene, HA and Gag-GFP, could be due to the random integration of the genes in the cell chromosomes, which can lead to different expression levels of the protein of interest. The transgenes can be inserted in zones of the chromatin more or less active and/or the insertion of “multiple gene copies can unpredictably cross-interact” (Li, Vijayasankaran *et al.*, 2010) influencing the protein expression (Wurm, 2004).

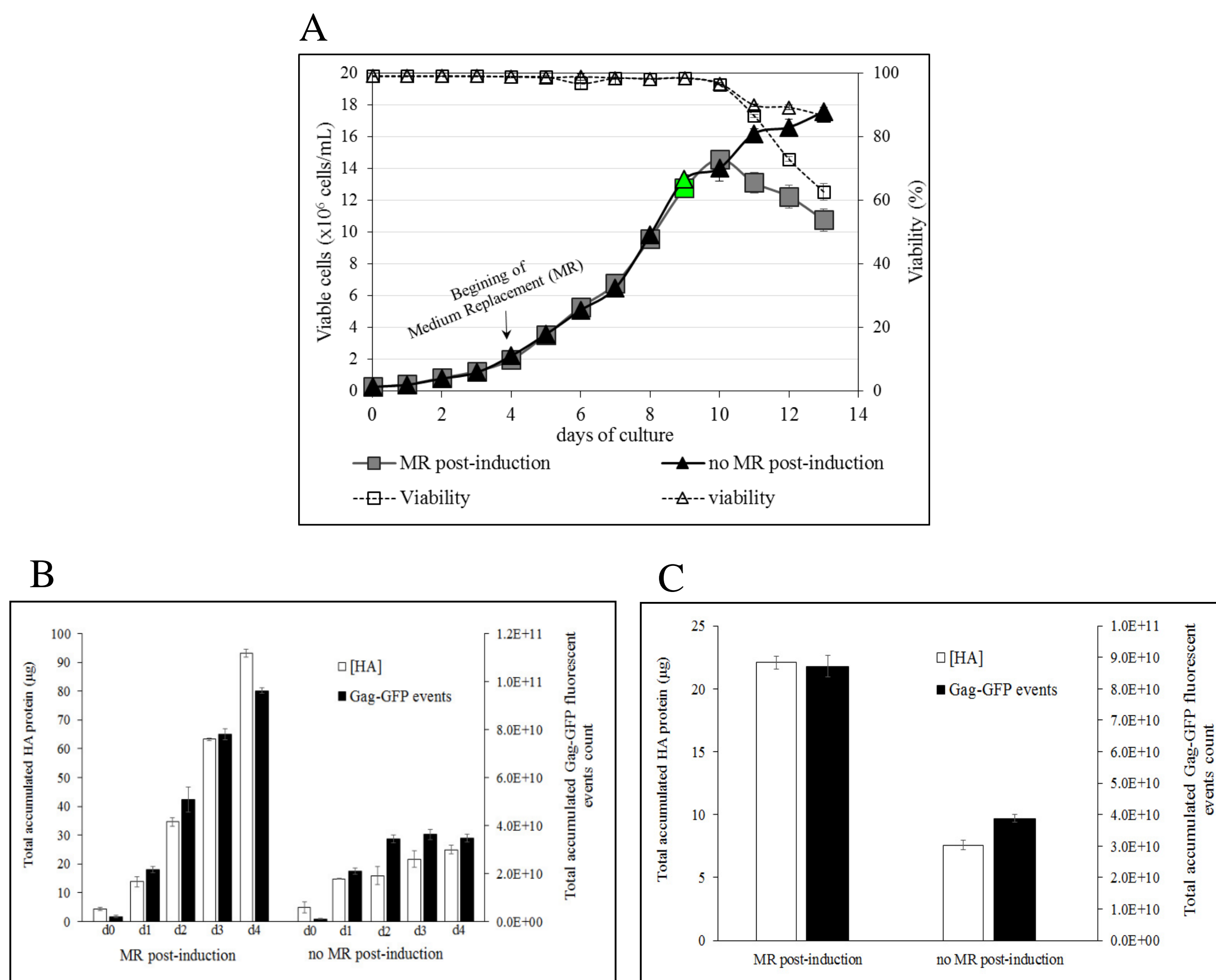

Figure 4-3 Production of VLPs in cultures with medium replacement (MR) at small scale A) Cell growth curves in medium HyCell-TransFx-H under two conditions: "MR post-induction" (the medium was exchanged from day 4 until the end of the culture) and "no MR post-induction" (the medium replacement was done on a daily basis from day 4 to 9 but not after induction). The MR consisted in an exchange of 75% of the culture volume with fresh medium by centrifugation at 300 xg 5min. The green marker indicates the time of induction. The amount of VLPs proteins in B) cell supernatant of days post-induction and C) 50X TFF concentrated harvests was monitored by flow cytometry and by Dot-Blot to report total Gag-GFP events count and total µg of HA produced. The error bars indicate the standard deviation for two replicates.

Table 4-1 Specific productivity, specific growth rate and productivity improvement factor from batch to perfusion

	Batch SFM4-TFx	Batch HyCell-TFx	MR post-induction	Perfusion bioreactor
Cell specific productivity (Gag-GFP events/cell based on viable cell density at the time of induction)	5	12	136	22
Cell specific productivity (µg of HA/cell based on viable cell density at the time of induction)	$8.9x10^{-9}$	$1.5x10^{-8}$	$3.5x10^{-8}$	$1.2*10^{-8}$
Cell specific growth rate before induction (h^{-1})	0.015	0.017	0.018	0.019
Volumetric yield (Gag-GFP events/L of culture)	$5.2x10^{9}$	$3.6x10^{10}$	$6.6x10^{11}$	$3.1x10^{11}$
Volumetric yield (HA µg/L of culture)	10	46	167	165
Volumetric yield improvement factor in comparison with the Batch control (for Gag-GFP events)	1	~7	~128	~60
Volumetric yield improvement factor in comparison with the Batch control (for µg of HA)	1	~5	~17	~17

4.4.4 Perfusion Bioreactor

Considering the significant improvements achieved using the medium replacement strategy at small scale, we aimed to operate a laboratory-scale bioreactor operated in perfusion mode with an acoustic filter. The perfusion rate was started on day 4 of the culture and was kept at a flow rate of 2L/day (1.3vol/day). The perfusion rate was maintained after the induction. The bioreactor culture reached similar cell densities compared to the medium replacement at small scale and the induction was also done at $14*10^{6}$ cells/ml (Figure 4-4A). The production in the supernatants reached a maximum of $6x10^{12}$ Gag-GFP fluorescent events and around 4 mg of HA at 96 hpi (Figure 4-4B). The total volume harvested from the bioreactor was around 9.5 L. This material was 50X concentrated by TFF as described in the following section. By doing perfusion in the bioreactor we could mirror the production obtained at small scale with medium replacement. Considering the concentration values obtained in the TFF semi-purified final material (Table 4-2) the volumetric yield was increased by 60-fold in terms of Gag-GFP events count and 17-fold for

μf of HA, with respect to the initial batch SFM4-TransFx condition. The higher productivity in terms of Gag-GFP events obtained in the medium replacement at small scale compared to the perfusion bioreactor might be due to the release of intracellular Gag-GFP as a result of the decrease in viability caused by the centrifugation steps to exchange the medium. The specific productivity estimated revealed an improvement of 4-fold (Gag-GFP events) in the perfusion process compared to the Batch-SFM4TransFx control (Table 4-1).

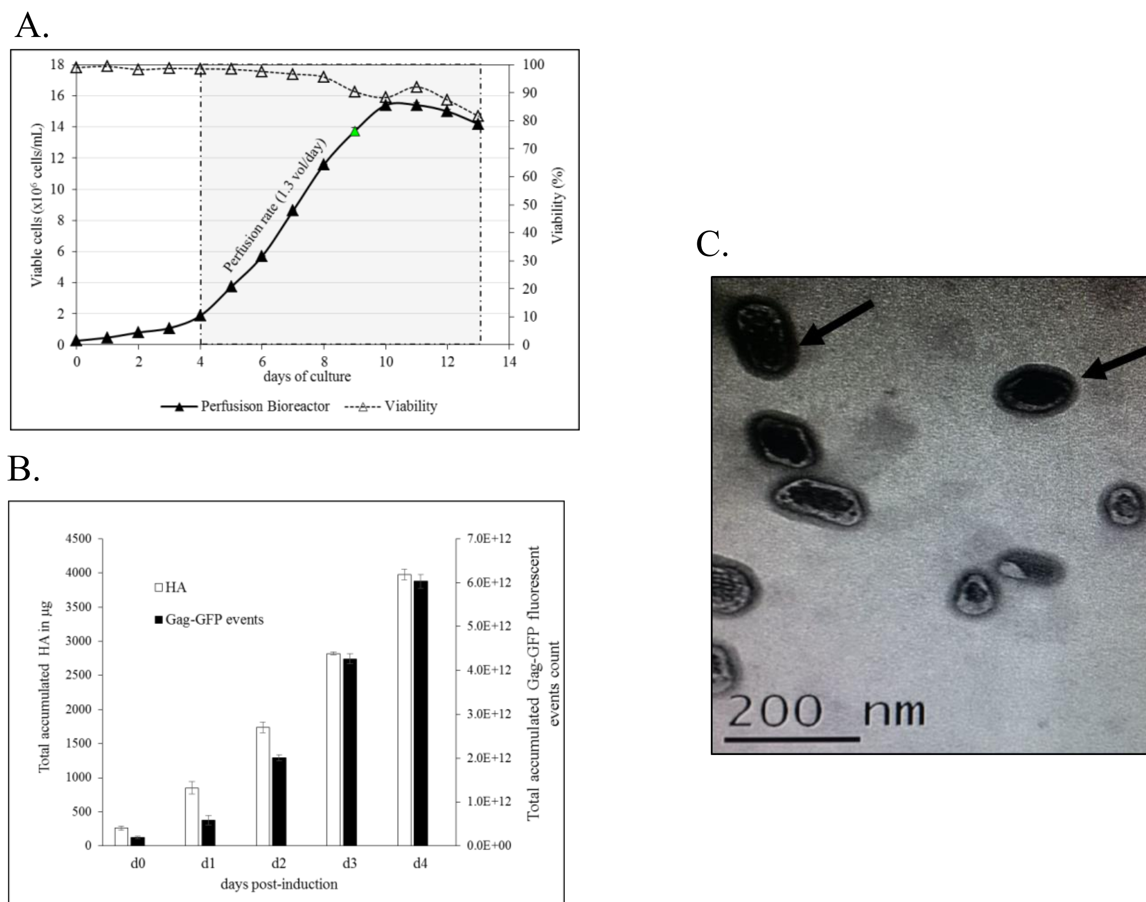

Figure 4-4 Production of VLPs in a perfusion mode 3L-bioreactor. A) Viable cell growth. The grey region indicates the continuous perfusion period at a rate of 1.3 vol/day. B) The production of VLPs was analyzed in cell supernatant at days post-induction by doing flow cytometry to measure Gag-GFP events and by Dot-Blot to monitor HA protein expression. The error bars indicate the standard deviation for three measurements. The values measured for the 50X TFF concentrated harvest are reported in Table 3-2 along with the recovery yields. C) Negative stained electron microscopy of VLPs recovered in the TFF retentate after diafiltration.

4.4.5 Tangential Flow Filtration (TFF)

The TFF is a gentle method to concentrate and semi-purify VLPs and viruses based on the size of the particles. We have shown in a previous study the efficiency of this method to remove cell debris and small extracellular vesicles from the sucrose cushion semi-purified material (Venereo-Sanchez *et al.*, 2016). However, having centrifugation steps during the downstream processing is not desirable due to the limitations and cost associated with scale-up. In this study, since the VLPs-containing harvest was collected after an acoustic filter separation system, very low amount of cells passed through the separation device ($0.15x10^6$ cells/ml). Therefore, no centrifugation steps were needed prior to TFF for most of the harvest (only the residual 2.6L left in the bioreactor were centrifuged to separate cells from the supernatant). Thus, the whole harvest supernatant of 9.5 L was pre-filtered through a fiber-glass to remove the cells and cell debris remaining in the harvest. Thereafter, another filtration through a 0.45 µm filter was performed and this clarified material was the feed (9.3L) for the TFF system.

Since enveloped viruses and VLPs are sensitive to shear stress, an important aspect to consider for TFF is to maintain low shear rate conditions during the run. Based on prior knowledge (Negrete *et al.*, 2014) and experience accumulated in our lab on ultrafiltration of enveloped viruses, a shear force lower than 4000 s^{-1} does not cause significant damage on the viral particles. Thus, the TFF to concentrate the 9.3 L VLPs-containing harvest was set-up using two cassettes of 0.1 m^2, with a cross flow rate of 1.2 L/min at a flux of 310 LMH. Under such conditions, the expected shear force is around 2000 s^{-1}. The schematic representation of the TFF set-up together with the permeate flow rate during the run is shown in Figure 4-5A, B. The overall recovery of the process is shown in Table 4-2. The higher loss observed, in terms of Gag-GFP events compared to HA after the clarification steps could be probably due to that some VLPs, exosomes containing Gag-GFP and/or Gag-GFP aggregates, which are bigger in size than HA protein, were retained in the fiber glass paper and/or the 0.45µm filter, while the HA free protein passed through. Actually, it can be observed that, after TFF, the loss in terms of Gag-GFP (5%) in comparison with HA protein (32%) was significantly lower because the cut-off used was 1000 KDa, which allows the free HA protein to pass through the membrane to the permeate. We have compared the Gag-GFP fluorescent events value obtained by flow cytometry in the final material recovered after TFF ($1.54*10^{10}$) with the VLPs/ml calculation from the HA units value by

hemagglutination assay of the same sample ($1.78*10^{10}$) and similar values were obtained. However, we chose to describe the flow cytometry data as Gag-GFP fluorescent events/ml instead of VLPs/ml, since small GFP positive extracellular vesicles or protein aggregates could interfere with the measurement.

The retentate VLPs were observed by transmission electron microscopy where particles of 100-150 µm with a typical dense core were identified as the influenza Gag-VLPs (Figure 4-4C). The VLPs semi-purified and concentrated by TFF were also quantified by Single Radial Immunodifusion (SRID) and hemagglutination assay revealing a concentration of 9.9 µg/ml of HA and 8913HAU/ml, respectively. The concentration of NA was also estimated by Dot-Blot as 2.1 µg/ml which means a NA/HA ratio on VLPs of 1/4. The host cell proteins (HCP) and host cell DNA in the final material were determined to be 29µg/ml and 3µg/ml, respectively.

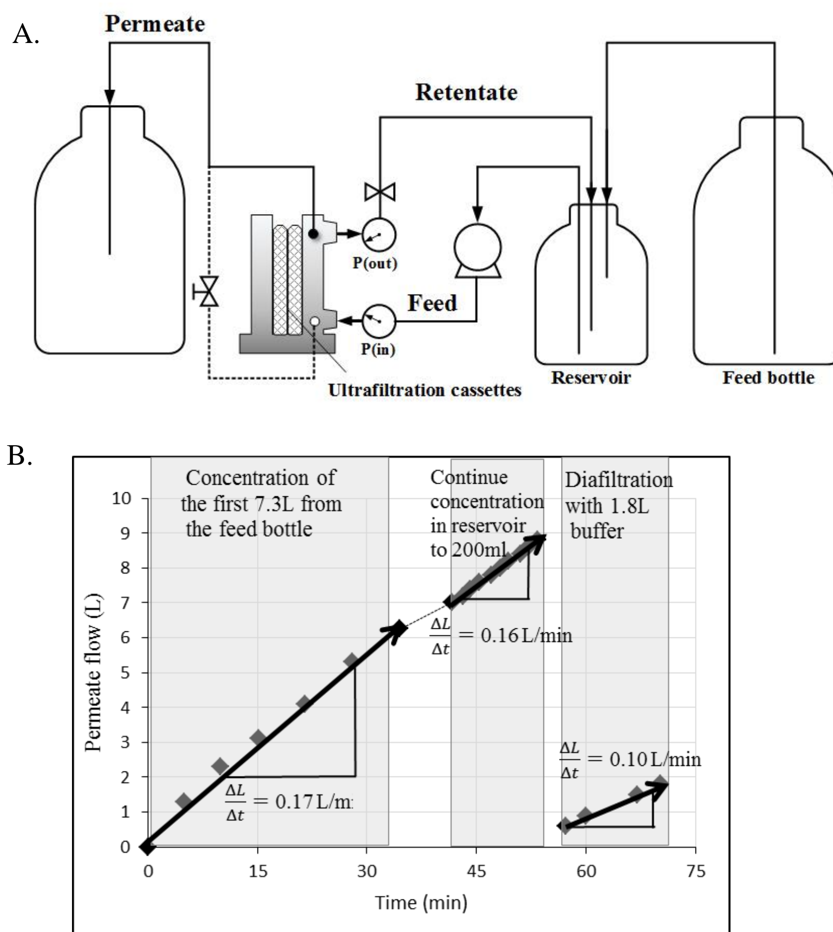

Figure 4-5 Tangential Flow Filtration (TFF) of VLPs produced in the 3-L perfusion bioreactor. A) TFF set-up. B) The 7.3L of harvest contained in the feed bottle were concentrated through the reservoir bottle (2L), such that the total harvest volume (9.3L) was concentrated 50X. The diafiltration consisted in the dilution of the retentate volume with 10 vol. of buffer 20 mM Tris-HCl, 2mM MgCl2, sucrose 5% pH 7.5, which was then concentrated.

Table 4-2 Recovery of VLPs during the downstream process of the perfusion bioreactor harvest in terms of Gag-GFP events/ml and HA concentration

	Total volume	Gag-GFP fluorescent events count by flow cytometry			HA concentration by Dot-Blot (µg)		
		Gag-GFP events/ml	Total Gag-GFP events	Recovery (%)	HA µg/ml	Total HA (µg)	Recovery (%)

Harvest broth	9502	6.6×10^8	6.5×10^{12}	100	0.43	4071.4	100
Clarified material	9300	3.3×10^8	3.1×10^{12}	49.5	0.31	2904.4	71.3
TFF retentate	186.5	1.5×10^{10}	2.9×10^{12}	44.1	8.4	1564.7	38.4

4.5 Declaration of interest

The authors declare no conflict of interest

4.6 Acknowledgements

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CHAPTER 5 PROTEOMIC CHARACTERIZATION OF INFLUENZA H1N1 GAG VIRUS-LIKE PARTICLES AND EXTRACELLULAR VESICLES PRODUCED IN HEK-293SF

One of the major concerns associated with the use of influenza virus-like particles (VLPs) as vaccine candidates is their heterogeneous composition. Enveloped VLPs take up the host cell membrane at the budding site carrying out with them not only the viral antigenic proteins but also host cell proteins. The intrinsic nature of the cells to produce membrane derived vesicles, which have similar size to the VLPs and can also contain the antigenic proteins, concomitantly to VLPs production makes the VLPs purification process challenging. Certainly, the expression system and the viral recombinant proteins employed will determine the nature of the proteins within the VLPs. To further characterize cell culture produced-influenza VLPs and contribute to enable their approval as vaccine candidates, the composition and biogenesis of VLPs needs to be better understood. In this chapter we have characterized, by nanoscale liquid chromatography tandem mass spectrometry (n-LC-MS/MS), influenza H1N1 Gag-VLPs produced in human embryonic kidney cells adapted to serum-free medium (HEK-293SF). The cells stably express HA and NA, and the VLPs production occurs following transient transfection with a plasmid containing the gag gene of HIV-1 fused to GFP. Extracellular vesicles (EVs) produced by the unmodified HEK-293SF were also characterized by n-LC-MS/MS. A total of 73 host cell proteins were identified in the VLPs, whereas 98 were detected in the extracellular vesicles. From that number, 32 host cell proteins were unique to VLPs while 41 proteins were found in both. Importantly, nucleolin was the most abundant host cell differential protein identified in VLPs while lactotransferrin and heat shock protein 90 were the most present in EVs. This study provides a detailed proteomic description of the VLPs and EVs produced in HEK-293SF as well as a critical discussion of the function of each protein incorporated in both nanoparticles species. The outcome of this research sheds light on unique target proteins that could potentially be used for purification of both nanoparticles produced in HEK-293 cells.

5.1 Introduction

Virus-like particles (VLPs) constitute an attractive platform to present a repetitive array of dominant antigens while preserving the protein native conformation. VLPs lack viral genome and their structure mimics wild-type viruses, therefore they are safer and more immunogenic than virus-based and subunit vaccines, respectively (Haynes, 2009). Similarly to viruses, enveloped VLPs bud from the cell membrane taking up the host cell lipid bilayer at the budding site (Thompson *et al.*, 2015) . In addition, the formation of VLPs relies on host cell protein machinery for gene transcription, translation, protein translocation throughout the cytoplasm, assembly and budding. Hence, released VLPs do not only contain the recombinant viral proteins of interest, but also several host cell proteins as well as genomic RNA and DNA (Vicente *et al.*, 2011). The expression system employed for VLP production will determine the nature of the host cell derivatives within the VLPs.

The production of influenza VLPs has been explored in several expression systems including insect cells (Pushko *et al.*, 2011), plants (D'Aoust *et al.*, 2010), and mammalian cells (Wu *et al.*, 2010). Within mammalian cells, the human embryonic kidney cell line HEK-293 seems attractive for VLP production since, in addition of offering human-like post-translational modifications, they are easy to transfect and show efficient growth in suspension culture in serum-free medium (Durocher *et al.*, 2002). However, there remain some concerns about the use of HEK-293 cells for vaccine manufacturing and/or viral vector production for gene therapy. Being a human derived cell line introduces a risk of contamination with human-specific viruses and a tumorigenic potential has been attributed to this cell line (Shen *et al.*, 2008; Stepanenko & Dmitrenko, 2015). Nonetheless, several biopharmaceuticals produced in HEK293 cells have been approved by the FDA for therapeutic use (Dumont, Euwart *et al.*, 2016).

Another aspect to consider when producing influenza VLPs in mammalian/human cells is the co-production of extracellular vesicles which are very similar in size to VLPs. The secreted vesicles are diverse in nature and can be differentiated in three types: exosomes, shedding microvesicles and apoptotic bodies (Ha, Yang *et al.*, 2016). The exosomes have an intracellular origin and a size in the range of 30 to150 nm. Shedding microvesicles bud from the cell membrane with a size ranging from 50 to 1000 nm and the apoptotic bodies (50-5000nm) have more irregular shapes

and arise from apoptotic cells outward membrane blebbings (Andaloussi, Mäger *et al.*, 2013). Exosomes play an important role in cell-cell communication and have received a lot of attention in the last decades for different applications as cancer biomarkers, as well as drug delivery vehicles and chemotherapeutic sensitization agents (Katakowski, Buller *et al.*, 2013; Nilsson, Skog *et al.*, 2009). They contain intra-lumen proteins and genomic material that snapshot the metabolic state of the secreting cell (Akers, Gonda *et al.*, 2013). The formation of exosomes starts with early endosomes that subsequently form multi-vesicular bodies (MVBs) containing intraluminal vesicles (ILVs). Then, the MVBs fused with the plasma membrane and the ILVs or exosomes are released to the extracellular milieu. The endosomal sorting complexes required for transport (ESCRT) mediate the formation and scission of MVBs (Ha *et al.*, 2016). Enveloped viruses, like HIV-1, "hijack" this cell mechanism for its viral budding (Subra, Laulagnier *et al.*, 2007).

In the light of these facts, and considering that the licensing process of human vaccines is becoming nowadays more and more rigorous; obtaining highly pure products with detailed specifications on vaccine composition is strictly required. Therefore, no component in the vaccine should be and/or have the potential to be hazardous for human health. Since enveloped VLPs are membrane-enclosed multimeric protein complexes, even after an intensive purification and polishing, host cell contaminants will not be completely removed since they are contained within and/or attached to the particles. Consequently, an extensive characterization of VLPs will provide more insights on the nature of their components and will allow to further elucidate the intracellular mechanisms (transport, assembly, and budding) associated with these nanoparticles. Furthermore, the analysis of the differential proteins content between VLPs and extracellular vesicles will help to identify targets for purification of both particle species. For this reason, in this study we have completed a proteomic analyses to characterize influenza H1N1 Gag-VLPs and extracellular vesicles produced in the human cell line HEK-293 cultured in serum free-medium.

5.2 Methodology

5.2.1 nLC-MS/MS of Tryptic Digests

VLPs and EVs samples were reduced with 4 mM dithiothreitol for 1 hour at 56 ^{o}C, and then

alkylated with 20 mM iodoacetamide for 1 hour at room temperature in the dark. The samples were subsequently digested with trypsin at a 30:1 protein to enzyme ratio overnight at 37 ºC. Peptides samples were then analyzed by reversed phase nLC-MS/MS, using a nanoAcquity UPLC (Waters) coupled to an Orbitrap XL mass spectrometer (Thermo Fisher) using electrospray ionization (ESI). First, the peptides were loaded onto a 50 mm x 300 µm C8 (Dionex) and a 20 mm x 180 µm C18 (Waters) trap columns in series, and then eluted onto a 100 mm x 100 µm C18 analytical column (Waters). The elution gradient was linear: 1%-45 % solvent B over 18 minutes, 45%-85% solvent B over 3 minutes, 85%-1% solvent B over 1 minute. The columns were finally re-equilibrated at 1% solvent B for 8 minutes. Solvent A was 0.1% formic acid in HPLC grade water; Solvent B was 0.1 % formic acid in acetonitrile. Data-dependent MS/MS analysis was performed, and the peak list files were searched against a database containing the Uniprot Human and influenza strain H1N1 A/PR sequences using MASCOT search engine (version 2.5; Matrix Science). Unique peptide matches were accepted when the ion score was greater than 30. The significance threshold was set to $p<0.05$.

5.2.2 Production of VLPs and EVs

The VLPs analyzed in this study were produced in a HEK-293 inducible stable cell line expressing hemagglutinin and neuraminidase of H1N1 subtype. The VLPs formation was mediated by transient transfection of a plasmid containing Gag protein of HIV-1 fused to GFP (Venereo-Sanchez *et al.*, 2016). The supernatant of cells, 72 hours post induction, was concentrated and semi-purified by ultracentrifugation through 25 % sucrose cushion. Subsequently, the recovered VLPs were ultrafiltrated through a membrane of 1000 KDa cut-off to remove smaller extracellular vesicles.

The extracellular vesicles were isolated from supernatant of HEK-293CymRrcTA cells (Venereo-Sanchez *et al.*, 2016) at 72 hours post-induction with cumate. The supernatant was 25X concentrated through a 25% sucrose cushion.

5.3 Results and Discussion

5.3.1 Proteins identified in VLPs

In the present study the proteins uniquely identified in the VLPs and EVs are presented and discussed in separate sections and tables. All proteins detected in VLPs sample are shown in Table 5-1 as differential and shared proteins with EVs. The proteins were grouped depending on their biological function. Mainly, proteins related to cell cytoskeleton, transport, RNA and DNA binding and processing, and post-translational modifications were identified in both species.

- **Heterologous proteins**

As differential VLPs proteins, the four recombinant proteins that mediate their formation were recognized within VLPs sample (HA, NA, Gag-GFP).

- **RNA binding and processing**

The nucleolin protein, one of the most abundant proteins in the nucleolus, was uniquely identified in VLPs with a MASCOT score of 324. This RNA-binding protein interacts with the nucleocapsid (NC) of Gag protein which is essential for the assembly (Bacharach, Gonsky *et al.*, 2000) and budding of HIV-1 particles (Ueno, Tokunaga *et al.*, 2004). Nucleolin is also expressed on the cell surface (Hovanessian, Soundaramourty *et al.*, 2010); cell-surface nucleolin and lipid rafts were shown to be involved in the virus entry process of HIV (Nisole, Krust *et al.*, 2002). The heterogeneous ribonucleoproteins (hnRNPs) C1, C2 and U were also incorporated in the influenza Gag-VLPs, but not in the EVs. The hnRNPs bind to pre-messenger RNA protecting the RNA from being degraded by endo- or exo-nucleases and transporting it to the ribosomes for translation. This protein complex comprises a large family of over 20 members, some hnRNPs do not shuttle between the nucleus and cytoplasm (e.g. C1, C2 and U), which are those differentially identified in our Gag-VLPs. Other proteins, like A1 and K, stay attached during RNA export before shuttling back into the nucleus (Monette, 2011). The interaction between nucleolin and hnRNP C with the 3’-UTR of the amyloid mRNA precursor has been reported (Zaidi & Malter, 1995). It is likely that Gag protein associates with these strong RNA binding proteins to guarantee the export of the vRNA into virions and reduce the release of empty virions.

- **Cytoskeleton associated proteins**

Our analysis also revealed the presence of several proteins related to the cytoskeleton, some of which were unique to VLPs (Tubulin β-3 chain, Utrophin, Filamin-C, Rho guanine nucleotide exchange factor 4, the FYVE, RhoGEF and PH domain-containing protein 5, ADP-ribosylation factor-like protein 13B) and others were shared between VLPs and EVs (Tubulin β-chain, Tubulin α-1B chain, Tubulin β-4B chain, Actin, cytoplasmic 1, Actin, alpha cardiac muscle 1). From the differential cytoskeleton proteins in Gag-VLPs, the last three mentioned are not directly part of the cytoskeleton, but rather act as regulators. The Rho guanine nucleotide exchange factors mediate the activation of Rho GTPases by exchanging guanosine diphosphate (GDP) for guanosine triphosphate (GTP). The Rho GTPases are involved in the regulation of actin-dependent process such as migration and adhesion, and are also associated with the microtubule cytoskeleton, intervening in cell polarity and membrane transport, among other functions (Schmidt & Hall, 2002). The FYVE, RhoGEF and PH domain-containing protein 5 also activates Rho/Rac GTPases family regulating actin cytoskeleton organization and cell shape (Nakada-Tsukui, Okada *et al.*, 2009). The ADP-ribosylation factor-like protein 13B, a member of ADP-ribosylation factor (ARF) family, is also essential in the stimulation of GTPases proteins being implicated in the intracellular vesicular trafficking (Brown, Gutowski *et al.*, 1993). The filamin protein has been previously identified in exosomes (Zhu, Chen *et al.*, 2015). In general, the cytoskeletal proteins have been implicated in the transport of viruses throughout the cell cytoplasm to the assembly and budding site. Gag protein directly associates with actin microfilaments; in fact, the HIV-1 virions release depends on an intact actin-cytoskeleton (Mouland, Mercier *et al.*, 2000; Sasaki, Nakamura *et al.*, 1995). In the present study, some cytoskeletal regulator proteins were uniquely identified in Gag-VLPs and not in the EVs, which could be due to a more active translocation process promoted by Gag and/or the use of differential pathway for budding.

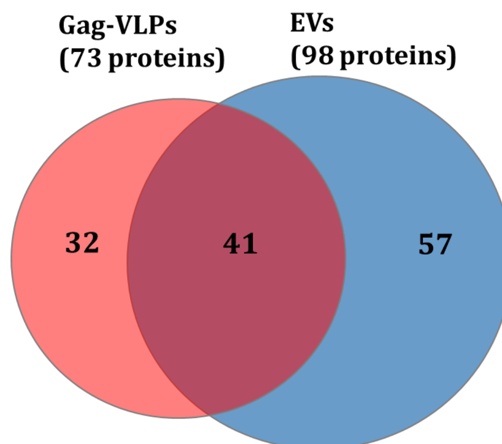

Figure 5-1 Differential and common proteins identified by nano LC-MS/MS in Gag-VLPs and extracellular vesicles (EVs).

- **Histones and Histone regulation proteins**

Several types of histones were identified within VLPs and EVs (Histone H4, Histone H2A, Histone H2B, Histone H3.2, Histone H1.2, Histone H2AX, Histone H2B type 1-J, and Histone H3) while histone regulator proteins (Protein SET similar protein, Protein SET, Histone deacetylase 4) were only found in VLPs. Histones are highly alkaline proteins rich in lysine and arginine that form a core around which DNA is wrapped, forming the nucleosome. In humans, this histone core is organized in octamers with a central tetramer of histones H3 and H4, flanked by two dimers of H2A and H2B. The histone H1 acts as a linker between two nucleosomes (Marsman, Zeerleder *et al.*, 2016). Histones play an essential role in regulating transcription and the condensation of chromatin. The presence of nucleosome fragments (histones and DNA) in the extracellular environment has been associated with cell death, cellular damage and apoptosis. However, it has been reported that different mechanisms are involved in the release of exosomes and apoptotic bodies and that both vesicle species are biochemically and morphologically different. The presence of histones in exosomes might be due to the co-production of apoptotic bodies in the cell culture (Théry, Boussac *et al.*, 2001). The acetylation and deacetylation of histones has been involved with the regulation of transcription. The removal of the lysine acetyl group by the enzyme histone deacetylase causes the condensation of the chromatin. Regions of the chromosome with low level of acetylation are enriched with silent genes (Marks, Richon *et al.*, 2000). The identification of histone deacetylase by LC-MS/MS has been previously reported in HIV-1 virions (Monette, 2011). The Su(var)3-9, Enhancer-of-zeste, Trithorax (SET)-domain

protein family mediates the methylation of histone lysines regulating gene expression. Depending on the specific position in the chromatin of the methylated lysine, the genes will be repressed or activated (Dillon, Zhang *et al.*, 2005; Nwasike, Ewert *et al.*, 2016).

- **Heat shock proteins**

The heat shock proteins (HSPs) (Heat shock 70 kDa protein 1B, Heat shock cognate 71 kDa protein) were identified in both VLPs and EVs. This family of proteins have been found to be released under cellular stress and they are highly conserved among different organisms. The HSPs act as chaperones, participating in protein synthesis, folding, translocation and preventing protein aggregation. They also mediate ubiquitination of aberrant proteins cooperating with the proteasome system during quality control (Richter-Landsberg, 2009). The presence of heat shock proteins has been previously identified in exosomes, HIV-virions, influenza VLPs and influenza virus produced in Vero cells and Moloney murine leukemia virus (Ha *et al.*, 2016; Monette, 2011; Segura, Garnier *et al.*, 2008; Shaw, Stone *et al.*, 2008; Wu *et al.*, 2010).

- **Post-translational modifications**

Furthermore, several proteins implicated in post-translational modifications were recognized in the VLPs and EVs (elongation factor 1-α 1 (EF1α), Eukaryotic initiation factor 4A-I, T-complex protein 1 subunit β, subunit γ, subunit θ, Peptidyl-prolyl cis-trans isomerase A), whereas other proteins such as Niban and Sia-alpha-2,3-Gal-beta-1,4-GlcNAc-R:alpha 2,8-sialyltransferase were uniquely identified in the VLPs. The EF1α is a crucial part of the cellular translation machinery. It is responsible for the GTP-dependent binding of aminoacyl-transfer RNA (aa-tRNA) to ribosomes, guaranteeing fidelity and rate of elongation during translation. The EF1α interacts with actin filaments of the cytoskeleton, and is also involved in cell proliferation and compartmentalization of translation in the cytoplasm (Condeelis, 1995). This elongation factor specifically interacts with Gag protein of HIV-1, while it is not incorporated in non-lentivirus virions like Moloney murine leukemia virus (Cimarelli & Luban, 1999). However, in our study, this protein was detected in both VLPs and EVs, which suggests that the interaction with the actin cytoskeleton results in incorporation of EF1α into both nanoparticles species. In the case of VLPs, the EF1α most probably also interacts with Gag. The eukaryotic initiation factor 4A-I (eIF4-A) is required for the binding of ribosome subunits 40S to the mRNA during the initiation of protein biosynthesis. It has a single-stranded RNA-dependent ATPase activity, which mediates

the unwinding of the 5'-proximal mRNA secondary structure facilitating the attachment to the ribosomes (Rozen, Edery *et al.*, 1990). The T-complex protein 1 is a hetero-oligomeric protein containing at least eight subunits that assists the correct folding and functional conformation of proteins in the cytosol of eukaryotic cells. It is involved in the folding of actin and tubulin (Kubota, Hynes *et al.*, 1995). The Peptidyl-prolyl cis-trans isomerase A, also known as cyclophilin A, catalyzes the cis-trans isomerization of peptidyl-prolyl bonds in oligopeptides, accelerating the folding of proteins (Göthel & Marahiel, 1999; Takahashi, Hayano *et al.*, 1989). The HIV-1 Gag protein specifically binds to cyclophilin A and B and this interaction appears to play an essential role in HIV-1 infection and replication cycle (Luban, Bossolt *et al.*, 1993). The cyclophilin A was indentified in our Gag-VLPs with a significantly higher MASCOT score compared to the EVs (Table 5-1). This is likely due to the interaction with Gag, which enhances the presence of the protein within the VLPs. The cyclophilin A has also been detected in influenza virus produced in Vero cells (Shaw *et al.*, 2008).

Niban and Sia-alpha-2,3-Gal-beta-1,4-GlcNAc-R:alpha 2,8-sialyltransferase were uniquely identified in Gag-VLPs. The Niban protein is commonly expressed in renal tumors, therefore it is considered a marker of this type of carcinogenesis (Adachi, Majima *et al.*, 2004). It regulates protein translation by phosphorylation of some eukaryotic translational initiation factors and is released under endoplasmic reticulum stress (Sun, Kobayashi *et al.*, 2007). The Sia α-2,3, Gal-β, 1,4-GlcNAc-R:α 2,8-sialyltransferase, also known as ST8SiaIII, catalyzes the polysialylation of membrane glycoproteins through the transfer of sialic acid from a cytidine monophosphate-linked sialic acid donor to an acceptor with alpha-2,8-linkages sialic acid ends (Volkers, Worrall *et al.*, 2015). The differential presence of the sialyltransferase in our VLPs could be part of the post-translational modifications needed for the two glycoproteins hemagglutinin and neuraminidase.

- **Proteins transport**

As part of the transport proteins identified in VLPs, the ATP-binding cassette sub-family A has been differentially detected. This complex protein family is implicated in the export and import of complex molecules across lipid membrane, such as amino acids, sugars and proteins (Dean, Hamon *et al.*, 2001). The ATP-binding cassette transporters superfamily has been involved with the assembly of HIV-1 virions (Zimmerman, Klein *et al.*, 2002), and may be involved in the transport of Gag protein throughout the cytoplasm. The GTP-binding nuclear protein Ran and

ammonium transporter Rh type B (RHGB) were identified in VLPs and EVs. The GTP-binding nuclear protein Ran or Ras-related nuclear protein participates in the import and export of RNA and proteins between the nucleus and the cytoplasm and also its interaction with other proteins seems to play a role in the mitotic cycle (Klebe, Bischoff *et al.*, 1995). The RHGB is a transmembrane protein mainly expressed in cells of organs related to the excretion, secretion of ammonia like the kidney. A previous study has shown the cellular localization of RHGB in the plasma membrane and intracellular granules (Liu, Peng *et al.*, 2001).

- **Signaling transducer proteins**

The signaling transducer proteins, growth factor receptor-bound protein 10 (Grb10) and Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit β-1 were uniquely identified in VLPs. The Grb10 is an adapter protein that links tyrosine kinases receptors via phosphorylation, including the insulin and insulin-like growth factor receptors modulating several signaling pathways (Kabir & Kazi, 2014). The Guanine nucleotide-binding protein subunit β is part of the heterotrimeric G protein complexes. These subunits possess GTPase activity mediating the hydrolysis of GTP to GDP. The G proteins are activated by G protein-coupled receptors and this association regulates several signalling cascades for protein transport, enzymatic functions and a wide range of cellular functions (Neves, Ram *et al.*, 2002). The unique presence of these transducer proteins in the Gag-VLPs is most likely related to a more organized cell membrane translocation and budding orchestrated by the viral heterologous proteins.

- **DNA related proteins**

The DNA-dependent protein kinase catalytic subunit, Tumor protein 63, Transcription factor ETV7, and THAP domain-containing protein 6 were uniquely detected in Gag-VLPs. The first two proteins mentioned are DNA-binding proteins that act as a response to cellular DNA damage stimulus. The transcription factor ETV7 (Tel-2) has been previously identified in human hepatocellular carcinoma being related with promoting chromosomal translocations in human cancers (Matos, Witzmann *et al.*, 2009). The thanatos (the Greek god of death)-associated protein (THAP) domain is a DNA-binding protein that contains a zinc finger domain. The THAP domain containing proteins play an important role in cell proliferation, apoptosis, chromatin modification and transcriptional regulation (Bianchetti, Bingman *et al.*, 2011). The N-glycosylase/DNA lyase

protein identified in VLPs and EVs is an enzyme that binds to damaged DNA and mediates DNA repair (Sharova, 2005).

- **Clearance of lipids**

Several proteins involved in the clearance of lipids were identified either in VLPs and EVs (Table 5-1). The clusterin protein is a heterodimeric disulfide-linked glycoprotein that is implicated in several biological events, such as lipid transport, apoptosis, membrane lipid recycling and also it acts as a chaperone for correct folding and aggregation of secreted proteins (Koltai, 2014). The mitochondrial metalloendopeptidase OMA1, is part of the membrane-embedded quality control system in the inner membrane of the mitochondria. This protease plays a role in degrading and removing misfolded proteins from the organelles, and also in regulating the metabolism of lipids (Käser, Kambacheld *et al.*, 2003; Quirós, Ramsay *et al.*, 2012). The lactadherin (MFGE8) is a protein abundantly released in mammalian glands but it has also been identified in other types of tissues, such as lung, brain and kidney. It binds to phosphatidylserine and has been detected on the surface of apoptotic bodies and exosomes produced from murine dendritic cell line (D1) and monkey kidney cell line, COS-7 (Oshima, Aoki *et al.*, 2002). The MFGE8 plays a role in the secretion of lipids.

- **Tetraspanins**

The tetraspanin member CD81 was found to be associated with the VLPs and EVs. Tetraspanins constitute a superfamily of proteins that have four transmembrane domains and two extracellular loops that are organized in the membrane as enriched-tetraspanin microdomains (TEMs). While tetraspanins are considered a biomarker for exosomes (Andreu & Yáñez-Mó, 2014), membrane zones enriched in tetraspanins are the areas from which HIV-1 assembles and buds (Thali, 2011). The tetraspanin CD81 is well known as a co-receptor for the entry of hepatitis C virus (Farquhar, Harris *et al.*, 2011) and it has also been identified in several virus such as Moloney murine leukemia virus (Segura *et al.*, 2008), feline immunodeficiency virus, canine distemper virus, influenza virus and influenza VLPs produced in Vero cells (Shaw *et al.*, 2008; Wu *et al.*, 2010). These findings suggest that these envelope viruses, VLPs and exosomes all follow similar path for budding through the TEMs spots.

- **Glycolytic enzymes**

Three enzymes involved in ATP generating steps of the glycolysis were identified in the VLPs and EVs (GAPDH, pyruvate kinase and α-enolase). The presence of these glycolytic enzymes in viruses, VLPs and exosomes has been previously reported (Arslan, Lai *et al.*, 2013; Monette, 2011; Shaw *et al.*, 2008; Wu *et al.*, 2010). It is still unclear why these proteins remain in the VLPs and exosomes, however alternative functions related to the regulation of virus RNA transcription have been reported for these proteins (Shaw *et al.*, 2008). In addition, it has been shown that the administration of mesenchymal derived-stem cells exosomes after myocardial ischemia/reperfusion injury increased ATP levels, decreased oxidative stress and activated cardioprotective pathways, thereby significantly reducing infarct size. The increase of ATP levels in the injured zone was claimed to be mediated by the glycolytic enzymes which reverted the depletion of ATP characteristic of this type of injury (Arslan *et al.*, 2013). Perhaps the presence of glycolytic enzymes in the EVs and VLPs has to do with the life span and/or bioactivity of these nanoparticles.

- **Other proteins**

Other proteins with diverse functions were identified in both VLPs and EVs. The fatty acid synthetase has been previously detected in influenza virions (Shaw *et al.*, 2008) and is a multifunctional enzyme that catalyzes the formation of long-chain fatty acids from acetyl-CoA, malonyl-CoA and NADPH. It has been reported that the infection with human cytomegalovirus (HCMV) upregulates the host cell metabolism (glycolisis, TCA and fatty acid biosynthesis) to provide energy for virus replication and the inhibition of fatty acid biosynthesis during HCMV and influenza A infection leads to the supression of viral replication (Munger, Bennett *et al.*, 2008). Lactotransferrin (LTF) was also detected in the influenza-Gag VLPs and EVs analyzed in this study. The protein has antiviral and antibacterial activity. Interestingly, LTF was the most abundant protein in the EVs evidenced by a highest MASCOT score among the proteins identified in extracellular vesicles. LTF has been previously identified in vesicles derived from urinary exosomes of both prostate cancer patients and controls (Øverbye, Skotland *et al.*, 2015). It is worth to note that the use of LTF as an additive in the medium 293-SFM4TransFx employed to grow the HEK-293 cells, was demonstrated by MS/MS after cutting a high intensity 80 KDa band from an SDS-PAGE run with the medium (data not shown). However, considering that the

extracellular vesicles were isolated by ultracentrifugation through sucrose cushion, there should not be free LTF protein remaining in the final pellet, unless the protein forms aggregates and/or gets attached to VLPs.

Table 5-1 Proteins identified by n-LC-MS/MS in VLPs sample

	Gag-VLPs unique proteins				Shared proteins between VLPs and EVs			
		MASCOT Score	Predicted MW (Da)	Sequence Coverage (%)		MASCOT Score	Predicted MW (Da)	Sequence Coverage (%)
Heterologous proteins	Pr55(Gag) [Human immunodeficiency virus 1]	3535	55894	70.6				
	Green fluorescent protein	229	26869	34.9				
	Hemagglutinin [Influenza A virus (A/Puerto Rico/8/1934(H1N1))]	148	63341	12.5				
	Neuraminidase [Influenza A virus (A/Puerto Rico/8/1934(H1N1))]	61	50111	4.2				
Cytoskeleton associated proteins	Tubulin β-3 chain	326	50400	20.7	Tubulin β-chain	641/1015	49639	40.3/62.2
	Rho guanine nucleotide exchange factor 4	99	108145	2.2	Tubulin α-1B chain	595/995	50120	39.9/49.4
	Utrophin	53	394220	0.5	Tubulin β-4B chain	488/1078	49799	36.9/62
	FYVE, RhoGEF and PH domain-containing protein 5	41	159791	0.8	Actin, cytoplasmic 1	724/752	41710	56/56
	Filamin-C	36	290841	0.5	Actin, alpha cardiac muscle 1	280/281	41992	22/24.9
	ADP-ribosylation factor-like protein 13B	35	48613	3				
RNA binding and processing	Nucleolin	324	76568	13.7	Heterogeneous nuclear ribonucleoprotein K	88/96	41781	3.2/3.2
	Heterogeneous nuclear ribonucleoproteins C1/C2	161	31929	12.8	Heterogeneous nuclear ribonucleoprotein A1	62/95	19460	5.2/17.9

	Heterogeneous nuclear ribonucleoprotein U	49	27387	3.3				
	U1 small nuclear ribonucleoprotein A	41	14635	7.8				
	Cleavage and polyadenylation-specificity factor subunit 1	49	23670	3.9				
Histones and Histone regulation proteins	Protein SET similar protein	54	33625	3.4	Histone H4	767/599	11360	51.5/51.5
	Protein SET	51	31105	12	Histone H2A	493/484	18470	37.9/27.2
	Histone deacetylase 4	45	118966	0.6	Histone H2B	386/637	13898	41.3/41.3
					Histone H3.2	217/238	15379	40.4/43.4
					Histone H1.2	176/386	21352	15/19.7
					Histone H2AX	366/221	15135	47.6/47.6
					Histone H2B type 1-J	307/690	13896	40.5/41.3
					Histone H2A.Z	242/390	13545	35.2/53.9
					Histone H3	113/144	14905	41.7/44.7
Heat shock proteins					Heat shock 70 kDa protein 1B	773/1292	70066	32.2/45
					Heat shock cognate 71 kDa protein	221/237	20000	21.2/9.7
Post-translational modifications	Protein Niban	43	103070	1.1	Elongation factor 1-alpha 1	136/189	47853	10.2/23.1
	Sia-alpha-2,3-Gal-beta-1,4-GlcNAc-R:alpha 2,8-sialyltransferase	43	43942	2.1	Eukaryotic initiation factor 4A-I	122/96	20675	6.9/6.9
					T-complex protein 1 subunit β	101/194	56771	4.2/6.9
					T-complex protein 1	65/158	55639	2.2/6.2

					subunit γ			
					T-complex protein 1 subunit θ	41/97	35457	3.4/4.6
					Peptidyl-prolyl cis-trans isomerase A	322/37	18001	44.8/32.8
Proteins transport	ATP-binding cassette sub-family A member 12	37	293049	0.3	GTP-binding nuclear protein Ran	94/78	26799	13.7/18.4
					Ammonium transporter Rh type B	44/53	10570	11.3/11.3
Proteins involved in clearance of lipids					Clusterin	157/222	52461	11.8/20.9
					Metalloendopeptidase OMA1, mitochondrial	48/42	37799	2.7/2.7
					Lactadherin	79/316	29149	4.9/30.7
Signaling transducer proteins	Growth factor receptor-bound protein 10	34	67189	3.4				
	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	33	25918	4.2				
DNA related proteins	DNA-dependent protein kinase catalytic subunit	42	468788	0.5	N-glycosylase/DNA lyase	41/46	19056	4.8/4.8
	Tumor protein 63	41	76736	1.8				
	Transcription factor ETV7	36	38973	4.4				
	THAP domain-containing protein 6	35	11087	10.2				
Tetraspanin					CD81	71/158	24423	7.1/16
Glycolytic enzymes					Glyceraldehyde-3-phosphate dehydrogenase	341/511	36030	36.1/51.6

					Pyruvate kinase	155/508	53011	8.9/24.1
					α-enolase	201/648	47139	9.9/29.3
Ribosomal proteins	40S ribosomal protein S20	46	5426	23.9	40S ribosomal protein SA	39/55	33293	2.7/7.7
Other proteins	Cyclin-dependent kinase inhibitor 2A	42	12076	18.1	Protein TRAJ56	49/50	2220	38.1/38.1
	Proline-rich basic protein 1	41	106852	1	Fatty acid synthase	76/102	273026	0.9/2.3
	Zinc finger protein 774	37	55032	2.7	26S proteasome non-ATPase regulatory subunit 2	67/43	100136	1.7/1.7
	Norrin	35	15033	16.5	Protein Dok-7	59/57	53064	3.6/3.6
	Pecanex-like protein 3	39	221898	0.4	Lactotransferrin	83/ 4003	76577	5/61.5
	Pecanex-like protein 2	30	237127	0.9	Creatine kinase B-type	99/256	24101	7.4/24.7
	Amine oxidase	30	15162	15.3				
	Protein N-terminal asparagine amidohydrolase	48	34656	2.6				

**MASCOT score and sequence coverage in “shared proteins between VLPs and EVs” are shown as VLPs/EVs value*

5.3.2 Extracellular vesicles unique proteins

The proteomic analysis of EVs revealed the presence of a total of 98 proteins and 57 unique proteins Table 5-2. From the differential proteins identified in EVs, the HSP90 was the most abundant based on the MASCOT score value and number of peptides identified. The HSP90 protein binds to a wide variety of client proteins being implicated in several biological processes and was identified in exosomes derived from different tumorogenic cell tissues (Hegmans, Bard *et al.*, 2004; Prodromou, 2016). It has been reported that the chaperone HSP70 is the one that is specifically incorporated in HIV-1 virions through interaction with Gag protein (Gurer, Cimarelli *et al.*, 2002; Segura *et al.*, 2008). Influenza viruses produced in Vero cells did not incorporate either HSP70 and/or HSP90 (Shaw *et al.*, 2008) while influenza VLPs produced in the same cell line incorporated both (Wu *et al.*, 2010).

Within the group of proteins involved in signalling transduction, members of 14-3-3 proteins (epsilon and theta) were differentially detected in the EVs. The 14-3-3 proteins are a family of highly conserved acidic proteins comprising several isoforms (α-η) (Aitken, 2006). They play a role as modulators of Ras signalling pathway (Ji, Erfani *et al.*, 2008). The Ras protein family are small GTPases involved in cellular signals transmission in the cytoplasm. Different members of the family have been identified in extracellular vesicles released from several cell types and it is considered an EV membrane marker in the Exocarta database.

In addition, typical proteins associated to the ESCRT complex were differentially detected in the EVs produced in HEK-293SF, such as the programmed cell death 6-interacting protein, alias ALIX from apoptosis-linked gene 2-interacting protein X. The ESCRT complex is formed by four components: ESCRT-0, I, II, and III. The ESCRT-0 complex is recruited to the early endosome which at the same time recruits ESCRT-I and ESCRT-II. The complexes ESCRT 0, I and II bind ubiquitinated membrane proteins to be degraded in lysosomes, recycled or released to the extracellular milieu. Then, the endosomal membrane undergoes an inward budding to generate ILVs which requires the ESCRT-III complex for the final membrane bend and scission. The ALIX protein interacts with the ESCRTIII complex via the subunit CHMP4 (charged multivesicular body protein 4a) to mediate the scission of the ILVs, inside MVBs. ALIX also interacts with the ESCRT-I subunit TSG101 (tumor susceptibililty gene 101 preotein), linking ESCRT-I and ESCRT-III (Bissig & Gruenberg, 2014). ALIX has also been implicated in the viral

budding of HIV-1. The fact that proteins related to ESCRT complex were not identified in the influenza Gag-VLPs suggests a budding mechanism independent of ESCRT.

Table 5-2 Proteins uniquely identified in extracellular vesicles produced in HEK-293 cells

	Protein Description and database	MASCOT Score	Predicted MW (Da)	Sequence Coverage (%)
Cytoskeleton associated proteins	MARCKS-related protein	71	19517	6.7
	Dystroglycan	50	97381	1
	PDZ and LIM domain protein 3	45	21741	5.6
	Fibrous sheath-interacting protein 2	40	789391	0.4
	Leucine-rich repeat and calponin homology domain-containing protein 2	42	84536	1.6
RNA binding proteins	Lupus La protein	107	21429	7.6
	Nucleolysin TIAR	39	14607	6.1
	Serine/arginine-rich-splicing factor 2	49	15518	6
	rRNA methyltransferase 3, mitochondrial	42	19683	12.4
Histones	Histone H2A type 1-A	218	14225	35.1
	Histone H1.3	251	22336	19
	Histone H2A type 2-B	206	13987	52.3
	Histone H1x	55	22474	5.6
Heat shock proteins	Heat shock protein HSP 90-alpha	523	84607	25.5
	Heat shock protein HSP 90-beta	490	83212	26.8
	Heat shock cognate 71 kDa protein	237	68763	9.7
	Putative heat shock 70 kDa protein 7	205	40220	18.3
Post-translational modifications	T-complex protein 1 subunit epsilon	223	55314	10.1
	T-complex protein 1 subunit alpha	145	46880	12.5
	T-complex protein 1 subunit zeta	38	57988	4
	Elongation factor 2	185	95277	9.3
	Elongation factor 1-gamma	103	50087	4.6
Transport proteins	Sodium/potassium-transporting ATPase subunit alpha	114	78857	2.5

lipid metabolism	2-oxoglutarate and iron-dependent oxygenase domain-containing protein 3	47	35624	2.8
	Prostaglandin F2 receptor negative regulator	47	98495	2
	Phospholipase B1, membrane-associated	34	18491	9.9
Signalling transducer proteins	14-3-3 protein epsilon	236	29155	22
	14-3-3 protein theta	194	27747	28.6
	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	95	45575	9.3
	Ankyrin repeat and SAM domain-containing protein 3	44	57992	2.8
	Citron Rho-interacting kinase	40	187298	0.4
DNA related proteins and transcription factors	X-ray repair cross-complementing protein 5	83	82652	7.4
	X-ray repair cross-complementing protein 6	77	64243	5.7
	T-box transcription factor TBX4	51	60166	2.4
	Poly (ADP-ribose) polymerase family, member 9, isoform CRA_b	32	45623	3
	Cyclic AMP-responsive element-binding protein 3-like protein 2	31	57379	10.6
Glycolytic and TCA enzymes	Phosphoglycerate mutase 2	78	28748	9.9
	ATP-citrate synthase	64	120762	1.4
	Phosphoglycerate kinase	45	44586	4.1
Ribosomal proteins	40S ribosomal protein S3	61	14994	9.6
	40S ribosomal protein S8	58	21866	5.9
ESCRT associated proteins	Ubiquitin-like modifier-activating enzyme 1	286	117774	10.8
	Coiled-coil domain-containing protein 180	53	190979	1.2
	Programmed cell death 6-interacting protein (ALIX)	52	95963	1.7
Cell-cell interaction and	Integrin alpha-X	42	128456	1.3
	Matrix extracellular	40	55630	3.2

intercellular signalling	phosphoglycoprotein			
	Protocadherin alpha-6	54	102652	1.9
	Basigin	46	20529	7.4
	Olfactory receptor 4F3/4F16/4F29	30	35050	10.3
Other proteins	Protein AHNAK2	115	616242	0.2
	Brain acid soluble protein 1	82	22680	22.9
	Adenosylhomocysteinase	48	47685	5.1
	Breast cancer type 1 susceptibility protein	39	69926	1.4
	RING finger protein 121 (Fragment)	32	16795	20.5
	Uncharacterized protein	383	88324	13.9
	F-box only protein 17 (Fragment)	36	12099	11.9
	Lamina-associated polypeptide 2, isoform alpha	32	75446	2.9

5.4 Conclusions

In a previous study we have shown the feasibility of producing influenza VLPs in HEK-293SF cells (Venereo-Sanchez *et al.*, 2016). In the present work, the proteomic composition of the influenza Gag-VLPs generated using an inducible HEK-293 producing cell line (Venereo-Sanchez *et al.*, 2016) and extracellular vesicles (EVs) produced from non-transfomed HEK-293SF was analysed by nano-LC-MS/MS. As expected, several host cell proteins were incorporated in influenza Gag-VLPs and EVs. The identified proteins are multifunctional proteins mostly involved in cell signalling pathways, protein translocation and folding, and were associated to the cell cytoskeleton and nucleic acids. The RNA-binding protein nucleoline was uniquely incorporated in the VLPs, probably through a specific interaction with the Gag protein. On the other hand, lactotransferrine was the most abundant protein in EVs, as well as the HSP90. In addition, important proteins associated to the endosomal sorting complexes required for transport (ESCRT) were identified in the exosomes, whereas they were not detected in VLPs. This observation could suggest a possible different mechanism where VLPs budding is independent of ESCRT. Further studies will be focused on determining the RNA composition within both nanoparticles species and *in vivo* experiments to confirm that there is no potential

hazardous components. In addition, proteins differentially identified either in VLPs and EVs will be exploited for the development of novel purification protocols to separate EVs from VLPs.

CHAPTER 6 GENERAL DISCUSSION

While VLPs have emerged as a promising influenza vaccine platform, there are still numerous challenges associated with their large-scale manufacturing and more work needs to be devoted to bioprocess development, purification and characterization. Mammalian cells offer several advantages as a platform for enveloped VLPs production, including superior post-translational modifications that are important for preserving the conformation and glycosylation of the antigens. In addition, mammalian cells are easy to grow in serum-free medium in suspension cultures allowing large scale production and high productivity yields. In this thesis, to address some of the significant difficulties associated to the large-scale manufacturing of VLPs, we have developed a production process to produce these nanoparticles in a human cell line. Human cell lines provide a glycosylation profile and folding consistent with human endogenous proteins, which alleviates the potential antigenicity of recombinant biopharmaceuticals for human use (non-human mammalian cell lines can introduce glycosylation patterns that can be antigenic in humans). Since the formation of influenza VLPs involved more than one protein, the VLPs production was driven in an inducible manner, such that there is no extra metabolic burden during the cells growth. We have proposed the use of a stable cell line for large-scale vaccine manufacturing, thus bypassing the need for transient transfection which can be technically cumbersome at large scale production.

The production of influenza VLPs is usually mediated by the co-expression of HA, NA, M1 and M2, although other studies were successful at producing VLPs by expression of HA, NA and M1 (Pyo, Masic *et al.*, 2012); HA and NA (Chen *et al.*, 2007); HA and M1(Quan *et al.*, 2007); HA (Chen *et al.*, 2007; D'Aoust, Lavoie *et al.*, 2008) ; NA (Lai *et al.*, 2010); M1 (Gómez-Puertas *et al.*, 2000); and M2 (Chen et al., 2007). After decades of intensive research on influenza virus assembly and budding mechanisms, it appears that these processes are mediated by a redundant interaction between the four proteins, and not by a single protein as for other types of viruses. The review of Rossman, and Lamb (2011) proposes a model for influenza assembly and budding where the accumulation of HA and NA in lipid raft zones may initiate the viral budding by altering the membrane curvature. Then, the cytoplasmic tails of HA and NA recruits the M1 protein at the budding site enhancing the budding process. The non-raft channel protein M2

interacts subsequently with M1, mediating the scission of the virions. NA mediates the final release of the virions from the cell surface by cleaving the sialic acid on receptors to prevent the binding of HA.

Considering the uncertainty as which proteins mediate virus budding in influenza and the need for a production process able to generate high yields of VLPs, we assessed the use of two proteins as driving force for viral budding: M1 matrix protein of influenza and Gag protein of HIV-1. At first, a stable cell line expressing HA and NA was transfected with the plasmid containing the corresponding proteins. Since the production of VLP proteins was significantly higher (7X) using Gag protein, the production process of VLPs was implemented in a lab-scale bioreactor by transient transfection with the plasmid containing Gag. The yields of HAU/ml (8913) and VLPs/ml ($1.78x10^{10}$) estimated in the material recovered from the ultrafiltration were higher than those reported by other studies producing VLPs either in insect cells (Thompson *et al.*, 2015) or mammalian cells (Cervera *et al.*, 2013). However, it should be emphasized that direct comparisons are difficult due to differences in the methodology and/or analytical techniques employed in each laboratory (Thompson *et al.*, 2013). Thus, the VLPs production process using transient transfection of Gag was our first approach to produce influenza Gag-VLPs and their immunogenicity and efficacy were demonstrated by *in vivo* experiments. The positive control group (CT+), included in the mice experiment, was immunized with a recombinant HA protein from the same strain H1N1 A/Puerto Rico/8/34. The levels of anti-HA specific antibodies IgG1 elicited in animals from CT+ were higher than mice vaccinated with the influenza Gag-VLPs. This immunoglobulin is related to a T helper (Th) 2 response, which is characteristic of immunization with soluble recombinant protein (Bright *et al.*, 2007). Interestingly, most of the mice vaccinated with the influenza Gag-VLPs elicited higher titers of IgG2a, which is an immunoglobulin characteristic of a Th1 response. This type of response is of great importance against viral infections since it promotes viral clearance and induces strongest protection (Huber, McKeon *et al.*, 2006). It has been shown that immunization with the whole influenza virion generates a significantly higher IgG2a in comparison with recombinant protein (Bright *et al.*, 2007). Furthermore, it has been reported that VLPs directly activate B cells and promote their differentiation in IgG2a producer plasma cells (Zhang, Cubas *et al.*, 2009). Although both mice groups (CT+ and influenza Gag-VLPs vaccinated) were protected against a challenge with the homologous strain, it appears that VLPs generate a more active immune

response capable of clearing the viruses and mediate a professional presentation of antigens that activate cellular immunity. Further studies should be performed in other animal models, like ferrets, to test different concentrations of VLPs for immunization and challenge animals with different strains to verify the capacity of the vaccine to elicit a broader response.

Once the proof of concept of HEK-293-produced influenza Gag-VLPs was demonstrated by *in vivo* experiments, the production process was reassessed, aiming to develop a strategy that can be suitable for large scale manufacturing. In the first production approach implemented, the formation of VLPs was mediated after transient transfection with a plasmid containing the Gag-HIV-1 protein. However, in the context of large-scale operation, the additional steps required for the transient transfection are not desirable. Taking into account that the Gag protein is the core protein of the VLPs, there is no need to do transient transfection for every bulk production. Therefore, a production process using a stable cell line expressing the three genes involved in the VLPs formation was developed.

The process was further optimized in an effort to increase the volumetric yield (without affecting the cell specific productivity) by increasing the cell density at the time of induction. Interestingly, we found that, by maintaining medium replacement after the induction at small scale, the total VLP proteins volumetric yield was increased by 128 fold (Gag-GFP events) and 17 fold (HA µg) compared to the batch control. This strategy was also found to improve the total volumetric yield by 2.9 fold (Gag-GFP events) and 2.2 fold (HA µg) compared to cultures without medium replacement after induction. Through medium exchange, toxic waste metabolites and growth-inhibitory cell factors are diluted and, simultaneously, the fresh medium added provides a constant supply of nutrients to avoid limitations (Hiller *et al.*, 2017). To achieve the same level of productivity, three bioreactor runs without medium replacement post-induction need to be performed, which has a significant impact on cost and labour. A reduction in cell viability and growth was observed in the shake flasks where medium replacement was maintained after induction due to a shear stress effect on cells as a result of the centrifugation steps. However, the productivity was not affected by this (Figure 4-3).

A 3L-bioreactor in perfusion mode was successfully implemented using the cell line 293-HA/NA/Gag-GFP. The protein HA productivity values were comparable to those of small scale cultures, whereas higher Gag-GFP event count was obtained in medium replacement condition at

small scale compared to the perfusion bioreactor. This could be due to the release of intracellular Gag-GFP associated with the decrease in viability resulting from cell centrifugation. The cell specific productivity, compared to the batch control, was increased for both genes by a factor of 4 for Gag-GFP events and by 500 fold for HA. The observed differences can be due to the region of insertion of the genes in the chromosome and how their expression is affected or favored by the high cell density (Wurm, 2004). The acoustic filter used for cell separation was highly efficient, as low cell counts were measured in the harvest stream. Therefore, no centrifugation steps were needed to clarify the harvest (only the residual 2.6L left in the bioreactor were centrifuged) which is highly advantageous when operating at large scale. Once the perfusion bioreactor was stopped, it took only 3 hours to process the 9.5 L harvest, including clarification and ultrafiltration/concentration. This scenario was significantly better to what we showed in our first production process approach where VLPs concentration was performed by ultracentrifigation through a 25 % sucrose cushion followed by a diafiltration.

With the aim to better compare the productivities obtained from our first process approach where VLPs were produced by transient transfection with Gag and the production process from the cell line stably expressing the three genes (293-HA/NA/Gag-GFP stable cell line), Table 6-1 shows the VLP protein yields based on different assays. The production of HA per liter of culture measured by dot-blot was higher in the transient transfection approach, while HA productivities based on SRID were higher in the stable cell line 293-HA/NA/Gag-GFP cultivated in a perfusion bioreactor. The differences can be due to intrinsic variability between the techniques or since SRID measures the antigenic conformation of HA (Li, Jaentschke*, et al.*, 2010), there might be less antigenic HA produced in the transient transfection production process approach. Since SRID is the established method used to quantify seasonal influenza vaccines, we relied on this method to compare the processes and calculate the vaccine doses produced. The expression of NA was higher in the production using the stable cell line 293-HA/NA/Gag-GFP leading to a HA:NA ratio of 1:4 which is the same ratio obtained with the sucrose cushion preparation of influenza virus H1N1 A/PR/8/34 produced in HEK-293 cells (Venereo-Sanchez *et al.*, 2016). The transient transfection approach was more efficient in the sense that similar VLP protein production was obtained at a lower cell density. However, 5-fold more total HA protein was produced from one single bioreactor run operated in perfusion mode with the stable cell line 293-HA/NA/Gag-GFP. The concentrations of host cell proteins (HCPs) and host cell DNA were

measured in the final material recovered from the two processes. The concentration of host cell DNA was similar between the two processes; however, a higher HCPs concentration was obtained in the VLPs produced from the stable cell line 293-HA/NA/Gag-GFP. The FDA provides guidelines with the purity requirements for vaccines establishing concentrations of DNA lower than 10 ng per dose. Considering that the dose in humans is 15 µg, with our current production we could expect around 5 µg of DNA in the final dose, which is considerably higher than the maximum residual DNA amount allowed. At the same time, very low amounts of HCPs concentration are accepted in the final product, and although biopharmaceutical companies do not release their lot specifications, more than 95% purity was required by the WHO for the late-stage VLP malaria vaccine from GlaxoSmithKline. For therapeutic proteins like antibodies, the specifications on HCPs levels are set below 100 ppm (Effio & Hubbuch, 2015). Certainly, future works on VLPs produced either by transient transfection or using stable cell lines need to address these important issues by implementing more refined purification and polishing methods that can effectively remove these product-related contaminants.

The electron micrographs obtained from sucrose cushion preparations that revealed the presence of small extracellular vesicles (Figure 3-3) and also the detection of total HCPs by ELISA underscored the need to characterize in more details the proteomic of VLPs and extracellular vesicles (EVs) produced by HEK-293 cells. The results of the nano-LC-MS/MS analysis revealed the presence of 98 and 73 proteins within EVs and VLPs, respectively, of which 41 were shared between them. Most of the proteins identified in both nanoparticles species are multifunctional proteins which play important roles in signaling pathways, intracellular trafficking of molecules, cytoskeleton organization, protein folding, transcription and translation. Based on the MASCOT scores, we found that nucleolin was the most abundant HCP within VLPs, while lactotransferrin and HSP90 were the majority in EVs. Nucleolin is an RNA binding protein that interacts with Gag-HIV during the HIV replication cycle. Cell-surface expressed nucleolin, together with lipid rafts have been implicated with the viral entry process of HIV in the host cell (Nisole *et al.*, 2002).

Table 6-1 Summary of the production performances of the two processes employed to produce VLPs

	Transient transfection (3L-batch bioreactor)	Stable cell line 293-HA/NA/Gag-GFP (3L-perfusion mode bioreactor)
Cell density at the time of induction ($x10^6$ cells/ml)	1	14
[HA] µg/L of culture by dot blot	243	165
[HA] µg/L of culture by SRID	138	194
HAU/ml of final product after TFF concentration	8913	8913
[NA] µg/L of culture by dot-blot	8	41
Gag-GFP events count by flow cytometry ($x10^{11}$ Gag-GFP events/L of culture)	6	3
Host cell proteins (µg/ml) final product after TFF concentration	0.49	29
Host cell DNA (µg/ml) final product after TFF concentration	2.46	3
Ratio HA/NA	1 :40	1 :4
Total HA produced (µ by SRID) in one single 3L-bioreactor run	370	1843
Human dose (15 µg of HA measured by SRID)	25	123

The fact that nucleolin is expressed on the cell surface makes this protein a potential attractive target for VLPs purification. On the other hand, the reason why lactotransferrin was abundantly incorporated in the EVs remains unclear. The protein has antimicrobial properties and has been identified in different types of exosomes, like urine exosomes (Øverbye *et al.*, 2015) and other secreted fluids like seminal plasma (Batruch, Lecker *et al.*, 2011) and saliva (Ambatipudi, Swatkoski *et al.*, 2012). The HSP90 is a chaperone commonly identified in exosomes isolated from different tissues (Prodromou, 2016). The protein ALIX, that plays an important role in assisting the ESCRT complexes, was identified in the EVs but not in the VLPs, suggesting that perhaps the VLPs are not using this pathway for budding. However, this would require further

investigation given the low MASCOT score of this protein identification and the fact that several proteins could interact with the particles during their formation inside the cell.

CHAPTER 7 CONCLUSION AND RECOMENDATIONS

7.1 Conclusions

The traditional production approach for manufacturing influenza vaccines in embryonated eggs has limited capacity, is labor-intensive and time-consuming. Furthermore, current seasonal vaccines do not provide complete protection in all age groups. Therefore, vaccines with more immunogenic efficacy manufactured in high-production capacity platforms need to be developed. Virus-like particles (VLPs) can be formulated into promising vaccines to prevent influenza infection. In addition to having a structure that mimics the wild type virus, VLPs are safe since they are devoid of viral genes and consequently are not infectious. The surface antigens of VLPs synthesized in mammalian cells, will have human-like post-translational modifications similar to the wild type virus, and therefore will trigger a potent and specific immune response to the pathogen.

In the first part of the thesis, a HEK-293 cell line stably expressing HA and NA under the regulation of a cumate inducible promotor was developed with the aim to produce influenza VLPs. Our observations after transfecting the 293HA-NA cell line with a plasmid containing M1 influenza matrix protein support the previous results (Chen *et al.*, 2007) that M1 by itself is not the driving force of influenza virus budding. M1 did not increase protein release in comparison with the co-expression of HA and NA alone. In contrast to M1, the potency of Gag of HIV-1 as a powerful protein to promote influenza VLPs formation in mammalian cells was demonstrated in the present thesis. The transfection of 293HA-NA cells with Gag increased the production of VLPs antigenic proteins by 7-fold compared to its counterpart M1. The VLPs production process using the stable cell line 293HA-NA with transient transfection of a Gag containing plasmid was validated in a 3L-bioreactor. The influenza Gag-VLPs produced in the bioreactor were efficiently semi-purified by TFF at 1000 KDa cut-off with a recovery efficiency of 45%. Ultrafiltration membranes with a pore size as large as possible, but still capable of fully retaining the product must be selected for the concentration and semi-purification of VLPs, since more contaminants can be eliminated in the permeate. The VLPs production process in the 3L-bioreactor after transient transfection of 293HA-NA stable cells with the Gag-containing plasmid yielded 138µg/L of HA, 6.5 µg/L of NA and $6x10^{11}$ Gag-GFP fluorescent events/L as measured by the

SRID, Dot-Blot and flow cytometry quantification values, respectively. The ratio of NA:HA in the VLPs was determined as 1:40. Finally, the intranasal immunization of mice with influenza Gag-VLPs induced strong antigen-specific mucosal and systemic antibody responses, and provided full protection against a lethal intranasal challenge with the homologous virus strain.

In the second part of the thesis, having demonstrated the immunogenicity and protective efficacy of the VLPs, we aimed to streamline and make the VLPs production process more scalable. To this end, the Gag-GFP gene was put under the cumate inducible promoter CR5 and was stably transfected in the cell line 293HA-NA. This obviated the need for the transient transfection step, which is technically cumbersome to perform at large scale. Once the best producer cell clone of 293-HA/NA/Gag-GFP was selected, the goal was to increase the cell density at the time of induction to improve the volumetric yield of VLPs. After culture media selection and assessing the impact of medium replacement at small scale, we performed a perfusion run in a 3L-bioreactor. By increasing the cell density to $1.4x10^7$ cells/ml at the time of induction, the volumetric yield of VLPs proteins increased by 60-fold (Gag-GFP fluorescent events/ml), 17-fold (HA µg/L) and 102-fold (NA (µg/L) compared to the batch control. The 9.5L harvest broth collected from the bioreactor was clarified by normal filtration without the need of centrifugation steps since the VLPs containing supernatant is directly separated from the cells through the retention device. This fact is highly advantageous for large scale manufacturing, since large capacity centrifuge can be costly. The yields of VLPs proteins obtained in the 3L-perfusion bioreactor were 194 µg/L in terms of HA concentration measured by SRID, $3.1x10^{11}$ Gag-GFP events/L and 41 µg/L of NA quantified by Dot-Blot. Performing one 3L-bioreactor in perfusion mode with the stable cell line 293-HA/NA/Gag-GFP allowed to generate 5-fold more VLP antigenic protein HA than what was produced with the 3L- batch bioreactor using transient transfection.

Finally, considering the great concern associated with the heterogeneous composition of enveloped VLPs containing endogenous HCPs, DNA and RNA and the co-production with extracellular vesicles (EVs) of similar size, in the present thesis the proteomic composition of VLPs and EVs produced from HEK-293 was identified by n-LC-MS/MS. A total of 73 host cell proteins were identified in the VLPs, while 98 were detected in the extracellular vesicles. From that, 32 host cell proteins were unique of VLPs and 41 proteins were common between the two

particles. The indentified proteins were mostly involved in cell signalling pathways, protein translocation, folding and were associated to the cell cytoskeleton and nucleic acids.

7.2 Recommendations for future studies

The work performed in this thesis has also highlighted some critical aspects that call for more in depth-investigations:

- Link reporter genes to viral recombinant proteins involved in VLPs formation. This will help to develop more straightforward methods for the online monitoring of VLPs by using sensitive probes.
- Perform *in vivo* immunization of VLPs in other animal models like ferrets, use of lower doses, test the effect of different adjuvants and assess the efficacy of the VLPs produced with the 3L-perfusion bioreactor. Ferrets are better than mice as animal models for influenza since they have similar lung physiology than humans, developing similar clinical signs and transmissibility of the virus.
- Perform further optimization of the production process by studying metabolic fluxes and designing a rational fed-batch strategy that can improve the production. Also, different cell densities at the time of induction can be studied in the perfusion process to evaluate the effect of this parameter on the VLPs production
- Characterize the RNA, DNA and lipid composition of VLPs and exosomes produced in HEK-293 cells in order to have a complete description of the uptaken elements and ensure the safety of our vaccine candidate.
- Perform biophysical characterization of the VLPs and EVs (Dynamic light scattering (DLS), Nanoparticles tracking analysis (NTA), Atomic force microscopy (AFM) or Scanning electron microscopy (SEM))
- Develop new affinity chromatography methods to purify VLPs and EVs by binding antibodies against the proteins uniquely identified for both nanoparticles species in the present thesis.(e.g. make an immunoaffinity matrix by immovilizing an anti-nucleoline antibody, then pass the VLPs concentrated by TFF, wash and elute to recuperate the VLPs)

- To speed up the production process, other different approaches can be used: 1) Develop a stable cell line using CRISPR technology such that the variable antigenic proteins can be inserted and removed without the need to develop a new cell line from the beginning. 2) Develop a stable cell line expressing Gag protein and construct a plasmid/plasmids with enzymatic restriction sites that allow a fast removal/insertion of the antigenic proteins.
- Use this VLPs platform, where Gag protein of HIV acts as a core protein and the VLPs are produced in HEK-293 cells, for presentation of antigens of other diseases and for presentation of universal epitopes against influenza
- Perform stability studies of the VLPs and test different buffer formulations for better protein and structure conservation.

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