Hugo Miguel Lambuça Ferreira

Graduated in Cell and Molecular Biology

Overcome challenges in influenza virus-like particles downstream process

Dissertation to obtain the Master Degree in Biotechnology

Supervisor: Dr. Cristina Maria da Costa Peixoto Lisboa

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Overcome challenges in influenza virus-like particles bioprocessing

Aos meus pais ♥

ACKNOWLEDGEMENTS

À Professora Paula Alves, pelo conhecimento, interesse e paixão pelo que faz e pelo que transmitiu nas aulas durante o mestrado, que de certa forma contribuiu para ter realizado a minha tese nesta unidade.

Ao Professor Manuel Carrondo, pelo seu conhecimento, espírito de liderança e rigor na Ciência que inspira qualquer jovem estudante.

À minha orientadora, Dr. Cristina Peixoto, pela oportunidade que me deu de poder realizar a minha tese. Agradeço também pela paciência, apoio e por todos os conselhos ao longo deste ano.

À equipa do DSP aqui vão os agradecimentos. Ao Dr. Ricardo Silva por tudo o que me ensinou, todos os conselhos e discussão sobre este trabalho, assim como pela disponibilidade de ajuda. À Sofia Carvalho pelo que me ensinou, assim como pela partilha de resultados e discussão dos mesmos. À Mafalda Moleirinho por estar sempre pronta a ensinar e ajudar. À Sara Rosa pela paciência que teve para ter aturado todas as minhas perguntas e pedidos. À Sofia Moreira pelo apoio, conselhos e danças ao longo do ano. À minha colega de secretária, Ana Quendera por todas as risadas e conselhos. Ao João Mendes por todas as gargalhadas sobre tudo e de tudo. E ao Tiago pela prontidão em ajudar e ensinar. Obrigado a todos pelo bom ambiente e disposição.

Agradeço também aos restantes membros do TCA pela ajuda nos momentos em que precisei ao longo de todo este ano.

Quero agradecer também aos meus colegas de mestrado, Bernardo, Gonçalo, Vânia, Patrícia, Mariana, entre outros pelos momentos que por muito curtos que fossem dava sempre para “dois dedos de conversa”. Pelo apoio e desabafos ao longo do ano. Estamos juntos!

Agradeço também a todos os meus amigos, padrinhos e afilhados de faculdade por estarem sempre dispostos a ouvir-me e dispostos sempre a ajudar.

Agradeço à família anTUNiA por todos os momentos de folia que me fizeram espairecer este ano e pelos fins-de-semana bem passados.

Obrigado especial ao TM, Flávio e Ticas por tudo. Pelos desabafos, pelas lamentações e pelas infinitas gargalhadas. Muito Obrigado por estarem lá sempre e por serem os amigos que muitos gostariam de ter.

Agradeço também às minhas manas, Mónica e Inês. Por serem tudo e tudo. Por não estarem por perto, mas eu ter 100 % certezas de que estão. Muito Obrigado por me ouvirem e por estarem comigo sempre e para sempre.

Agradecimento muito, muito especial à minha Bárbara, por tudo o que partilhámos, pela força, incentivo, motivação e gargalhadas. Obrigado por todo o teu apoio. Sem ti, teria sido uma história bem diferente.

Agradeço à minha família, a de sempre e a nova. Aos meus padrinhos, primos, avô Lambuça e avó Quina por estarem lá sempre em cada fim-de-semana de visita e por me receberem sempre de braços abertos. Aos pais, tios e primos da Bárbara, em especial à afilhada e minha sobrinha Matilde.

À minha avó Lucinda e ao meu avô Ferreira. Onde quer que estejam estão sempre ao meu lado, dia-a-dia e sei que fizeram de tudo para esta tese acabasse bem.

Agradeço, por fim aos meus pais por terem confiado em mim ao longo destes 5 anos. Um especial obrigado pelo que me puderam proporcionar e por serem pessoas excecionais e espetaculares. Muito, muito obrigado. (Apesar de estar em inglês), É a vós que dedico este meu trabalho.

ABSTRACT

The development of new vaccines for influenza virus introduced a new generation of vaccines using virus-like particles (VLPs). The lack of genetic material, possibility of production on cell lines and presence of antigens with immunogenicity are the main advantages over the traditional vaccines. The development of a cost-effective downstream process while maintaining the high purity, potency and quality of VLPs is a challenge. In this thesis, several purification steps – clarification, concentration, chromatography, polishing and sterile filtration – were studied to develop a new downstream proves for influenza VLPs.

In clarification step, a strategy using D0HC followed by Opticap XL SHC filters presented the best result. For concentration step, the cassette with *cut-off* of 300 kDa presented a higher yield on hemagglutinin recovery and the lowest process time. For chromatography step, the membrane Sartobind® Q and the resin HiTrap Q HP were evaluated, concluding that resin HiTrap presented higher dynamic binding capacity and better resolution on elution. For polishing step, size-exclusion chromatography and multimodal chromatography operate in flow-through mode were compared. The last presented higher recovery yield on hemagglutinin and it was select due to the non-limitation for scale-up. Different materials were analysed for the final sterile filtration.

A proof of concept run was performed were the optimized conditions and best devices were evaluated. In the end of process, it was obtained influenza VLPs with concentration and quality enough to advance for animal *in vivo* studies and for clinical phase I.

Additionally, a new tool – magnetic sulphated cellulose particles – was evaluated with the goal to obtain purified and concentrated samples to use in characterization techniques.

Overall, this thesis contributes to introduce a new tool and a novel cost-effective downstream purification process with high purity, potency and quality for the next generation of influenza vaccines - VLPs.

Keywords: purification process; influenza vacines; vírus-like particles; magnetic sulphated cellulose particles.

RESUMO

O desenvolvimento de novas vacinas para o vírus de influenza introduziu uma nova geração de vacinas utilizando partículas semelhantes a vírus (VLPs). A ausência de material genético, possibilidade de produção em linhas celulares e presença de antigénios com imunogenicidade são as principais vantagens em relação às vacinas tradicionais. O desenvolvimento de um processo de purificação de baixo custo mantendo a elevada pureza, potencia e qualidade das VLPs é um desafio. Nesta tese, alguns passos de purificação – clarificação, concentração, cromatografia, polimento e filtração estéril final – foram estudados para desenvolver um novo processo de purificação de VLPs de influenza.

Na clarificação, a estratégia usando os filtros D0HC seguido do Opticap XL SHC apresentaram os melhores resultados. Na concentração, a cassete com *cut-off* de 300 kDa apresentou um maior rendimento na recuperação de hemaglutinina e o mais baixo tempo de operação. Na cromatografia, a membrana Sartobind® Q e a resina HiTrap Q HP foram avaliadas, concluindo-se que a resina apresenta maior capacidade de ligação dinâmica e maior resolução na eluição. No polimento, a cromatografia de exclusão molecular e a cromatografia multimodal, operada em *flow-through* comparadas. Esta última apresentou valores superiores de recuperação de hemaglutinina sendo escolhida por não conter limitações no escalamento. Diferentes materiais foram analisados na filtração estéril final.

Na realização da corrida de prova de conceito as condições ótimas e os melhores materiais foram estudadas. No final do processo, obteve-se VLPs de influenza na concentração e qualidade suficiente para avançar para estudos em animais *in vivo* e para fase clínica I.

Adicionalmente, uma nova ferramenta – partículas magnéticas de celulose sulfatada – foram estudadas com objetivo de obter VLPs purificadas e concentradas para utilização em técnicas de caracterização.

Em geral, esta tese contribuiu para introduzir uma nova ferramenta e um novo processo de purificação mais económico com elevada pureza, potência e qualidade, para a nova geração de vacinas - VLPs.

Palavras-chave: processo de purificação; vacinas de influenza; partículas semelhantes a vírus; partículas magnéticas de celulose sulfatada.

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LIST OF ABBREVIATIONS

CCI – Cell concentration at infection

CF – Concentration factor

CIP – Cleaning-in-place

CV – Column volumes

DBC – Dynamic Binding Capacity

DEAE – Diethylaminoethyl

DF – Diafiltration

DLS – Dynamic light scattering

FDA – Food and Drug Administration

FT – Flow-through

GMP – Good manufacturing practices

HA – Hemagglutinin

HBV – Hepatitis B virus

HCP – Host-cell protein

HEV – Hepatitis E virus

HFs – Hollow fibers

HIC – Hydrophobic interaction chromatography

hpi – hours post-infection

HPLC – High performance liquid chromatography

HPV – Human papilloma virus

IC/BEVS – Insect cells/Baculovirus expression vector system

IEX – Ion exchange chromatography

MF – Microfiltration

MOI – Multiplicity of infection

MSCPs – Magnetic sulphate cellulose particles

MTT – Microculture tetrazolium assay

MWCO – Nominal molecular weight *cut-off*

NA – Neuraminidase

NFF – Normal flow filtration

NMR – Nuclear magnetic resonance

NTA – Nanoparticle tracking analysis

PES – Polyethersulfone

PS – Polysulfone

PVDF – Polyvinylidene fluoride

Q – Quaternary amine

qPCR – real-time quantitative polymerase chain reaction

rBVs – Recombinant baculovirus

RC – Regenerated cellulose

RT – Room temperature

S – Sulfonic acid

SEC – Size exclusion chromatography

SFCA – Surfactant-free cellulose acetate

TEM – Transmission electron microscopy

TFF – Tangential flow filtration

TMP – Transmembrane pressure

UF – Ultrafiltration

VLPs – Virus-like particles

WHO – World Health Organization

yP – Permeate flux

CHAPTER 1

INTRODUCTION

1.1. Vaccines

According to World Health Organization (WHO), a vaccine "*is a biological preparation that improves immunity to a particular disease."* It *"contains an agent that resembles a disease-causing microorganism, and is often made from weakened or killed forms of the microbe, its toxins or on of its surface proteins.*"[1]. Improvement of immunity is one of the most important roles of vaccines. When administrated on humans or animals allow their immune system to recognize these particles and answer with a specific immune response. If a pathogenic microorganism causes an infection, the immune system, that already recognizes the pathogen, will be faster to induce the specific and right response to eliminate it and prevent any kind of pathology.

Edward Jenner gave the first concept of the vaccine in the 18th century when smallpox virus was a serious world threat. With the advance of technologies all over the years, vaccines had an evolution on manufacturing. Most of all are based on live-attenuated or chemical-inactivated infectious viral strains[2]. However, the new generation of vaccines are raising: recombinant proteins, viral vectors, DNA-based vaccines and virus-like particles (VLPs)[3]. This last one is described as the promising alternative to actual vaccines[4–6]. Three vaccines, based on VLPs, are already on market with successful application for hepatitis B virus (HBV), human papilloma virus (HPV)[2,6–9] and hepatitis E virus (HEV)[9]. They were accepted by Food and Drug Administration (FDA) in 1986, 2006 and 2011, respectively[4,9].

Vaccines are very important as a market health-care tool due to: (i) the emerging of markets that search for new and more efficient vaccines; (ii) the significant importance to solve problems where medical need are unperformed; and (iii) the possibility of treating illness without cure that can be prevented with vaccination because of the advance on health areas like immunology and microbiology[10,11].

The magnitude of infectious diseases' challenge calls the attention of pharmaceutical industry for investment on vaccines development – considered the major tool to prevent it. However, the stringent guidelines from the regulatory authorities (FDA) demand that the product respect some

criteria as purity, potency, quantity, and others[12]. For purity, several tests need to be performed along the manufacture of the product. Two types of impurities exist, product-related (product aggregates, molecular variants, degraded products) and process-related (Host-cell protein (HCP), host cell DNA, media components, enzymes/chemicals). For potency, the tests are related to the cell-based, which is measure the biochemical or physiological response at the cellular level or animal-based which the measure the biological response from organism to the product respectively. The quality is related to the protein content (in mass) that should be determined using appropriate assays[12].

1.1.1. Influenza vaccines

Native influenza virus belongs to the *Orthomixoviridae* family that presents segmented RNA genome and it is composed of surface glycoproteins, Hemagglutinin (HA) and Neuraminidase (NA). It is surrounded by lipid membrane – coming from virus budding process on infected cells – and that's why it is characterized as an enveloped virus. The core proteins are M1 protein – confer structure to the envelope and virus capsid – and M2 protein – an essential ion-channel.

Every season, influenza virus causes around 5-15% of infection on northern hemisphere's population, according to WHO[13]. Therefore vaccination plays a major role in the prevention of influenza virus' infection[8,14]. For several decades (and even nowadays), influenza vaccines were made from live-attenuated or chemical-inactivated virus obtained by infection of chicken fertilized eggs[2]. This manufacturing process is so complex and slow that in case of a pandemic event it cannot answer in time to prevent or fight the virus infection[3,6,8,11,15].

Influenza vaccines are compromised by the weak initial immunogenicity and efficacy, mainly because of two facts: the virus strains present on vaccine (do not have a good match with the circulating virus) and the antigenic drift of influenza virus[6,8]. This last one promotes the change of the proteins of virus' surface specially the most abundant – HA[5,8]. Consequently, a new strain of influenza virus appears every year[5,9]. One example is the pandemic H1N1 influenza virus in 2009 that was caused by genetic reassortment between different species of avian, swine and human origin[9,16,17].

The challenge is developing vaccines and producing-platforms with more efficiency than the actual ones[3,8] never forgetting the main goal on vaccinology – getting an "universal" influenza vaccine with the capacity to prompts an immunity response, and that can respond to all influenza strains, be administrated just one time in life and can be produced fast and in controlled conditions[3]. In answer to that, a new generation of vaccines are approaching, and some examples are summarized in Fig. 1.1.

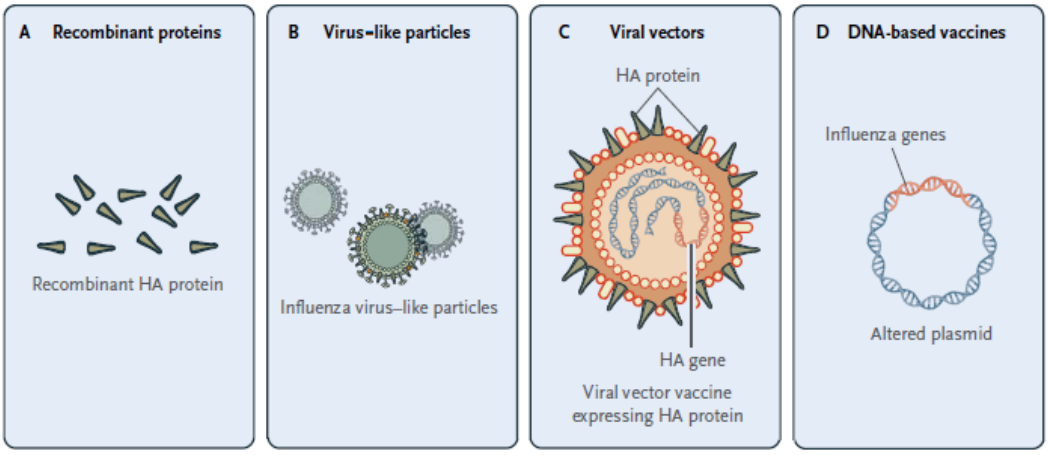

Figure 1.1 - New generation of influenza vaccines. (A) Recombinant proteins, (B) Virus-like particles (VLP), (C) Viral vectors, (D) DNA-based vaccines. (Adapted from literature[3])

These technologies achieve a revolution step because of their manufacturing process, that are made in cell-based technology, replacing the egg-based one[3,11]. Some new generation of vaccines are already in early stages of development with the aim of manufacturing time-reduction and cost-effective as well as efficacy and safety improvement.

1.1.2. Influenza virus-like particles

VLPs are self-assembly structures that can be similar especially on structure namely in the organization and conformation to virus particles[18]. However, VLPs shows many advantages: (i) do not have viral genome inside the particle – preclude the replication; (ii) can be produced in cell lines – bacteria, yeasts, plants, mammalians and insects; (iii) own active antigens – have immunogenicity response that does not need adjuvants[8,19]. The focus of this thesis will be influenza VLPs.

Influenza VLPs presents the same size (80-120 nm[18]) and a lipid envelope alike the virion. On this envelope are contained the antigens - HA and NA - that stimulate the antigenic response from immune system to VLPs. That is one of the reasons why VLPs can be used for vaccine application. However, on VLPs structure just HA exists due to the low immunity response of NA on vaccines[5,20]. Besides that, the combination between HA and M1 is sufficient to generate a functional VLPs with immunogenic properties[21].

The influenza VLPs' envelope allows these ones to follow the methodology of releasing from cell – by the budding process. Instead of a lytic process, where virus lysis the cell where it was produced, a budding process where cells are not lysed, and VLPs will get lipid bilayer derived from cells plasma membrane[22]. This allows VLPs to get some characteristics like the recombinant baculovirus (rBVs) - used on VLPs production (have the same releasing process of insect cells).

Some new tries to produce VLPs were studied with different cells (Since Sf-9, High Five, CHO, HEK293, *Escherichia coli*, *Saccharomyces cerevisiae* to Potato and Tobacco), expression systems (Insect Cells/Baculovirus Expression Vector System (IC/BEVS), Yeast, Mammalian cells, Bacteria and Transgenic plants), and specifics recombinant proteins (several antigens from different virus)[4]. Focus on production of influenza VLPs are, in all founded examples, described on Insect cells (Sf-9 and High Five cell lines)[4].

The high complexity of influenza VLPs, the presence of lipid envelope and the need of post-transcriptional modifications requires a eukaryotic host[23,24]. Sf-9 insect cells are from *Spodoptera frugiperda* pupal ovarian tissue and are very useful for the generation and amplification of rBVs. In another hand, High Five™ insect cells are from cabbage looper ovary of parental *Trichoplusia ni* and are more efficient to produce recombinant proteins using IC/BEVS with higher yields[25] and higher complexity[24]. About the influenza recombinant proteins, this production is based just on the required proteins to generate an immune response – HA and M1 proteins[4,19,26,27].

IC/BEVS is a powerful weapon used for influenza VLPs production[28]. It presents advantages: not able to replicate in mammalian cells and are approved by regulatory authorities[8,28,29]; easy to scale-up on influenza VLPs production in answer to a pandemic scenario or to a new emerging strain every year[25]; able to perform post-transcriptional modifications[4,19] (however not similar to the mammalian cells[6,8,15]); able to grow in serum- and protein-free media without CO_2[7] and have capacity to accommodate and express large and multiple foreign protein genes[30,31]. However, as bottlenecks are the viral stock maintenance where infectious particles titer decreases gradually with time[32], as well as, after the downstream process, the presence of rBVs on the final vaccine product that is still a big challenge to overcome[15,19,25,27].

1.2. Downstream process of virus-like particles

The downstream process is an important step for biopharmaceutical however and compare with the upstream step, it is the most cost-intensive step due to the values that can reach 70 % of total process[33–35], what make the combination with cost and yields/impurities reduction an important aspect to be in count. The main goal is to remove several product-related and process-related impurities, with several examples: media-components, host cells impurities (as cells debris, proteases, DNA, proteins, among the others), other components added during the upstream or downstream process (as for example, nucleases) and rBVs. Essentially some efforts were done to minimize the difficulty on the downstream process, as the usage of serum-free media mainly because of animal-derived supplements[4] even as the usage of a nuclease (Benzonase® endonuclease) on culture bulk[4,23,36,37].

Nonetheless, the utilization of disposable membrane technology (including tangential flow filtration (TFF)), as well as traditional techniques like size-exclusion, ion exclusion and affinity

chromatography is the new trends for utilization on downstream process[4]. However, when it is necessary to design a train for downstream process, the main goal should remain on get a product with high purity (without all impurities related to the production and process), high potency (reach a high concentration sufficient for a vaccine dose) and high quality (all particles are in suitable conditions for inducing immunogenic response). With that, the process should be robust and scalable.

In order to get the desired product and process for influenza VLPs, a downstream process train (see figure 1.2.) is evaluated, on this thesis, where new approaches are performed and characterized for this kind of biological material (VLPs). Due to the centrifugation scale-up and high-cost limitations, the train starts with the clarification step where is evaluated depth filtration, microfiltration and centrifugation (for comparison). On concentration step is evaluated the concentration factor and diafiltration volumes optimal for getting a concentrated sample. Afterwards, chromatography step is performed by ion exchange chromatography (IEX) with a resin chromatography column. In another hand, and instead of concentration step, influenza VLPs can be purified by an IEX performed by membrane adsorber (chromatography step – right side on figure 1.2). After each chromatographic step, a polishing step will be performed with two different approaches - size-exclusion chromatography (SEC) and multimodal chromatography. For the last, the sterile filtration step where will be evaluated different types of filters.

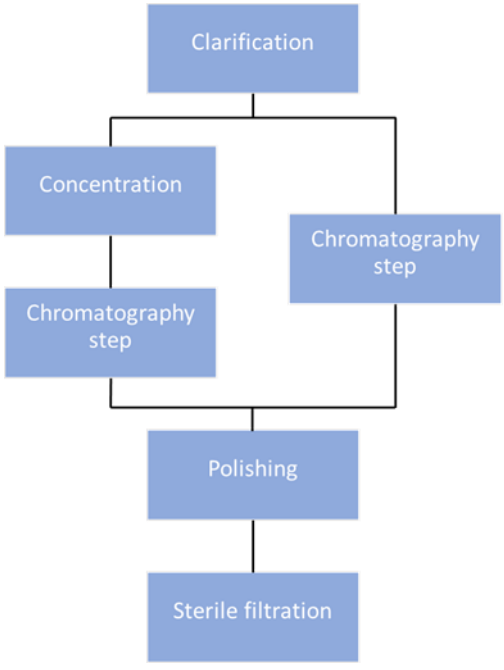

Figure 1.2 – Downstream process train evaluated for influenza VLPs.

1.2.1. Clarification

Clarification is considered for some authors a part of upstream process[36] and for others is considered the first step on a downstream train[38], so it is important that some bulk characteristics (as cell density and viability, nature of product release through budding process or cell lysis[15,36]) should be considered to get satisfactory yields and high impurities removal. This step has on its base the separation of the final product from all cells debris, solids and some process impurities (DNA and HCP) present on harvest bulk[23]. In clarification, some technologies can be used as examples, centrifugation, depth filtration and membrane filtration (including microfiltration (MF))[23,36,39]. Normally, this step is composed by two phases: the first one is essential to remove cells debris and large solids from the product; the second one is important to remove some others small impurities[36].

The productions of VLPs by IC/BEVS have some characteristics: (1) the rBVs has rod-shaped form (60-80 nm of width and a length of 300-400 nm) – they are similar in size to VLPs; (2) the production can reach high cell culture densities; (3) the large quantities of host cell nucleic acids; (4) the large quantities of proteins come from the culture medium and cells. All of these characteristics present some challenges in clarification step. Nevertheless, it should guarantee the possibility of removal of some impurities (HCP, DNA and rBVs)[36].

The addition of Benzonase® – an engineered endonuclease – is normally used on cells culture to digest nucleic acids existents into smaller ones, with the aim to facilitate the clarification[23,36,40,41], despite that it is a very expensive step. At the same time, the addition of antiproteases is essential, mainly for the safety of the desired product, in this case, the influenza VLPs[39].

In cases of bulks with high cell density[36] and high viscosity (like influenza virus prevenient from chicken embryos eggs[36,42]), centrifugation is sometimes performed on the first phase of clarification step[23]. The good recoveries of product and the good removal of DNA and proteins are one of value point of this methodology. However, the high costs and the difficulty to implement this technology on scale-up are the main disadvantages for this technology[36].

MF is an alternative to centrifugation[43,44]. The greatest advantages of this technology are the easiest scale-up, very cost-effective and the high product recoveries. This technology operates in TFF with a pore size range of 0,1-0,65 μm and it is preferable for primary clarification step[36]. Some devices as flat membranes and hollow fibers (HFs) is normally used for this step[39,43], as performed and indicated for HPV VLPs[23]. Moreover, MF can be operated in continuous mode[44]. In another hand, depth-filtration operate in normal flow filtration (NFF) (dead-end mode) and it was utilized for rotavirus-like particles[45] and canine adenovirus[37]. Some advantages of this filtration method are: it is a disposable technology; it is easily to scale-up and it has the potential to retain some impurities like DNA because of the membrane is positive-charged[36,43,46].

Furthermore, some of these membranes are constituted as multi-layer structures with a pore size range of the filter in a way to optimize the removal of cell debris and other impurities already mentioned[43] and confer a high capacity to the filter that can present an advantage for this technology[36].

1.2.2. Concentration

After clarification, a large volume is obtained and it needs to be processed and purified. For concentrated clarified bulk, “traditional” methods like utilization of ammonium sulphate and polyethylene glycol[23] are described for several VLPs. But, because of the several disadvantages presented, like time-consuming, cost-intensive, the difficulty to scale-up and others, the volume reduction – ultrafiltration (UF) – is normally performed. UF is followed by a change for formulation buffer – diafiltration (DF) – that is important to guarantee that VLPs are on the desired buffer to be utilized in the further downstream steps. UF/DF can be made by using devices like flat sheet cassettes and HF cartridges. The membranes of these devices can be constructed from a wide variety of polymers: polyethersulfone (PES), polyvinylidene fluoride (PVDF), polysulfone (PS) and regenerated cellulose (RC)[47].

UF/DF is performed in TFF, it means that the flow is perpendicular to the membrane (cross-flow filtration), which avoid the retention of retained particles[48]. This step is normally performed with transmembrane pressure (TMP) (see equation 2.1) constant. However, permeate flux (yP) and pore size are parameters to be in count when the goal remains on to get high yields on VLPs concentration. The pore size of membranes is mainly given as nominal molecular weight *cut-off* (MWCO) defined as the molecular weight of solute that has a retention on membrane with a coefficient of 90%. A large range – between 100 and 1000 kDa – are available on market and described for several VLPs purification/concentrations as HIV gag-VLPs, rota-VLPs, cytomegalo-VLPs and enterovirus 71 VLPs[23]. To scale-up this step it is important to remain constant the TMP, feed flux and concentration profiles along the length of the filtration module[43,49].

Considering that VLPs and viruses are sensitive particles, the shear rate is a parameter to be in consideration to get an efficient and higher-yield process[40]. The higher shear rate can increase the risk of deformation or disassembly of particles[23,50,51] due to it is a characteristic that is directly associated to the cross-flow on the membrane and to the feed flow paths[40,43]. So, comparing HFs to cassettes, the lowest shear rate is described on HFs[40,43], what makes it preferable to process this step on “live vaccines”[40]. But, cassettes can present a less processing time and a large flow channel that allow a fast-clean step and avoid (in comparison with HFs) gel layer formation and concentration polarization[40,43].

1.2.3. Intermediate purification

Intermediate purification is accomplished by using chromatographic methods based on adsorption methods that include IEX and hydrophobic interaction chromatography (HIC). These kinds of chromatography operate in bind-and-elute or a new approach – in FT mode[52,53]. With that, and as an alternative to concentration step and after it, an IEX is an option to consider. IEX and HIC matrices have functional groups positively charged that promote the adsorptions of the virus and VLPs. Some examples present on market are quaternary amine (Q), diethylaminoethyl (DEAE) and sulfonic acid (S). In literature, IEX is described as an excellent method to capture (bind-and elute mode) the influenza virus[54,55]. Based on that, functional group Q is evaluated on this thesis, not only on membrane adsorbers (in the alternative to concentration step) but also on resin (after concentration step) (see figure 1.2.).

In the market exists some devices as membrane adsorbers, monoliths and resin beads that can be used for capture VLPs. Some characteristics are important to guarantee a high-yield purification, highlight the morphology (membrane adsorbers, monoliths and resin beads) and pore size (that can be from 13 nm to 6 μm – depending on manufacturer – on membrane adsorbers and monoliths; to 10-100 nm in case of resins beads)[40] . Another characteristic is the mode of operation that can be in positive mode – VLPs adsorbing to the medium – or negative mode – VLPs do not adsorb to the medium and are present on flow-through (FT)[54].

For scale-up an IEX step, it is necessary to determine the dynamic binding capacity (DBC). DBC is the amount of VLPs that binds to the medium – at the moment when the outlet reaches the initial concentration of VLPs – per volume of resin or membrane[56]. This characteristic allows to get minimal losses of VLPs on FT, that can be described in 1 % or 10 %. In membrane adsorbers and monoliths it is an advantage mainly because of the high value of DBC due to the convective material transport (that make DBC independent of flow rate value[57]) with no diffusion limitations[40]. Even so, the DBC of monoliths is in general higher than membrane adsorbers[40]. However, due to the high diffusion limitation and internal surface area, for virus particles, beads have lower DBC values[57].

A new approach for capture the influenza virus particles with magnetic sulphate cellulose particles (MSCPs) was developed and further compare with other techniques such as centrifugation[58] and previously tested for inducing immunization on mices[59]. In this thesis, this methodology will be evaluated as capture step for influenza VLPs after clarification step.

1.2.4. Polishing step

In the last step of the downstream train for influenza VLPs the polishing and the sterile final filtration steps are included. The main goal is getting the most purify product with high quality to

clinical applications. The several impurities that still remain on influenza VLPs (proteins, DNA and rBVs) must be removed on this step. Chromatography or UF/DF is usually performed. These steps include the conditioned to final formulation buffer, where VLPs are more stable and potency and quality are not compromise.

In chromatography, SEC[42,54] or multimodal chromatography[37] operating in negative mode could be used. As influenza VLPs are large molecules[54], the purification on SEC is determined by the differences in the size of VLPs and impurities. In multimodal chromatography, where a SEC combined with hydrophobic and positively charged octylamine ligands is perform, the VLPs will be eluted in FT while impurities will link to the ligands. However, due to the similar sizes and/or surface charge at given pH, the rBVs are still a challenge in the downstream process of VLPs[23,39].

One challenge of SEC is the difficulty to scale-up, mainly because with increasing the sample volume, higher column volume is used (because the feed volume should not exceed 10% of total column volume). Furthermore, it is high time-consumption and low cost-effective. However, continuous mode of operation with SEC is finding on literature for adenovirus[60] and influenza virus[61]. Instead of SEC, UF/DF is easy to scale-up due to in case of an increase of sample volume, it is possible to upgrade membrane area (with a continuous process or with other devices with large membrane area existents on market). In case of antibodies purification, the UF/DF is where the final conditions (final concentration and formulation buffer) are achieved[62].

Multimodal chromatography operates in FT mode (negative mode), where the exclusion limit and the core composed of functionalized beads with octylamine ligands are the main characteristics of these devices[63]. They are mainly described on literature related to purification steps of virus and VLPs[38,53,64,65]. In the examples, the impurities are separated from the final product mainly because that can access to the core and are adsorbed to the ligands until virus and VLPs pass in FT[63].

The final sterile filtration is composed of 0,22 μm filters that confer the sterility of the final product. This is a demand of FDA, that in several cases just can be achieved through this step. The polymer that composes these filters can be PES, PVDF or RC[43].

1.3. Aim of the thesis

The optimization, design and development of a downstream process for influenza VLPs are still a challenge to overcome, mainly on chromatographic steps that are consider the major bottleneck. Nevertheless, on this thesis a new purification train for influenza VLPs was developed, from clarification to final sterile filtration, using different strategies and different devices.

Starting in clarification it was evaluated different approaches, mainly depth filtration and centrifugation. In concentration step, it was evaluated different devices existents on market (like

HFs and flat sheet cassettes) to achieve a fast, high-yield and cost-effective step. In both cases, recovery of HA, impurities removal and others parameters were determined. To overcome the bottleneck on chromatography step, two different devices (membrane adsorbers and resins) were evaluated in different stages of VLPs purification. In both cases, some critical parameters were determined, as DBC, elution and load condition. For the lasts steps, polishing by SEC was compared with a new strategy – by multimodal chromatography. On sterile filtration, the evaluation of different filters was evaluated have in consideration the different polymers that compose the filters and the filtration area. All over the process the removal of process-associated impurities was monitored by different analytical tools, in a way to guarantee the purity of the final product.

In parallel, a new tool using MSCPs was evaluated for influenza VLPs. The goal was getting a final sample that can be used for several analytical tools for characterization, as dynamic light scattering (DLS), transmission electron microscopy (TEM), high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) and others. For that, it was evaluated the buffer that confers better recovery yields and the better ratio between beads mass and influenza VLPs concentration.

This thesis aims to develop and evaluate a new downstream train for purification of influenza VLPs, passing through developing new strategies and determine the better conditions to improve productivity, reduce costs of the whole downstream train, focus on getting a process scalable and obtain a final product with high purity, potency and quality.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell maintenance

Gibco® Sf9 cells cloned from parental IPLBSF-21 (Sf21) cell line that derived from the pupal ovarian tissue of *Spodoptera frugiperda* (Sf9 cells) (11496015, Gibco™) were used for rBVs expansion in Sf-900™ II SFM medium (10902104, Gibco™). Cells were passaged at a concentration between 2-3 x 10^6 cells mL^{-1} and were inoculated at 5 x 10^5 cells mL^{-1}.

For influenza VLPs production, High Five™ Cells (BTI-TN-5B1-4) from parental *Trichoplusia ni* cell line (B85502, Gibco™) were used in Insect-XPRESS™ Protein-free Insect Cell Medium with L-glutamine (BE 12-730Q, Lonza Group Ltd). Cell passage occurs when cells achieve cell concentration between 2-3 x 10^6 cells mL^{-1} and were reinoculated at 3 x 10^5 cells mL^{-1}.

For cell counting and viability determination, a Fuchs-Rosenthal haemocytometer (Brandt, Germany) was used, following the trypan blue exclusion dye method with 0.1 % trypan blue (111732, Merck Millipore). Cedex HiRes Analyzer (Roche Diagnostics GmbH, Germany) was also used for the same purpose above mentioned.

2.2. Recombinant baculovirus expansion

The rBVs used was kindly provided by EDUFLUVAC partners. rBVs stock was expanded using Sf9 cells in Sf-900™ II SFM medium (10902104, Gibco™) in a working volume of 50 mL. Cells were infected with rBVs at a cell concentration at infection (CCI) of 1 x 10^6 cells mL^{-1} and multiplicity of infection (MOI) of 0.1 infectious particles per cell. The harvest was performed by centrifugation using Centrifuge 5810 R (Eppendorf AG, Germany) at 200x *g*, 4 ºC for 10 minutes. The rBVs stock was obtained after supernatant collection and stored at 4 ºC covered with aluminium foil to be protected from the light.

2.3. Recombinant baculovirus titration

rBVs titration was performed by Microculture tetrazolium assay (MTT) following the protocol described elsewhere[66]. Briefly, it was used Falcon® 96-well Cell Culture Microplate (353072, Corning Inc.) for two different purposes: to be cultured with cells and implemented in the assay; The others microplates were utilized to prepare the rBVs dilutions. Triplicates of each plate were performed. Firstly, on cells microplates, it was inoculated 5.0 x 10^4 of Sf9 cells per well. These plates were incubated at 27 ºC for 1 hour to guarantee that cells adhered to the bottom. Serial dilutions in a range between 10^{-1} to 10^{-11} of rBVs stock were made with Sf-900™ II SFM medium (10902104, Gibco™). On cells microplates, the culture medium was removed and added 100 μL of corresponding rBVs dilution. In the end, plates were incubated at 27 ºC for 6 days.

Thiazolyl blue tetrazolium bromide solution was prepared by dissolution of the powder (M5655, SIGMA-ALDRICH®) in ultrapure H_2O (Mili Q water - 18.2 MΩ cm^{-1} at 25 ºC) at a concentration of 5 mg mL^{-1} and filtrated with Acrodisc® Syringe Filter 0.2 μm (PN4612, Pall Corporation). 10 μL were added to each well. The plates were incubated for 4 hours at 27 ºC. Then, the medium was removed and 150 μL of dimethyl sulfoxide (D4540, SIGMA-ALDRICH®) were added to each well. The plates were agitated in Wellmix shaker WM-506 (Denley, Needham) for 10-20 minutes to dissolve the crystals and the absorbance at 570 nm and 690 nm was read by Infinite® 200 PRO NanoQuant (Tecan Group Ld, Switzerland) multimode microplate reader. Data were analysed with GraphPad Prism software (GraphPad Software, Inc) to obtain the rBVs titration.

2.4. Influenza virus-like particles production

For influenza VLPs production, High Five™ cell culture was used. The cells were thawed in Insect-XPRESS™ Protein-free Insect Cell Medium with L-glutamine (BE 12-730Q, Lonza Group Ltd) and maintained in culture. The production of influenza VLPs was performed on a 3000 mL Erlenmeyer (431252, Corning Inc.) with a working volume of 1250 mL. To achieve this volume, a pre-inoculum in 2000 mL Erlenmeyer (431255, Corning Inc.) with a working volume of 300 mL was used. The cell culture was infected with rBVs from virus stock as a CCI of 2 x 10^6 cells mL^{-1} and a MOI of 1 infectious particles per cell.

At 60 hours post-infection (hpi), cOmplete™ EDTA-free Protease Inhibitor Cocktail (05056489001, Roche Diagnostics GmbH) at a final concentration of 1 tablet per 50 mL of cell culture were dissolved in 25 mL of culture media Insect-XPRESS™ Protein-free Insect Cell Medium with L-glutamine (BE 12-730Q, Lonza Group Ltd). This mixture was filtered in 0.22 μm Stericup-GP (SCGPU02RE, Merck Millipore) and then Benzonase® (101656, Merck Millipore) was added at a final concentration of 50 U mL^{-1}.

The harvest occurred when cell viability reached 50-60 % and the concentration of HA was higher or equal to 0.7 μg mL^{-1}.

2.5. Influenza virus-like particles downstream processing

2.5.1. Clarification studies

The bulk (1250 mL) was clarified in two different steps. For the first step, Millistak+® Depth Filter in μPod™ Format (MD0HC23CL3, Merck Millipore), Sartopore® PP3 SartoScale 47 (5055306PS--FF--MM, Sartorius) and centrifugation were used. For the second step, Opticap® Sterile XL Millipore Express® SHC (KHGES015FF3, Merck Millipore) and Sartopure® 2 SartoScale 47 XLG (5445307GS--FF--M, Sartorius) were utilized.

Centrifugation was performed on Centrifuge 5810 R (Eppendorf AG) at 200x *g* and 4 ºC for 10 minutes. Regarding filtration, a Tandem 1081 Pump (Sartorius Stedim Biotech) was used to control the feed flow rate, as well as a MasterFlex® 16 tubes (MasterFlex Group) with 3.1 mm internal diameter. To control pressure through the filters, in-line pressure transducer (080-699PSX-5, SciLog) was used. The filtered volume was monitored over time using a technical scale (TE4101, Sartorius Stedim Biotech). All of the filtrations were performed at constant feed flow rate, as described in table 2.1.

Table 2.1 – Description of the area, pore and feed flow rate of all used filters on clarification studies.

Filter name	Filter area (cm^2)	Filter pore (μm)	Feed flow rate ($mL\ min^{-1}$)
Millistak+® Depth Filter in μPod™ Format	23	0.45	23
Sartopore® PP3 SartoScale 47	17.3	0.45	17.3
Opticap® Sterile XL Millipore Express® SHC	140	0.5/0.2	140
Sartopure® 2 SartoScale 47 XLG	17.3	0.8 + 0.2	17.3

It is important to emphasize that first, all filters were wet with ultrapure H_2O (Mili Q water - 18.2 MΩ cm^{-1} at 25 ºC). The equilibration step and clarification operation were performed using a buffer (50 mM HEPES, pH 7.4). Also, in the beginning, and at the end of the process, turbidity was measured by 2100Qis Portable Turbidimeter (2100QIS01, HACH®).

2.5.2. Concentration studies

Concentration and diafiltration of influenza VLPs were evaluated by two types of membranes modules designs: HFs and cassettes. The HFs used were MidGee Ultrafiltration Cartridge with 1.0 mm internal diameters fibers and 0.0016 m^2 fibers area (500000 MWCO pore size (UFP-500-

E-MM01A) and 750000 MWCO pore size (UFP750EMM01A), GE Healthcare). The cassettes used were Pellicon XL Ultrafiltration Module Biomax 0.005 m^2 membrane area (300 kDa (PXB300C50) and 500 kDa (PXB500C50), Merck Millipore). Both cassettes and HFs were set up accordingly with the manufacturer's instructions. A Tandem 1081 Pump (Sartorius Stedim Biotech) was used, with MasterFlex® 16 tubing (MasterFlex Group). The retentate was recycled to the feed container and, at the same time, the permeate was collected separately. To control and set up the TMP (see equation 2.1), an in-line pressure transducer (080-699PSX-5, SciLog) was used. The TMP was controlled over time by adjusting the retentate flow rate using a flow restrictor valve. Feed/retentate and permeate volumes were monitored over time using technical scale (TE4101, Sartorius Stedim Biotech).

$$\mathrm{TMP} = \frac{(\mathrm{P_{feed}} + \mathrm{P_{retentate}})}{2} - \mathrm{P_{permeate}} \quad (2.1)$$

Every device was firstly washed with ultrapure H_2O (Mili Q water - 18.2 MΩ cm^{-1} at 25 ºC) to eliminate trace preservatives. Then, each one was equilibrated with buffer (50 mM HEPES, pH 7.4). The initial volume to perform UF and DF of influenza VLPs clarified bulk was carried out considering a ratio of 3 mL of feed volume per each cm^2 of membrane area. In all experiments, the volume of bulk was reduced 5-fold and diafiltrated other 5 times. DF was performed with the same buffer of equilibration (50 mM HEPES, pH 7.4).

Before starting, bulk recirculated (capped the permeate side of membranes) for 5 minutes. Over the process of UF/DF, the TMP was adjusted to 0.3 bar for HFs and 0.8 bar for cassettes, as well as, some samples were taken to analyse the recovery of HA quantity and the removal of impurities. At the end of the process, all the devices were submitted to cleaning-in-place (CIP) procedure. For HFs 0.5 M NaOH was added, followed by a 60 minutes incubation, and for cassettes, 0.1 M NaOH was added and subjected to the same time of incubation.

All these procedures were made at room temperature (RT) (20-22 ºC) and the final product of UF/DF was injected at chromatography column.

2.5.3. Chromatography studies

The chromatography studies were performed by two different devices, a membrane and a packed-resin.

Membrane chromatography step was performed by Sartobind® Q nano 1 mL (96IEXQ42DN-11--A, Sartorius) membrane adsorber operated in positive mode (elution) at RT (20-22 ºC). This membrane adsorber was functionalized with a Q ligand. The runs were performed on ÄKTA Explorer 100 liquid chromatography system (GE Healthcare) equipped with UV and

conductivity/pH monitors. System operation and data gathering/analysis were done using the UNICORN™ 5.01 software (GE Healthcare).

For DBC determination, the FT was analysed to guarantee that membrane adsorber was saturated. For this analysis, a buffer (50 mM HEPES, pH 7.4) was used for equilibration step and for running of experiments. The flow rate was set to 2 membrane volume per minute. To ensure the recuperation of all particles, an elution buffer (50 mM HEPES + 2 M NaCl, pH 7.4) was used. VLPs concentration along these fractions was determined by HA assay (see section 2.6.1.). Regeneration step was performed by 1 M NaOH.

In packed-resin chromatography step, HiTrap Q HP (17-1154-01, GE Healthcare) with 5 mL bed volume was used. It was functionalized with a Q ligand and operated at positive mode (elution). The runs were performed on ÄKTA Explorer 100 liquid chromatography system (GE Healthcare) equipped with UV and conductivity/pH monitors. System operation and data gathering/analysis were performed using the UNICORN™ 5.01 software (GE Healthcare).

UF/DF product was injected to determine DBC of the column. The runs were performed at a flow rate of 150 cm h^{-1} and at RT (20-22 ºC). Buffer (50 mM HEPES, pH 7.4) was used in equilibration step and during the experiment, while elution buffer (50 mM HEPES + 1 M NaCl pH 7.4) was used to recover all HA linked to the resin.

The FT fractions were collected over time and HA assay was performed (see section 2.6.1.) to understand when the resin was already fully saturated. Elution was executed in gradient over 20 column volumes (CV) and fractions were analysed to evaluate at which NaCl concentration influenza VLPs start to elute off the resin. All those fractions were analysed to quantify the major impurity present. Regeneration of HiTrap Q HP was made with 1 M NaOH at same feed flow rate and temperature performed on runs.

2.5.4. Polishing studies

Polishing studies were evaluated through Sepharose™ 4 Fast Flow resin (17014901, GE Healthcare) based on SEC and HiScreen™ Capto™ Core 700 (17-5481-15, GE Healthcare) with the principle of multimodal chromatography. The runs were performed on ÄKTA Explorer 100 liquid chromatography system (GE Healthcare) equipped with UV and conductivity/pH monitors. System operation and data gathering/analysis were performed using the UNICORN™ 5.01 software (GE Healthcare). Sepharose® 4 Fast Flow was packed on XK 16/20 Column (28988937, GE Healthcare) with 33.9 mL of bed volume.

The samples used for injection on both polishing column were collected from HiTrap Q HP column or Sartobind® Q nano 1 mL (see section 2.5.4.). The equilibration and experiment run buffer was the same (50mM HEPES, pH 7.4) for both columns. The flow rates performed were

different and column dependent. For Sepharose® 4 Fast Flow a flow rate of 119 $cm.h^{-1}$ was performed while for HiScreen™ Capto™ Core 700 a flow rate of 258 $cm.h^{-1}$ was used. Due to the characteristics of this last column, an elution buffer was used (50 mM HEPES + 1 M NaCl, pH 7.4).

All fractions (from FT and elution) were collected and analysed for HA quantification and impurities presence through corresponding analytical methods (see section 2.6.).

2.5.5. Final sterile filtration studies

On the final sterile filtration step, several syringe filters were evaluated (table 2.3). The various filters devices are described by filter area and filter material: RC, hydrophilic PES, hydrophilic PVDF and surfactant-free cellulose acetate (SFCA).

Table 2.2 – Syringe filters devices used with correspondent filter area.

Syringe filters devices	**Filter Material**	**Filter area (cm^2)**
Whatman® SPARTAN® RC 30 syringe filters pore size 0.2 *µ*m (10 462 960, GE Healthcare)	RC	5.7
Millex-GP Syringe Filter Unit, 0.22 *µ*m (SLGP033RS, Merck Millipore)	Hydrophilic PES	4.5
Millex-GV Syringe Filter Unit, 0.22 *µ*m (SLGV033RS, Merck Millipore)	Hydrophilic PVDF	4.5
Minisart® NML Syringe Filter, 0.2 *µ*m (16534--------GUK, Sartorius)	SFCA	6.2
Minisart® High Flow Syringe Filter, 0.22 *µ*m (16532----------K, Sartorius)	PES	6.2
Acrodisc Syringe Filters, Sterile, 0.2 *µ*m (PN4602, PALL)	PES	1.0
Acrodisc PF Syringe Filters, 0.8/0.2 *µ*m (PN4658, PALL)	PES	5.8
Acrodisc Syringe Filters, Sterile, 0.2 *µ*m (PN4907, PALL)	PVDF	2.8

For these studies, the HA recovery evaluation and impurities removal were performed with samples from polishing step. In each filter, the tested volume was correspondent to the ratio of 1 $mL\ cm^{-2}$.

2.5.6. Proof of concept

On proof of concept run, some modifications were performed. It was produced 2500 mL of influenza VLPs and for clarification step Millistak+® HC Pod Depth Filter (MD0HC021H1, Merck Millipore) with 270 cm^2 of filter area was used with the difference on tubes, where MasterFlex® 35 tubes (MasterFlex Group) with 7.9 mm internal diameter was utilized. The last modification was on concentration step, a Pellicon® 2 Mini Ultrafiltration Module Biomax® 0.1 m^2 membrane area and 300 kDa (P2B300C01, Merck Millipore) was used in Sartocon Slice 200 Holder (17525--01, Sartorius Stedim).

On final sterile filtration step, the filters used were Millex-GV Syringe Filter Unit, 0.22 μm (SLGV033RS, Merck Millipore), Millex-GP Syringe Filter Unit, 0.22 μm (SLGP033RS, Merck Millipore), Acrodisc Syringe Filters, Sterile, 0.2 μm (PN4602, PALL) and Acrodisc Syringe Filters, Sterile, 0.2 μm (PN4907, PALL) with specifications described on table 2.2.

2.5.7. Magnetic Sulphated Cellulose Particles studies

MSCPs were kindly provided by Max Planck Institute for Dynamics of Complex Technical Systems Magdeburg, Germany. The production of MSCPs is described elsewhere[58].

All experiments were done in 50 mL tubes and the agitation of them was performed by hand following the protocol illustrated at figure 2.1. MSCPs were equilibrated 5 times (duration of 1 minute each) with equilibration buffer. The addition of sample – Influenza VLPs – was followed by a binding step of 10 minutes. The MSCPs were washed 3 times (duration of 1 minute each) with the equilibration buffer ensure that unlinked particles were completely removed. The elution step was performed with 2 different concentrations of NaCl. Each one had the duration of 5 minutes. In all those steps (except equilibration), a sample was collected for analysis. In the end, the MSCPs were regenerated with 1 M NaOH for 10 minutes, then washed with equilibration buffer until pH was the same as the buffer pH. Lastly, they were stored at 4 ºC in 20 % ethanol. All mentioned buffers are referred in table 2.3.

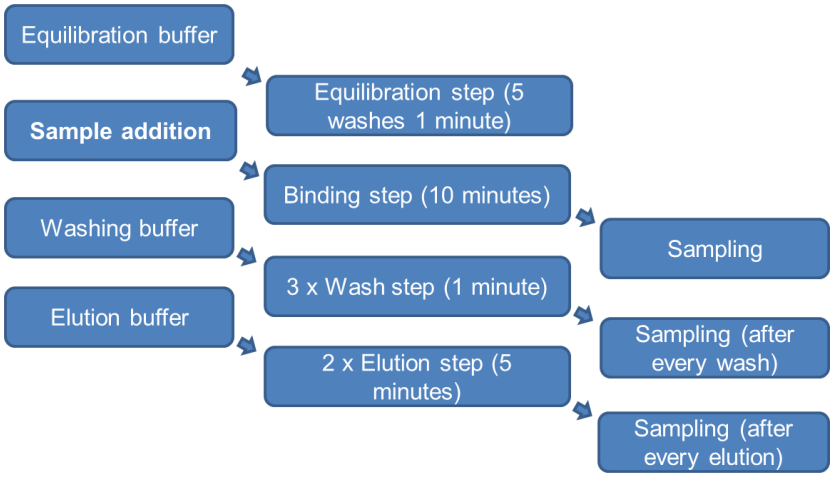

Figure 2.1 – Magnetic Sulphated Cellulose Particles (MSCPs) experiment steps.

In these experiments, the ratio between HA quantity and mass of beads was evaluated. The used mass of MSCPs was (i) 0.8 g; (ii) 1.2 g; (iii) 1.5 g; (iv) 2 g, but the initial quantity of HA was always the same. The sample was previously prepared for these experiments by being desalted on HiPrep 26/10 Desalting (17508701, GE Healthcare Life Science) on ÄKTA Explorer 100 liquid chromatography system (GE Healthcare). The applied flow rate was 170 cm h^{-1} and running buffer was the same as the one used on MSCPs experiments at both equilibration and washing steps.

Table 2.3 – Buffers for equilibration, washing and elution steps for Magnetic Sulphated Cellulose Particles experiments.

Equilibration and washing step	Elution steps	
Phosphate Buffer Saline (PBS) (-/-), pH 7.4	PBS (-/-) + 1 M NaCl, pH 7.4	PBS (-/-) + 2 M NaCl, pH 7.4
PBS* (-/-), pH 7.4	PBS* (-/-) + 1 M NaCl, pH 7.4	PBS* (-/-) + 2 M NaCl, pH 7.4
10 mM TRIS + 50 mM NaCl, pH 7.4	10 mM TRIS + 1 M NaCl, pH 7.4	10 mM TRIS + 2 M NaCl pH 7.4

PBS* - Phosphate Buffer Saline made with low NaCl concentration (20mM).

2.6. Analytical methods

2.6.1. Hemagglutination assay (HA assay)

HA concentration on each sample was obtained by HA assay, based on the protocol described elsewhere[67] with some changes. Briefly, in 96-wells microtiter plates with V bottom (611V96, Thermo Scientific) the samples were initially diluted (1:2 and 1:3) in D-PBS (14190-094, Gibco™). Two-fold serial dilutions to take a range of 11 concentration values, with a final volume of 50 μL, were made. As a positive control, a commercially available vaccine (ISTIVAC®, Sanofi

Pasteur MSD) was used. Finally, 50 μL of 1 % chicken erythrocytes (LOHMANN TIERZUCHT GmbH, Germany) was added to all wells. The plates were incubated at 4 °C at least 30 minutes.

The results were analysed by visual inspection to check for a positive or negative response on each well. A red dot on the bottom of the well means that erythrocytes did not agglutinate, representing a negative result. A positive result, where a red dot does not appear, means that erythrocytes agglutinate.

The highest dilution with a positive result was determined for each sample. For the vaccine, it corresponds to the known concentration of HA. Thus, and taking in consideration which dilution was made, the concentration of HA on all samples were determined.

2.6.2. Total protein quantification

To quantify total protein and according to the manufacturer's instructions, Pierce™ BCA Protein Assay Kit (23225, Thermo Scientific) was used. Samples were added to a 96-well microplate (260895, NUNC, USA) and serial dilutions with D-PBS (14190-094, Gibco™) between 2 and 256-fold were made. Bovine Serum Albumin (23209, Thermo Scientific) was used for the calibration curve. Absorbance at 562 nm was measured on Infinite® 200 PRO NanoQuant (Tecan Group Ld., Switzerland) multimode microplate reader.

2.6.3. Total dsDNA quantification

Total dsDNA quantification was performed using Quant-iT™ PicoGreen® dsDNA Assay Kit (P11496, Invitrogen™) following the manufacturer's instructions. A 96-well, black, clear bottom microplate (3603, Corning Inc.) was used. Standard dsDNA and samples were diluted with TE buffer between 2 and 256-fold. Fluorescence at 480 nm – excitation wavelength – and at 520 nm – emission wavelength – was measured on Infinite® 200 PRO NanoQuant (Tecan Group Ld., Switzerland).

2.6.4. Particles concentration and size distribution

To measure particles' concentration and size distribution the NanoSight NS500 (Malvern Instruments Ltd., United Kingdom) was utilized. The samples were diluted with D-PBS (14190-094, Gibco™) to be on instrument's linear range limits (10^8-10^9 particle mL^{-1}). It was necessary a manual adjustment of the particles' focus. Then, several frames during 60 seconds (called sample video) were taken and analysed by the software Nanoparticle Tracking Analysis (NTA) 2.3 (only particles between 70 and 150 nm were selected).

2.6.5. Immunoblotting

The presence of HA and M1 proteins on VLPs samples was evaluated by Western Blot. A SDS-PAGE gel (4-12% polyacrylamide NuPAGE (NP0321BOX, Invitrogen™)) was used. NuPAGE® LDS Sample Buffer (NP0007, Novex™) was added to the samples and they were incubated at 70 ºC for 10 minutes. The gel was loaded with 10 μL of sample and a SeeBlue Plus 2 (LC5925, Novex) molecular weight marker was utilized. H1 protein (11684-V08H, SinoBiological) and M1 protein (40010-V07E, SinoBiological) from Influenza H1N1 strain were added as a positive control. NuPAGE® MOPS SDS Running Buffer (NP0001, Novex™) was used as running buffer. A constant voltage of 200 Volts was applied for 50 minutes.

The gel was transferred into a PVDF membrane (IB401031, Novex™) using iBlot dry blotting system (Invitrogen™). Membranes were blocked with 5 % (w/v) of dry milk (115363, Merck Millipore) in TRIS-buffered saline with 0.1 % (w/v) of Tween 20 (T-TBS buffer) for 1 hour. Primary antibodies – anti-HA mouse (kindly provided by EDUFLUVAC partner) and anti-M1 goat antibody (Abcam ab20910) – were diluted 1:2000 in 5 % (w/v) of dry milk in T-TBS buffer and incubated overnight. Membranes were washed with T-TBS buffer for 2 hours. Secondary antibodies – anti-mouse (A9917, SIGMA) and anti-goat (A5420, SIGMA) – both conjugated with Horseradish Peroxidase, were diluted like primary antibodies (1:2000) and incubated for 1 hour. Membranes were washed with T-TBS for 1 hour. At least, membranes were revealed on ChemiDoc™ XRS+ System (Bio-Rad) with Amersham™ ECL™ Prime Western Blotting Reagent (RPN2232, GE Healthcare) – 5 photos during 300 seconds were taken using ChemiHi Sensitive software's option.

2.6.6. Baculovirus quantification

rBVs DNA extraction and purification was performed by High Pure Viral Nucleic Acid Kit (11858874001, Roche Diagnostics GmbH) as described on manufacturer's instructions. All samples were previously diluted 1:10. At the end, 50 μL of extracted DNA was obtained.

The number of rBVs DNA copies was obtained by real-time quantitative Polymerase Chain Reaction (qPCR) as described elsewhere[68] with some modifications. Shortly, DNA samples were diluted 1:100 in water PCR grade (03315932001, Roche Diagnostics GmbH). In each well of LightCycler® 480 Multiwell Plate 96 white (04729692001, Roche Diagnostics GmbH) 15 μL of a master mix and 5 μL of each diluted sample were added. For the master mix, 10x of Light Cycler® 480 SYBR Green I Master (04707516001, Roche Diagnostics GmbH) and 0,5 μM of each primer were prepared. LightCycler® 480 Instrument II (Roche Molecular Systems, Inc.) was used to execute and analyse qPCR reactions on the plate.

2.6.7. Transmission Electron Microscopy analysis

Through a Hitachi H-7650 120 Kv electron microscope (Hitachi High-Technologies Corporation), the samples were analysed on presence, integrity and morphology (shape and size) of influenza VLPs. 5 μL of samples was adsorbed onto a formvar coated 150 mesh copper grid from Veco (Science Services) for 2 minutes. The grid was washed 5 times with sterile filtered dH_2O. Then, during 2 minutes, it was soaked in 2 % uranyl acetate and dried in air at RT (20-22ºC).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Clarification studies

Clarification studies were performed to select which filtration device or centrifugation itself is the best option for clarification step in downstream train for influenza VLPs. In this train clarification was performed in 2 steps: the first one was the evaluation of a depth filter and centrifugation; the second one was the evaluation of filter with final pore size of 0.2 μm.

3.1.1. Influenza VLPs recovery

The recovery of influenza VLPs was determined by the percentage of HA recovery as illustrated in figure 3.1.

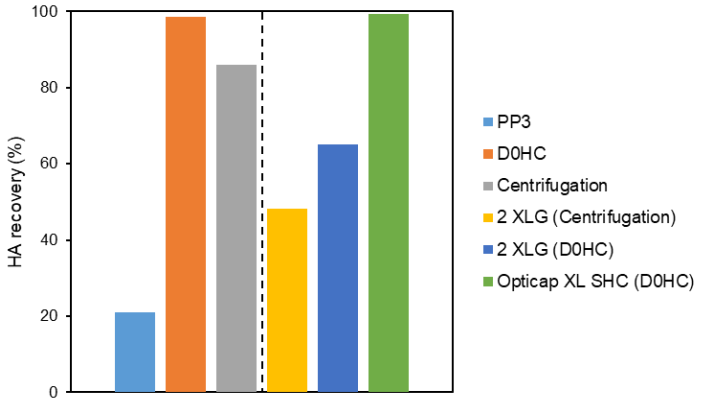

Figure 3.1 – HA recovery (%) after each filter and centrifugation evaluated for clarification studies. PP3, D0HC and centrifugation was evaluated for first step. 2 XLG and Opticap XL SHC was evaluated for second step.

For the first step, the best option is D0HC filter where the recovery is about 100 %. This result was expected due to the recovery yields of enveloped VLPs and viruses described on literature[37,45,69]. Relatively to PP3 filter the result is only 20 % recovery of HA. In centrifugation, the recovery from the process is approximately 85 % – a value already expected[36].

For the second step, Opticap XL SHC reaches an HA recovery of around 100 %, while 2 XLG present recoveries of around 50 % and 70 %, in both experiments. One where sample was prevenient from centrifugation and other where samples was prevenient from D0HC filter.

3.1.2. Impurities removal

The total protein and dsDNA removal were determined and the results are present in figure 3.2. Moreover, the baculovirus removal, turbidity and capacity of filters were evaluated and are summarized in table 3.1.

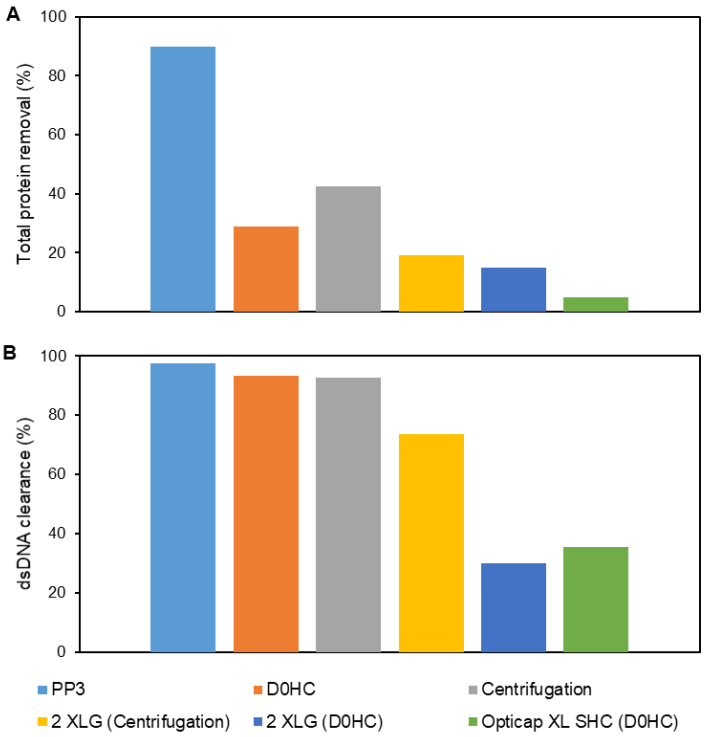

Figure 3.2 – (A) Total protein removal (%) and (B) dsDNA clearance (%) after each filter and centrifugation evaluated for clarification studies. PP3, D0HC and centrifugation was evaluated for first step. 2 XLG and Opticap XL SHC was evaluated for second step.

Table 3.1 – Baculovirus reduction (value by log reduction), turbidity and capacity of each filter and centrifugation evaluated on clarification studies. PP3, D0HC and centrifugation was evaluated for first step. 2 XLG and Opticap XL SHC was evaluated for second step.

	rBVs (Log Reduction)	Turbidity (NTU)	Capacity (mL/cm^2)
Initial	-	894	-
PP3	1.6	34.8	2.1
D0HC	1.7	60.3	42.4
Centrifugation	1.3	100.2	-
2 XLG (After Centrifugation)	1.6	10.0	7.7
2 XLG (After D0HC)	0.9	13.2	11.2
Opticap XL SHC (After D0HC)	1.2	8.8	5.5

When evaluating all impurities removal, turbidity and capacity, the D0HC filter is the best option, for first clarification step, due to high capacity conjugated with rBVs and dsDNA removal. The clearance of dsDNA was expected due to the positively-charged membrane of this filter[36]. The turbidity achieved was 60.3 NTU, a lower value than the ones founded on literature for clarification of recombinant proteins prevenient of mammalian cells (90 NTU)[70].

Despite PP3 filter presented a good removal of impurities, mainly the total protein, that was almost 100 %, the HA recovery is very low comparably to D0HC filter.

On centrifugation, removal of impurities was around 43 % for total protein, 93 % for dsDNA and a log reduction of 1.3 for rBVs – results expected, following literature[36]. Despite, for influenza VLPs, centrifugation is not a good choice for clarification step due to the high turbidity achieve and all disadvantages already mentioned (see section 1.2.1). However, centrifugation belongs to some clarification trains like rotavirus like-particles[45], rotavirus[71] and baculovirus[72] as the first step of clarification step.

For the second filter, the removal of impurities by Opticap XL SHC was only 5 % of total protein and 35 % of dsDNA. However, it reached a HA recovery of around 100 %. In another hand, 2 XLG filter had a similar behaviour as Opticap XL SHC due to the removal impurities, although, it presented low HA recovery comparatively to Opticap XL SHC. In conclusion, Opticap XL SHC is considered the best choice for the second clarification step of this downstream train.

3.2. Concentration studies

In these studies, on HFs and cassettes devices, influenza VLPs recovery and impurities removal were evaluated to select which is the better device for the concentration step of the downstream train for influenza VLPs. The scouting of TMP was evaluated only for HFs. For cassettes, it was already optimized in the laboratory (0.8 bar) (data not shown).

3.2.1. Hollow-Fibers

3.2.1.1. Scouting Transmembrane Pressure

To start the concentration studies with the HFs, it is necessary to determine the optimal TMP. The figure 3.3 illustrate the evaluation of the yP at different TMP along the same feed flux for HFs of 500 and 750 MWCO, respectively.

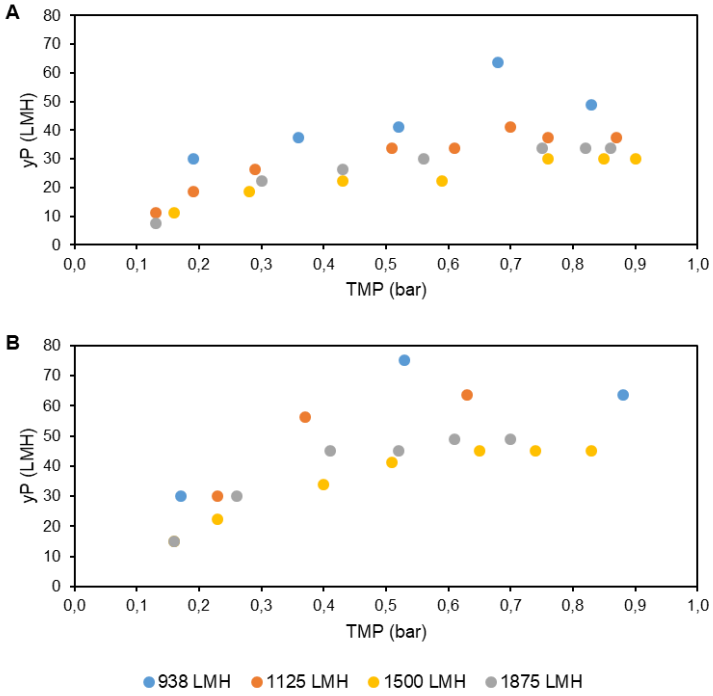

Figure 3.3 – Determination of optimal TMP for concentration studies with HFs, at different flow rates, determining the yP. (A) 500 MWCO HFs and (B) 750 MWCO HFs.

The optimal TMP value was reached through understand where the yP value start to stabilize. The start points of curve where yP stabilize means that the TMP is optimal. This value, for both HFs evaluated was 0.3 bar.

3.2.1.2. Shear rate studies

The recovery of influenza VLPs on UF/DF step processed by HFs was evaluated for two different shear rates: 2122 s^{-1} and 4244 s^{-1}. Samples were collected at 2, 3 and 5 concentration factor (CF) during UF and after 1, 3 and 5 DF volumes added and processed. The recovery of influenza VLPs along all step and for both HFs cartridges evaluated is shown in figure 3.4.

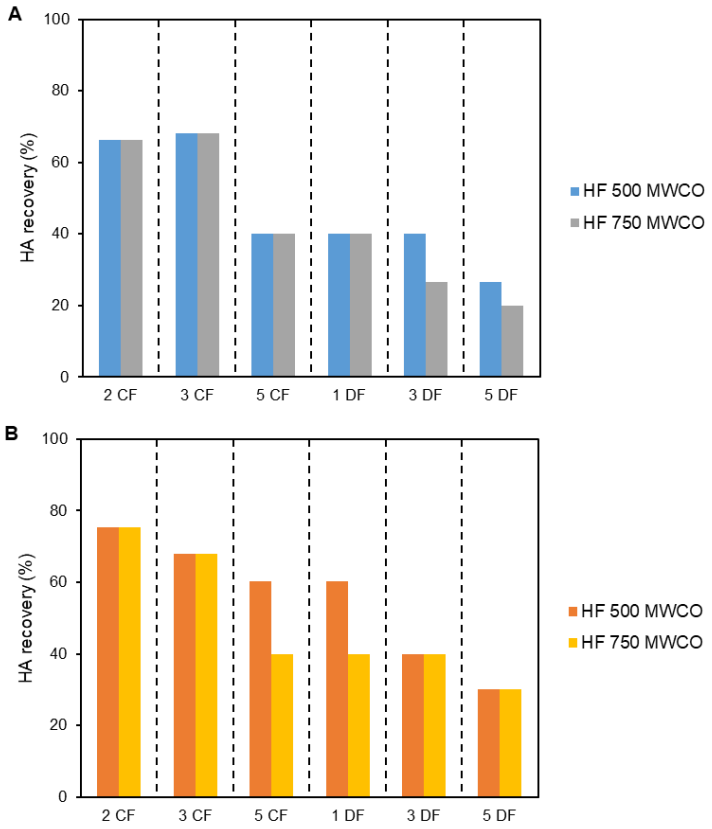

Figure 3.4 – HA recovery (%) as a function of CF and DF volume for both evaluated HFs with shear rate values of (A) 2122 s^{-1} and (B) 4244 s^{-1}.

The shear rate of 4244 s^{-1} (fig. 3.4 B) present higher HA recovery for both HFs (30 %). However, for a shear rate of 2122 s^{-1} (fig. 3.4 A), the recovery reached values between of 20 and 26 % for 500 MWCO HF and 750 MWCO. According to the results, the shear rate of 4244 s^{-1} is preferable than 2122 s^{-1}. However, HA recovery results were not close to the studies found, where HFs with 750 kDa was described with 106 % of recovery for influenza virus[69], while HFs with 500 kDa was described with 101 % of recovery for retrovirus vector[73].

The processing time is also a critical parameter. Table 3.2 summarize the recovery HA and the processing time of UF/DF step.

Table 3.2 – HA recovery (%) and processing time (min) of all UF/DF process in function the different processed shear rate of each HFs evaluated.

Device	MWCO	Shear rate (s^{-1})	HA recovery (%)	Processing time (min)
Hollow fiber	**500**	2122	20	156
		4244	30	141
	750	2122	26	131
		4244	30	185

The devices operated with high shear rate, that means high feed flow rate had low processing time, however it was just observed in HFs of 500 MWCO device. The shear rate of 2122 s^{-1} for the HFs with 750 MWCO has less processing time. This can be explained by the large pore size than HFs 500 MWCO.

HFs with 500 MWCO was preferable and taken in account for influenza VLPs downstream process. The operation shear rate of 4244 s^{-1} is the best option.

3.2.1.3. Impurities removal

The reduction of impurities was evaluated along the UF/DF, these results can be observed in figure 3.5 and table 3.3.

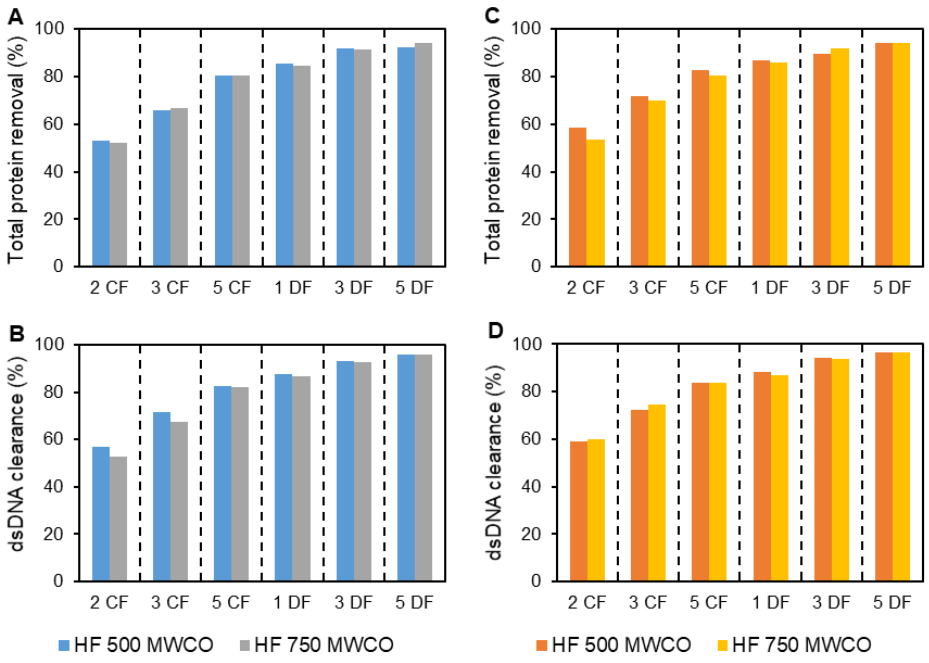

Figure 3.5 – (A) Total protein removal (%) and (B) dsDNA clearance as a function of CF and DF volume with 2122 s^{-1} of shear rate value. For 4244 s^{-1} of shear rate, (C) total protein removal (%) and (D) dsDNA clearance as function CF and DF volume.

Table 3.3 – Baculovirus removal (value by log reduction) comparable with the initial value from all experiments of evaluated HFs samples.

Device	Baculovirus (Log Reduction)
HFs 500 MWCO (2122 s^{-1})	0.5
HFs 500 MWCO (4244 s^{-1})	0.5
HFs 750 MWCO (2122 s^{-1})	0.5
HFs 750 MWCO (4244 s^{-1})	0.3

The removal of total protein and dsDNA was gradual along both CF and DF samples. It reached values of 92-94 % for total protein and 95-97 % for dsDNA. However, this removal was more evident along DF volumes. The rBVs removal results were not significant in any one of the devices and conditions evaluated.

These results are in agreement with the ones founded on literature, where 90 % of total protein and dsDNA removal was achieved with HFs of 750 kDa and 500 kDa, the authors further add that 500 kDa HFs shows better recovery of adenovirus[74]. The same result is shown here. Which could be concluded that HFs with 500 MWCO was considered the best option for concentration step on influenza VLPs downstream train.

3.2.2. Cassettes

3.2.2.1. Influenza VLPs recovery

To compare these results with the HFs results, the same conditions were applied, except the evaluation of different shear rates. Samples were collected at 2, 3 and 5 CF during UF and after 1, 3 and 5 DF volumes added and processed. The recovery of influenza VLPs along all step and for both cassette devices are illustrated in figure 3.6.

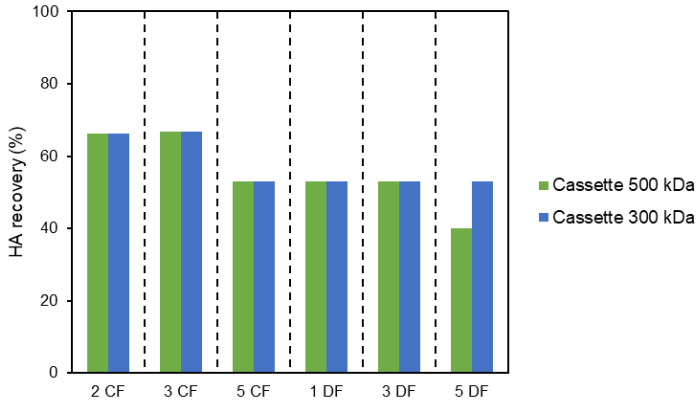

Figure 3.6 – HA recovery (%) as a function of CF and DF volume for both evaluated cassettes.

HA recovery was better performed in a cassette with 300 kDa with a value of 53 % while cassette with 500 kDa had a recovery of 40 %. In this approach, the better condition is the cassette with 300 kDa.

To complement, the processing time was evaluated and is summarized in table 3.4.

Table 3.4 – HA recovery (%) and processing time (min) of all UF/DF process in function the different cassette devices evaluated.

Device	HA recovery (%)	Processing time (min)
Cassette 500 kDa	40	75
Cassette 300 kDa	53	77

For both cassettes, the processing time is similar, however the HA recovery is much better on cassette of 300 kDa. This result of HA recovery yield can be explained by the most retention of influenza VLPs due to the smaller pore size than cassette 500 kDa.

3.2.2.2. Impurities removal

The removal of impurities is present in figure 3.7.

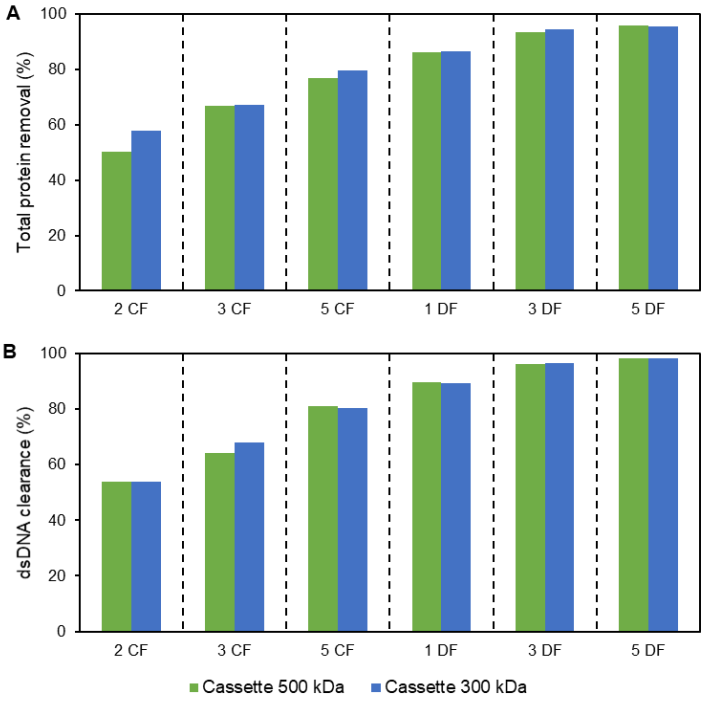

Figure 3.7 – (A) Total protein removal (%) and (B) dsDNA clearance as a function of CF and DF volume for each evaluated cassettes.

Both cassettes devices had an equal removal of dsDNA and total protein, around 98 and 96 % respectively. In another hand, there was not any removal of rBVs, what can present a difficulty in further steps of influenza VLPs downstream purification train.

For the concentration step, the cassette of 300 kDa presents the best results for being selected for influenza VLPs downstream purification train. However, on literature are examples

of influenza virus recovery around 100 % concentrated by a cassette with 300 kDa and 100 kDa[75]. Once more, these results could be explained due to the small pore size on those devices.

To conclude, the better results for HA recovery, impurities removal and processing time were achieved with cassettes devices. Which can be confirmed the best option for influenza VLPs downstream train – cassette with 300 kDa. Moreover, cassettes are cheaper than HFs which is an important characteristic to be in consideration due to the possibility of scale-up.

3.3. Chromatographic studies

The chromatographic studies are divided into two parts, the membrane chromatography and the resin chromatography. The membrane chromatography was evaluated as a capture step after clarification step. The resin chromatography is performed after the concentration step (in influenza VLPs downstream train – see figure 1.2).

3.3.1. Membrane Chromatography

3.3.1.1. Dynamic Binding Capacity determination

The membrane chromatography was performed by Sartobind® Q nano 1 mL. DBC at 10 % breakthrough was calculated through figure 3.8.

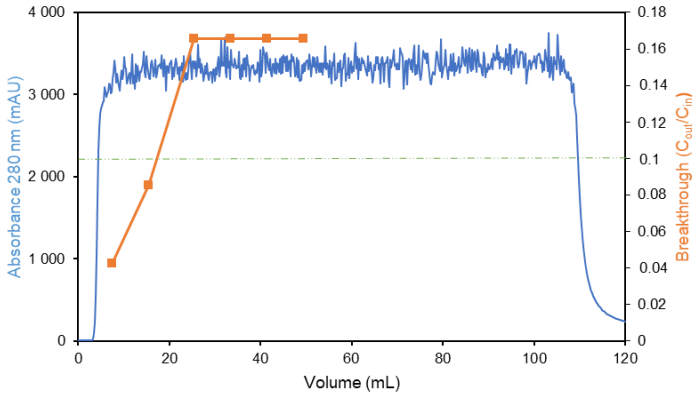

Figure 3.8 – FT section of membrane chromatography for DBC determination through the breakthrough curve (calculated by C_{out}/C_{in} of each fraction collected).

$$DBC_{10\%} = V_A \times \frac{C_0}{V_C} \quad (3.1)$$

To calculate the DBC is necessary to follow the equation 3.1, where V_A corresponds to feed volume applied up to the break point (mL), C_0 is the concentration of HA in the feed (μg mL^{-1}) and V_c is the total column bed volume (mL)[76].

For feed volume applied up to the break point it was necessary to discount the void volume of the system, that was 1.5 mL. However, due to the break point at 10 % was not exactly reached it was made an approximation to the value. So, the feed volume applied was 16.4 mL. The concentration of HA in the feed was 2.11 μg mL^{-1}. Total column bed volume was 1 mL so DBC of Sartobind® Q nano 1 mL at 10 % of breakthrough was 34.6 μg (HA) mL^{-1} of column bed volume.

In literature, the calculation of DBC with different breakthroughs refers to the process impurities, as an example – DNA[77]. One example was found with the determination of the dynamic capacity of Sartobind® Q MA75 for influenza virus[55].

After DBC determination, influenza VLPs was eluted (data not shown) and the recovery of HA reached 88 %. It was an expected result due to results on literature, where recovery of influenza virus with Sartobind® Q MA75 was described as 86 %[54,55].

3.3.1.2. Elution condition evaluation

To optimize the elution of influenza VLPs on Sartobind® Q it was evaluated the elution by steps (figure 3.9) and determined where the VLPs start to elute and if the impurities could be removed.

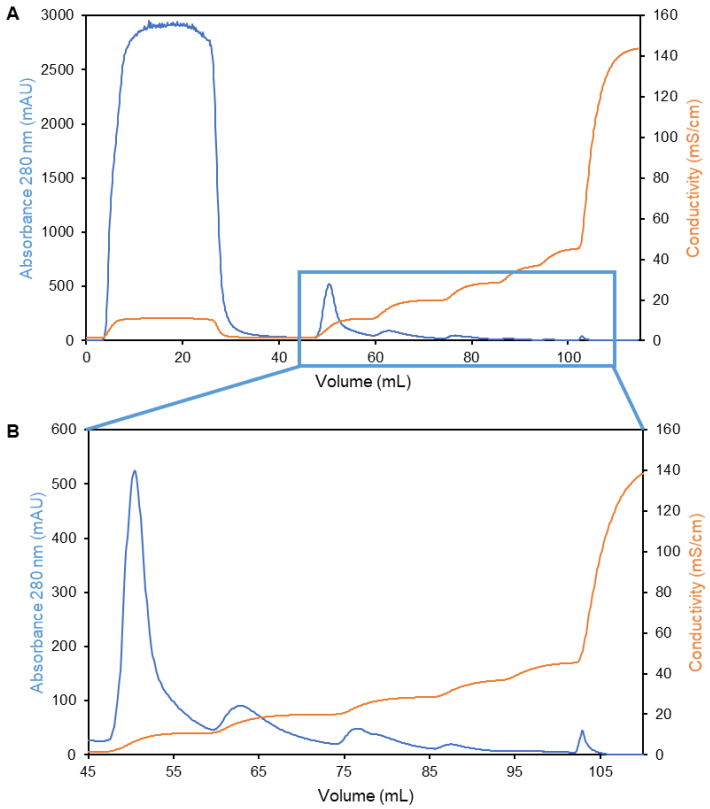

Figure 3.9 – Elution condition evaluation for membrane chromatography. Steps of 100 mM of NaCl were performed. Fractions of peaks were collected and analysed. (A) total chromatogram and (B) elution step chromatogram.

After analysing all fractions, the peak eluted at 100 mM of NaCl did not present any HA, what means that a step of 100 mM of salt should be done for separate influenza VLPs from impurities (with an HA recovery of 67 %). With an elution step with 100 mM of NaCl, around 97 % of total protein was removed. The dsDNA removal was reached at 44 % over several peaks. These results are concordant with the literature, where around 77 % of total protein are removed in Sartobind® Q MA75, however the authors affirm that dsDNA is not possible to separate from influenza virus[55].

3.3.1.3. Run and load condition evaluation

To perform this evaluation, the conductivity (concentration of salt (NaCl)) were changed: on binding condition and load condition (sample). On the first one two runs were performed with two different binding conditions: 100 and 200 mM NaCl. On the second one, two different salt concentrations were evaluated: 150 and 200 mM NaCl (final concentration of the injected sample). On these last, the binding condition were the same as injected sample. In table 3.5 is shown the HA recovery and loss of each condition evaluated. (The control load condition run was performed with elution conditions optimized.)

Table 3.5 – HA loss on flow-through (%) and HA recovery on elution (%) due to the evaluated different binding and load conditions of NaCl concentration (mM).

[NaCl] (mM) – binding condition	[NaCl] (mM) – load condition	Recovery HA (Elution) (%)	Loss HA (FT) (%)
0	Control	66.7	0
100	---	49.7	15.4
200	---	11.6	45
150	150	17.6	28.2
200	200	11.6	40.2

In all four evaluated runs, the increase of NaCl concentration means a decrease on HA recovery and a consequent increase of HA loss.

In runs where binding condition was evaluated, the conductivity of the injected sample was lower than the conductivity evaluated (100 or 200 mM NaCl). The conductivity on membrane, when the sample was injected, decrease (because the mixture of sample conductivity with the buffer of equilibration), what make to get a higher result on HA recovery on 100 mM NaCl than 200 mM NaCl.

On load condition runs, the injected sample had the same conductivity of membrane. However, the results shown that the recovery of HA was below 20 % on each condition.

These results can help to understand that ionic strength influences the way that influenza VLPs linked to the ligand Q of membrane adsorber. Which can be conclude that this step needs to be performed with a buffer without any NaCl concentration for membrane equilibration.

3.3.2. Resin Chromatography

3.3.2.1. Elution condition evaluation

The resin chromatography was performed by HiTrap Q HP column with 5 mL bed volume. The chromatogram is illustrated in figure 3.10. The injected sample is prevenient from UF/DF best condition.

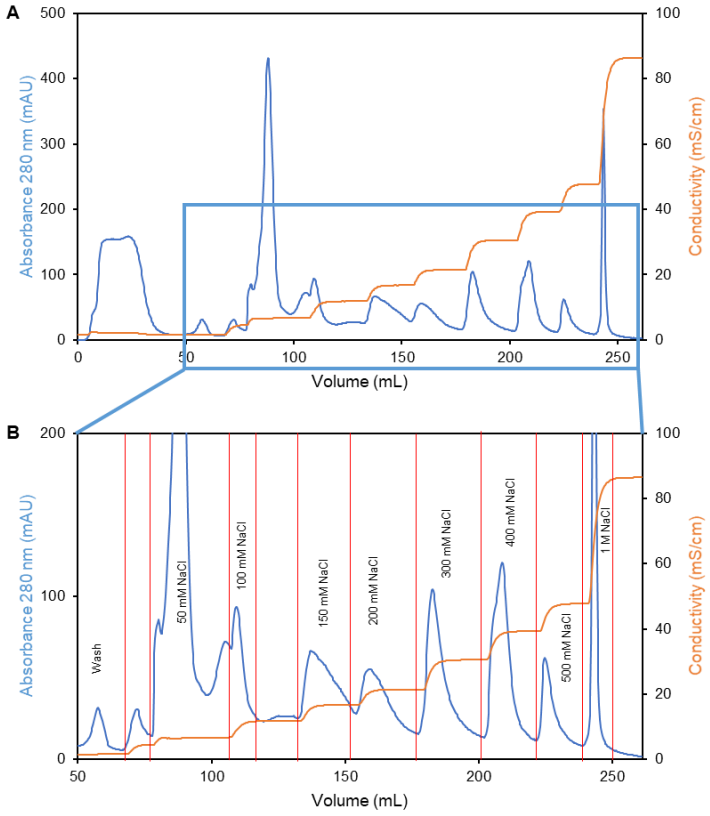

Figure 3.10 – Elution condition evaluation for resin chromatography. Fractions of peaks were collected and analysed. (A) total chromatogram and (B) elution step chromatogram with reference to which concentration of salt (NaCl) exist on each step.

HA and impurities (dsDNA and total protein), were analysed for all the fractions (figure 3.11).

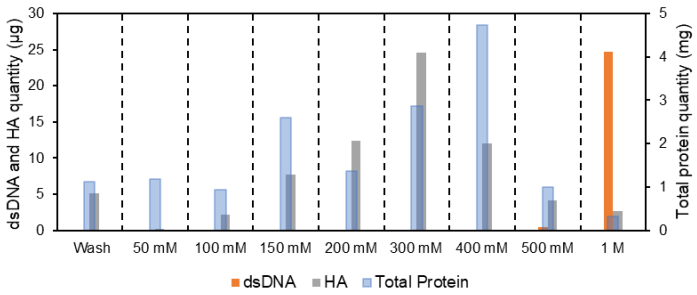

Figure 3.11 – Quantity of HA (μg) and impurities (dsDNA (μg) and total protein (mg)) along the elution evaluation step of resin chromatography.

The results indicate that HA was eluted between 150-500 mM NaCl. On other hand, the dsDNA was eluted after 500 mM NaCl while total protein was eluted with a step of 50 mM NaCl without HA. These results indicated that elution should be performed in 2 steps, at 50 mM NaCl and at 500 mM NaCl (to elute influenza VLPs). In validation run (data not shown) these conditions were evaluated and was obtained an HA recovery on 500 mM NaCl fraction of 160 %, total protein removal of 66 % and dsDNA removal of 94 %.

3.4. Polishing and Sterile Filtration studies

3.4.1. Multimodal Chromatography

Multimodal chromatography was performed using HiScreen™ Capto™ Core 700 column with a bed volume of 4.7 mL. The operation of this column is in negative mode. Influenza VLPs are collected on the FT, while impurities are eluted with 1 M NaCl (see figure 3.12).

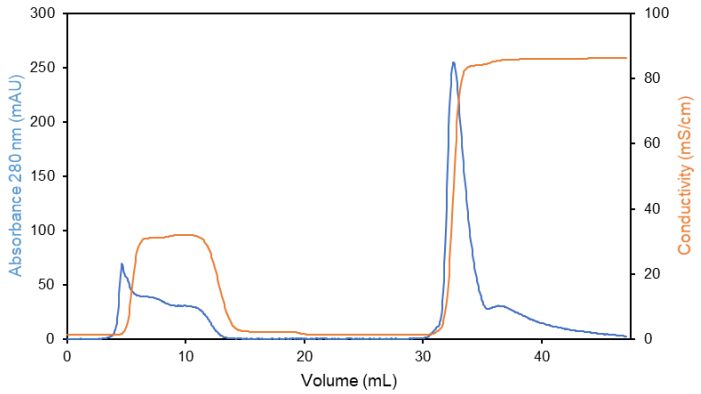

Figure 3.12 – Chromatogram representative of multimodal chromatography run. The FT and elution fractions were collected for analysis.

It is expected that influenza VLPs should be present on FT fraction, and major or total of other particles should be present on elution fraction. The results of the quantity of HA and impurities present in each fraction is shown in figure 3.13.

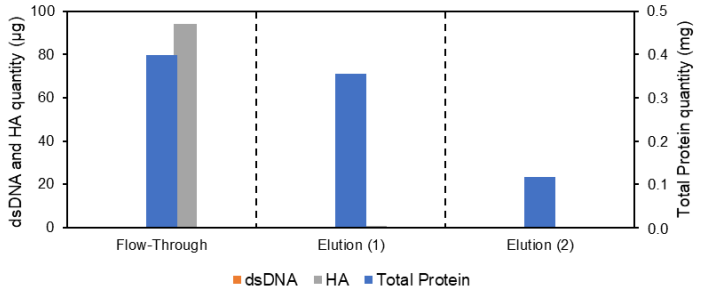

Figure 3.13 – Quantity of HA (μg) and impurities (dsDNA (μg) and total protein (mg)) along the run of multimodal chromatography. dsDNA has not representation due to samples had less than 0.0078 μg mL^{-1} (minimal detection limit by method).

According to the results, HA quantity is presented on FT fraction (94 μg). However, the HA recovery on this step was 60 %. The presence of high quantity of total protein on FT fraction due to the high quantity of influenza VLPs. The existence of HA on Elution (1) means the existence of some free HA in the initial sample. The removal of total protein is 95 %. The dsDNA removal was not determined since the concentration is below of 0.0078 μg mL^{-1} (minimal detection limit by method).

3.4.2. Size-Exclusion Chromatography

SEC was performed by Sepharose® 4 Fast Flow column with a bed volume of 33.9 mL. Influenza VLPs is collected on void volume of the column (15 mL). The chromatogram with experiment is illustrated in figure 3.14.

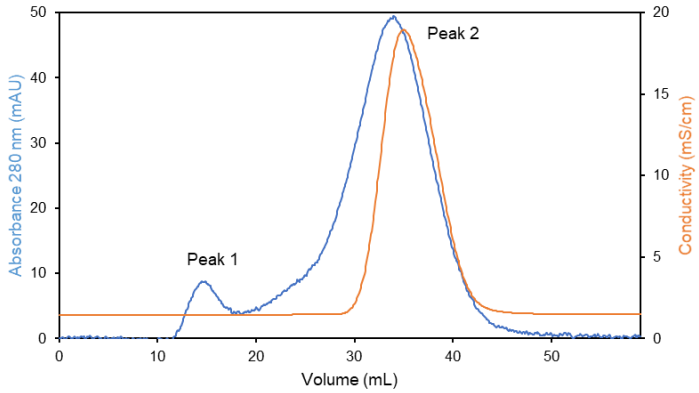

Figure 3.14 – Chromatogram representative of SEC run. The peak 1 was collected in the void volume of the column. Both fractions (peak 1 and 2) were collected and analysed.

HA and impurities were quantified for both fractions and are illustrated in figure 3.15.

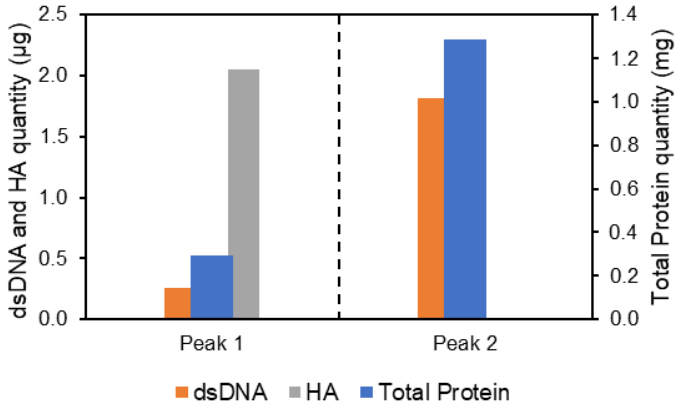

Figure 3.15 – Quantity of HA (μg) and impurities (dsDNA (μg) and total protein (mg)) of SEC samples.

On these results, the HA recovery on this chromatography run is 48 %. Yet in this run, the removal of total protein and dsDNA is 41 % and 65 % respectively. The removal of impurities in this step is lower comparatively to the other alternative for polishing step (multimodal chromatography). Moreover, in others SEC runs performed (data not shown) the recovery of HA reached was between 33-48 %, which is still lower than multimodal chromatography.

In literature, multimodal chromatography and SEC were evaluated as an intermediate step of purification. The HA yield reached was 104 % and 73 % for multimodal chromatography and SEC respectively. The impurities removal was also described. Total protein and dsDNA removal was 83 % and 58 % respectively for multimodal chromatography, while for SEC, total protein and

dsDNA removal was 92 % and 80 % respectively. However the authors concluded that use of multimodal chromatography (after a resin chromatography) in the downstream train for influenza virus is the best option to remove total protein and dsDNA[76]. Influenza VLPs produced in bacteria were also purified by multimodal chromatography in intermediate step, achieving a recovery of 89 %[38]. In conclusion, the utilization of multimodal chromatography is the best option to select.

3.4.3. Sterile Filtration

All filters for sterile filtration step are described in table 3.6. The filters were operated at 1 mL cm^{-2}. The filtration area of each filter is summarized in table 2.2 – section 2.5.6.

Table 3.6 – Representation of all filters utilized for sterile filtration steps with the correspond HA recovery (%).

	Filters	HA Recovery (%)
1	Whatman® SPARTAN® RC 30 syringe filters pore size 0.2 μm	66 %
2	Millex-GP Syringe Filter Unit, 0.22 μm	64 %
3	Millex-GV Syringe Filter Unit, 0.22 μm	66 %
4	Minisart® NML Syringe Filter, 0.2 μm	66 %
5	Minisart® High Flow Syringe Filter, 0.22 μm	66 %
6	Acrodisc Syringe Filters, Sterile, 0.2 μm	67 %
7	Acrodisc PF Syringe Filters, 0.8/0.2 μm	64 %
8	Acrodisc Syringe Filters, Sterile, 0.2 μm	66 %

Besides the HA recovery, impurities (total protein and dsDNA) removal was evaluated. However, total protein and dsDNA concentration are below the detection limits of the methods (0.0250 mg mL^{-1} and 0.0078 μg mL^{-1}).

3.5. Proof of concept

A proof of concept was performed with all of the selected devices and conditions. The volume of 2500 mL of influenza VLPs was produced and processed. At chromatographic step performed by HiTrap Q HP column with 5 mL bed volume, the DBC was determined. The figure 3.16 schematize the downstream train with all techniques and devices utilized.

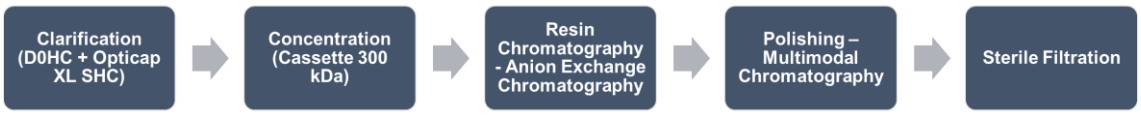

Figure 3.16 – Downstream train of influenza VLPs with all selected strategies and devices.

3.5.1. Clarification

On clarification, D0HC and Opticap XL SHC filters were selected. The results of HA recovery and impurities removal are shown in figure 3.17.

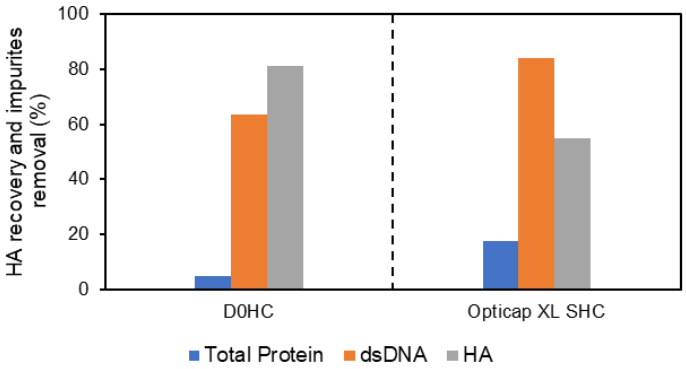

Figure 3.17 – HA recovery and impurities removal (%) on both clarification filters used (D0HC and Opticap XL SHC).

The HA recovery in this step was a little bit lower than the result achieved on clarification studies. This was not observed in impurity removal, where the same removal values were achieved. The rBVs log reduction was 0.15 and 0.75 for D0HC and Opticap XL SHC filter, respectively.

These results, mainly low HA recovery and rBVs removal can be explained by the use of an oversized D0HC filter which as 10 times-fold bigger while the processing volume was increased 2 times-fold.

3.5.2. Concentration

For concentration step, cassette with 300 kDa was selected. The filtration area was increased because of an increase in processing volume. The results of HA recovery and the impurities removal are shown in figure 3.18.

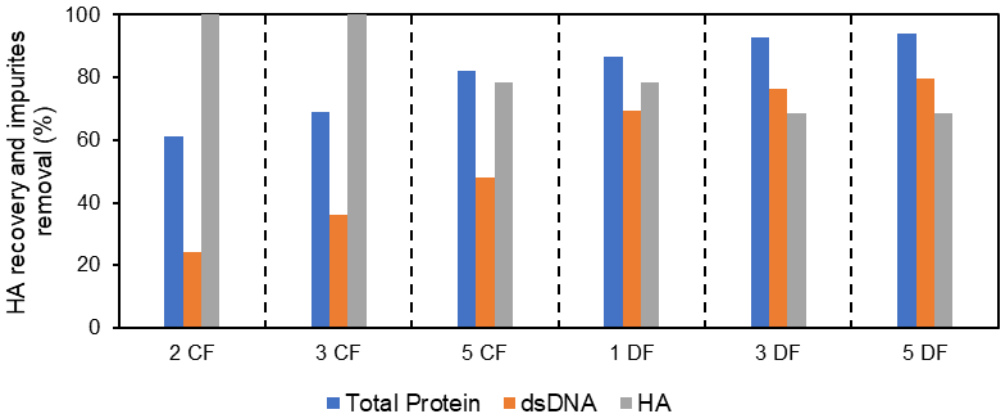

Figure 3.18 – HA recovery and impurities (dsDNA and total protein) removal as function CF and DF volumes of concentration step.

Cassette used on proof of concept run has 1000 cm^2 of filtration area. It represents a 20 times-fold than cassette module used in preliminary studies. The results obtained show that HA recovery was around 80 %, which is superior to results obtained from previous studies (section 3.3.2.1.). Relatively to the impurities removal (dsDNA, total protein and rBVs) the achieved values were identical to the results obtained on the studies. On this experiment, the removal of total protein and dsDNA was, respectively, 90 % and 80 %. There was not any removal of rBVs.

These results show that the main achievement of this step was the successful scale-up using 300 kDa cassette device, which is an important step to transfer in the future this downstream train to good manufacturing practices (GMP) facility.

3.5.3. Resin Chromatography – Anion Exchange Chromatography

The determination of DBC value for the chromatography column was performed in proof of concept run. The DBC at 10 % of the breakthrough curve was calculated through figure 3.19.

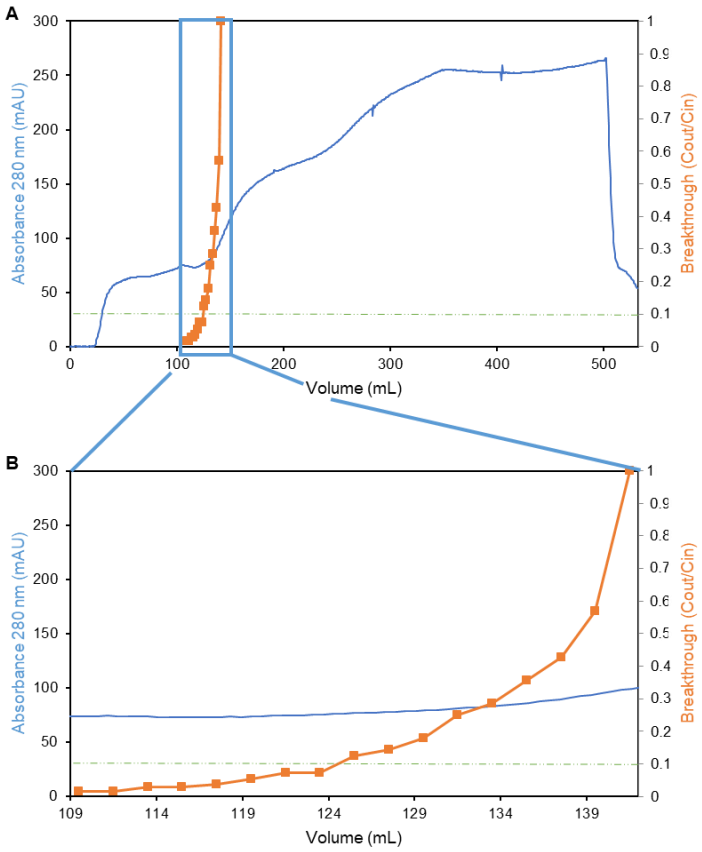

Figure 3.19 – (A) FT section of resin chromatography for dynamic binding capacity determination through the breakthrough curve (calculated by C_{out}/C_{in} of each fraction collected) and (B) selected area zoom.

In this chromatogram is possible to observed that breakthrough curve can reaches value 1, which means that column is fully saturated. The DBC at 10 % of the breakthrough curve was calculated by equation 3.1 (see section 3.3.1.1.).

The feed volume applied was 123.6 mL and the concentration of HA in the feed was 2.455 μg mL^{-1}. DBC of HiTrap Q HP at 10 % of breakthrough is 60.7 μg (HA) mL^{-1} of column bed volume. This result is higher than the one DBC value achieved for membrane adsorber Sartobind® Q nano 1 mL.

Figure 3.20 shows the elution step from the HiTrap Q HP, after the determination of DBC.

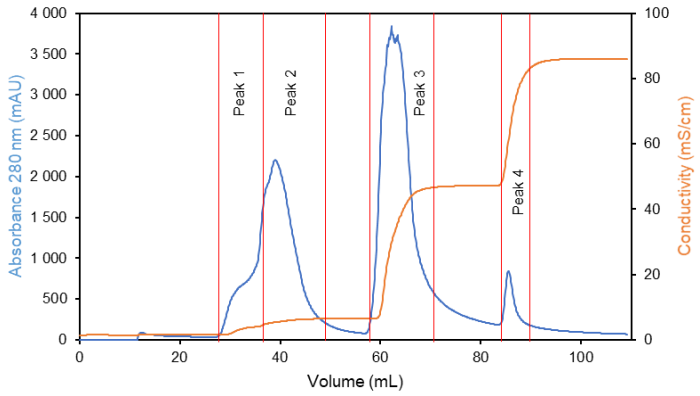

Figure 3.20 – Elution of resin chromatography step - proof of concept run. The 3 steps of conductivity correspond to the optimal NaCl concentration for elution step. All peaks identified were collected and evaluated.

The elution step was performed with steps of 50 mM, 500 mM and 1 M of NaCl. The quantity of HA and impurities is shown in figure 3.21.

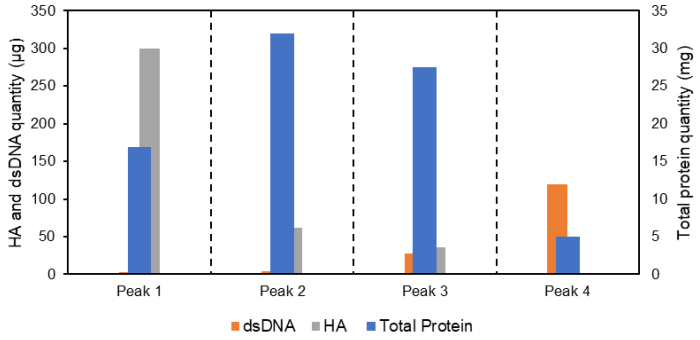

Figure 3.21 – Quantity of HA (μg) and impurities (dsDNA (μg) and total protein (mg)) from the elution step of resin chromatography.

Apparently, the results obtained in previously chromatographic studies (see section 3.3.2.1) are not in agreement whit the ones obtained here, mainly on HA recovery. The dsDNA removal on peak 3 was around 55 %, which was previously reached on studies. However, the existence of a large quantity of HA on peak 1 was unexpected.

On peak 2, corresponding to 50 mM NaCl, a large quantity of total protein was found where a lower quantity of HA was achieved. This result was not in agreement with the results previously obtained, where large quantity of HA was eluted in peak 3 (and not on peak 1).

The rBVs reduction of each peak was determined and is shown in table 3.7.

Table 3.7 – Baculovirus reduction (value by log reduction) for each peak of chromatography step – proof of concept run.

Peak	rBVs reduction (log reduction)
1	1.62
2	2.10
3	1.55
4	2.06

At the desired and optimized peak (peak 3), the rBVs reduction was lower than the others. Additionally, peak 1 does not demonstrate the larger quantity of rBVs removal. The results of this step show the challenges of removing the rBVs from influenza VLPs.

As a conclusion, the doubt about where are influenza VLPs still remains, since the HA assay only indicates the existence of all HA (including free HA in the system). The existence of peak 1 can be explained through the possibility of unspecific linkage of HA to the column or to other proteins that with a lower NaCl concentration can be eluted.

Peak 3 was selected to continue the proof of concept run due to results obtained on chromatographic studies. Moreover, the peak 1 was also selected due to the results obtained in this run.

3.5.4. Polishing (multimodal chromatography) and Sterile Filtration

The polishing step of this proof of concept run was performed by HiScreen Capto Core 700, and the chromatograms of both samples prevenient from the last step are shown in figure 3.22.

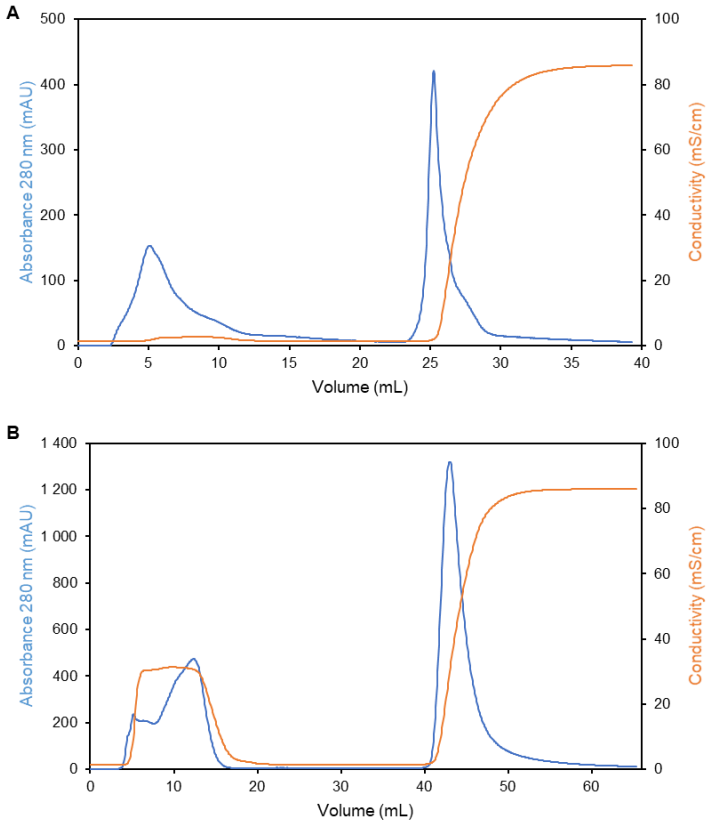

Figure 3.22 – Multimodal chromatography chromatograms – proof of concept run. (A) Corresponds to peak 1 prevenient from resin chromatography step and (B) correspond peak 3 prevenient from resin chromatography elution step.

For both cases, VLPs are detected in FT sample. The recovery of HA on this step was 49 % for the chromatogram A and 56 % for the chromatogram B of figure 3.22. Moreover, for chromatogram A, the removal of total protein was 89 % and the removal of dsDNA was not significant due to the initial low quantity on the injected sample (peak 1 of figure 3.21). In chromatogram B, the removal of dsDNA was 59 % and for total protein it was achieved 90 % of removal. The removal of rBVs was not significant on this step.

For sterile filtration, both FT samples were utilized and tested independently. Were used 4 filters from the original table (see table 2.2 – section 2.5.6.). Two of them made with PVDF and the other two with PES.

The filters selected are summarized in table 3.8 with the recovery of HA of both 2 experiences.

Table 3.8 – Filters selected for evaluation on proof of concept run. HA recovery of both experiments. SF (A) corresponds to FT fraction of chromatogram A from polishing step. SF (B) corresponds to FT fraction of chromatogram B from polishing step.

	Filter	Material	HA recovery (%) (SF(A))	HA recovery (%) (SF(B))
1	Millex-GV Syringe Filter Unit, 0.22 μm	PVDF	50	78
2	Millex-GP Syringe Filter Unit, 0.22 μm	PES	49	78
3	Acrodisc Syringe Filters, Sterile, 0.2 μm	PES	74	77
4	Acrodisc Syringe Filters, Sterile, 0.2 μm	PVDF	50	70

On SF(A) sterile filtration samples – prevenient from peak 1 of chromatographic step – the dsDNA and total protein removal was not possible to determine because samples were below of detection limits of the methods (0.0250 mg mL^{-1} and 0.0078 μg mL^{-1}).

On SF(B) sterile filtration sample – prevenient from peak 3 of chromatographic step – the dsDNA removal for all samples achieved values around 60 % while for total protein the removal was around of 30 %.

The removal of rBVs was not significant in any filter. However, the filter 3 was considered the best in both experiments. For SF(A) achieved 1 rBVs log reduction (when the other one reached only 0.5). For SF(B) achieved a 0.5 rBVs log reduction (while the other one do not remove significantly rBVs).

As a conclusion, the filter 3 made with PES can be considered the selected filter for influenza VLPs sterile filtration step, because of the removal of rBVs and the HA recovery achieved with this filter.

3.5.5. Characterization of Product

Characterization of the product is an important step for understanding the stability and quality of influenza VLPs after the all downstream process. The final samples were characterized by TEM imaging and Immunoblotting.

HA and M1 proteins from influenza VLPs were identified in immunoblotting. However, the true conformation of influenza VLPs was seen through TEM images, as well as the existence of rBVs. These results and images are presented in figure 3.23.

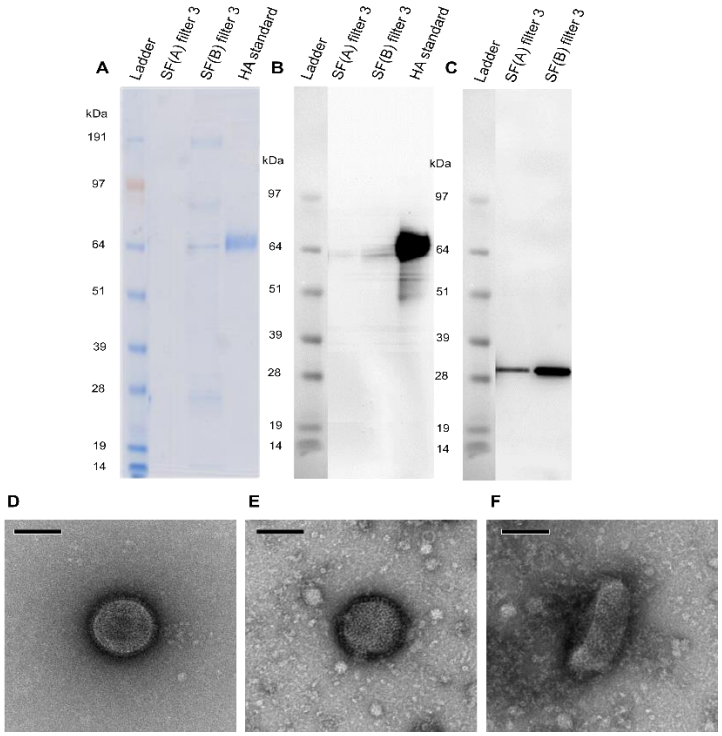

Figure 3.23 – (A) Analysis of total protein through the SDS-PAGE present on SF(A) and SF(B) filter 3 samples. Immunoblotting analysis (B) for identification of HA protein and (C) for identification of the M1 protein. TEM images of Influenza VLPs prevenient from (D) polishing step – peak 1 from chromatography step and (E) final sample prevenient from SF(B) of filter 3. (F) TEM image of baculovirus present on the final sample from SF(B) of filter 3. Scale bars represent 100 nm.

The presence of HA and M1 was proven by immunoblotting analysis, where it is possible to understand that final samples had the HA and M1 proteins after filter 3. On SDS-PAGE results, the sample SF(A) filter 3 does not have any presence of proteins. However, and with the immunoblotting results, it can be confirmed the presence of influenza VLPs but in a lower concentration than SF(B) filter 3 sample.

Thorough TEM images it can be observed the existence of influenza VLPs with desired conformation and with the HA spikes on the membrane. The dimensions of these particles vary between 80 and 120 nm, which are in agreement with literature[18]. Nevertheless, on final samples, it is seen the existence of rBVs, what it is still a challenge to overcome.

On sample SF(B) filter 3, the ratio of quantity HA/total protein and quantity HA/dsDNA was determined and the results are 6.71 μg (HA) mg^{-1} (total protein) and 11.46 μg (HA) μg^{-1} (dsDNA). Commercial influenza vaccines had 45 μg of HA quantity per 1 dose. So, if the commercialization of this sample happened, the quantity of dsDNA achieved per dose corresponds to 3.9 μg. In another way, the total quantity of total protein in this dose corresponds to 6.7 mg. Both results are higher than authorities' specification, mainly dsDNA where per dose the quantity of host cell DNA should be less than 10 ng and for total protein the value should be less than 270 μg per dose

(with 45 μg of HA). The quantity of HCP per dose is also considered for authorities. Nevertheless, this work shows that with these downstream train we are close to the “purity targets” demands by authorities.

3.6. Magnetic Sulphated Cellulose Particles studies

MSCPs were evaluated as a new approach to getting a maximal purified and concentrated sample to use in characterization techniques (like DLS, TEM, HPLC, NMR and others) of influenza VLPs. The samples collected are shown in figure 3.24. The 2 final samples (elution 1 and 2) are considered the desired samples.

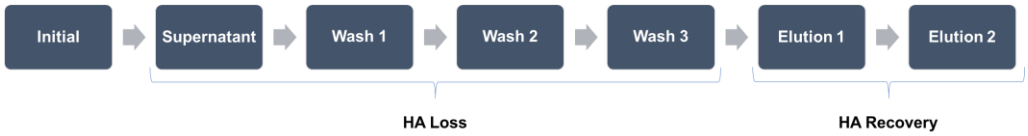

Figure 3.24 – Scheme of all samples collected from MSCPs experiments with an indication where HA is loss or recovered.

3.6.1. Desalted buffers evaluation

Aiming a high yield of recovery, the buffer of the desalting step was evaluated. Were made 3 runs of each condition. The recovery in percentage was determined to have in consideration the average of the 3 runs in all steps. The desired results are: 0 % of HA recovery in ‘HA loss’; 100 % recovery in ‘HA recovery’ (see figure 3.24). All results are summarized on table 3.9.

Table 3.9 – HA recovery yields (%) of each step for each buffer evaluated. (HA recovery was calculated to have in consideration the initial quantity of HA).

	HA recovery yields (%)			
Samples	**Control bulk**	**Bulk desalted for PBS (-/-), pH 7.4**	**Bulk desalted for PBS* (-/-), pH 7.4**	**Bulk desalted for 10 mM Tris pH 7.4 + 50 mM NaCl**
Supernatant	49.9	75.7	37.6	29.1
Wash 1	17.5	18.6	11.9	8.6
Wash 2	11.1	7.1	3.8	3.8
Wash 3	7.0	0.0	0.0	0.0
Elution 1	34.9	14.8	29.5	58.1
Elution 2	8.8	5.7	16.7	29.1

PBS* - Phosphate Buffer Saline made with low NaCl concentration (20mM).

The recovery of captured influenza VLPs where PBS (-/-) was used as buffer shows that the loss on supernatant step (after 10 minutes of incubation) is larger than 75 %. In contrast with

PBS* (that have just 20 mM of NaCl), the loss of HA on supernatant step is less than 40 %, which indicates that high ionic strength can influence the linkage of the HA to the MSCPs. This conclusion is contested in other study where clarified bulk of influenza virus where initial diluted to decrease the concentration of salt[58].

It was evaluated the buffer 10 mM Tris pH 7.4 + 50 mM NaCl and selected as the desired and optimal buffer for this process of capture influenza VLPs. The loss on supernatant is around 29 % and the recovery is 58 % and 29 % for elution 1 and 2, respectively.

3.6.2. Beads mass evaluation

After selected the buffer, it was evaluated the beads mass necessary for reach high recovery yields on elution and fewer losses on the steps before elution. For that, the same concentration and volume were desalted with the best buffer already determined. As the previous experiments, were made 3 runs, in this case with a different mass of beads (0.8, 1.2, 1.5 and 2 g) of each condition. The calculations were performed in the same way. The percentage of HA recovery yields are shown in table 3.10.

Table 3.10 – HA recovery yields (%) in each step for each different mass of beads evaluated. Bulk desalted for 10 mM Tris pH 7.4 + 50 mM NaCl.

Beads	0.8 g	1.2 g	1.5 g	2 g
		HA recovery yields (%)		
Supernatant	12.5	29.2	11.4	17.5
Wash 1	7.5	9.8	5.7	9.6
Wash 2	5.4	5.7	4.4	8.2
Wash 3	4.3	3.5	2.5	4.3
Elution 1	34.5	30.6	25.1	17.1
Elution 2	31.4	27.8	33.5	25.2

Through these results, the better mass of beads to get a more concentrate sample is 0.8 g of beads. The opposite result, where higher losses were seen, was presented on 1.2 g beads. The evaluation of impurities (mainly total protein and dsDNA) will be studied on the next point. Only after that it can be concluded which are the best conditions to achieve the main propose of this methodology.

3.6.3. Impurities removal

The total protein and dsDNA clearance over the experiments of beads mass evaluation is shown in figure 3.25.

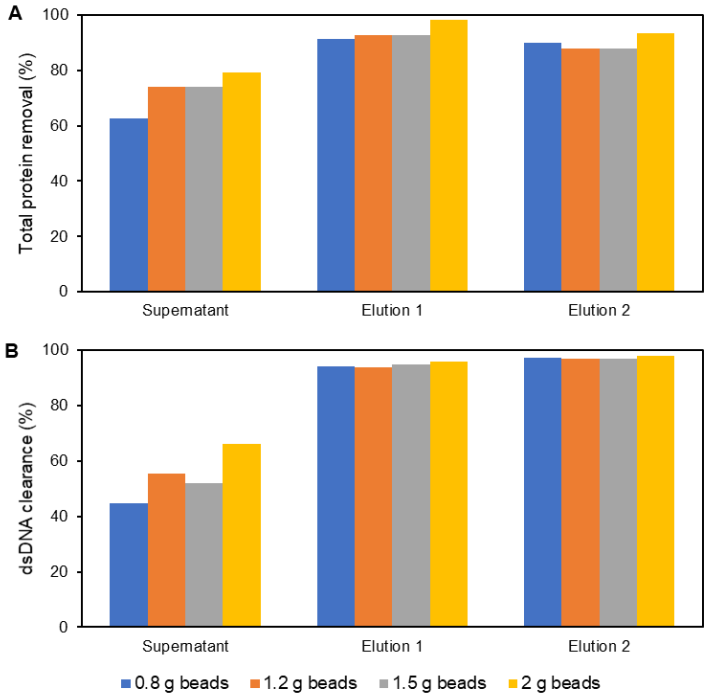

Figure 3.25 – (A) Percentage of total protein removal and (B) Percentage of dsDNA clearance in the three steps (Supernatant, Elution 1 and Elution 2) of MSCPs mass of beads evaluation experiments.

The removal of total protein and dsDNA is around 90 % and 95 % respectively. On supernatant sample, the loss of HA can be explained due to the linkage of these impurities to MSCPs. It can be confirmed by the lower removal percentage of dsDNA and total protein.

These results indicate that 0.8 g of beads and 0.7 μg of HA quantity from clarified bulk desalted with 10 mM Tris pH 7.4 + 50 mM NaCl are recommended to get high recovery of influenza VLPs and low presence of impurities in the final sample.

The main conclusion achieved is that instead of a long-time process to get a purified and concentrate influenza VLPs samples, MSCPs could be an easy-friendly tool that takes around 30 minutes to get the final desired product. Furthermore, this tool was already described as 7-fold faster than centrifugation in the purification of influenza particles[58].

CHAPTER 4

CONCLUSIONS AND FUTURE PERSPECTIVES

Some efforts have been recently made to improve the downstream process for virus and VLPs, due to the importance of these particles in the biopharmaceutical industry. Among of several examples, influenza VLPs can be considered the future approach for the next generation of influenza vaccines. To achieve a final VLPs product with high purity, quality and potency it is necessary to get an efficient downstream train scalable and robust.

Based on all work accomplished in this thesis, the main conclusion achieved was the development of a downstream process train to obtain influenza VLPs for animal *in vivo* studies and most probably for clinical phase I. On clarification step, the results showed that with the combination of two depth filters a reduction more efficient of turbidity is obtained. The utilization of filters instead of centrifugation was demonstrated and well succeeded. The recovery of HA presented results around 100 %. However, the removal of impurities as total proteins and dsDNA was not effective. These results showed that D0HC followed by Opticap XL SHC filters should be selected for both steps of clarification. As proof of concept a scale-up was performed, but on the first filter was not efficiently performed (with low HA recovery achieved), due to the large filter area compared to the large volume process. That indicates that in the future it is necessary to study more about the membrane filtration area on clarification – as an example use more than one filter in series or parallel - in other to guarantee the maximal recovery and impurities removal.

On concentration step, the utilization of cassettes instead of HFs was preferable, due to the HA recovery obtained in the end of the step, as well as the lower processing time achieved. As proof of concept, the scale-up on cassette was the most valuable achievement, with good results where HA recovery achieved 80 %. However, in all studies, DF volumes were added in batch. In the future, the influence of DF volumes added on continues mode must be studied, which is an important characteristic for transference of this downstream train to GMP.

At chromatographic step, DBC value for membrane chromatography was 34.6 μg (HA) mL^{-1}. On other hand, for resin chromatography, the DBC value was of 60.7 μg (HA) mL^{-1}. On membrane chromatography, the parameters of load and condition were established and the recovery of HA was 67 %. Otherwise, on resin chromatography, the elution conditions were determined and a

removal of impurities was achieved. Due to these results, it is concluded that resin chromatography is the best choice for intermediate purification. However, more studies on this step are needed.

For polishing step, SEC and multimodal chromatography were evaluated. I was obtained a recovery of 48 % and 60 %, respectively. Considering the high costs associated to SEC scale-up comparing with multimodal chromatography, it was concluded that the second one should be performed on downstream train. On proof of concept run, similar results were achieved. On final step, a filter with PES material was chosen and performed on proof of concept run, where HA recovery achieved values of 77 %.

A fast tool was also implemented with the primary goal of having purified and quickly concentrated influenza VLPs for characterization methods. With MSCPs was optimized the utilization buffer and the ratio between the quantity of HA and mass of beads that can be applied to get higher HA recovery and impurities removal. As future studies, the evaluation of efficiency of this technique, mainly on application – the samples should be analysed on several techniques as DLS, TEM, HPLC, NMR and others – should be done. Additionally, MSCPs should be scale-up and applied on a chromatography column to be evaluated as an alternative for already studied chromatography columns and others. In conclusion, others VLPs could be studied in this new approach of chromatography.

As the main conclusion, a downstream train and a new and fast tool – MSCPs – were evaluated for influenza VLPs purification, overall with successes. However, future studies should be performed for scale-up on clarification step. Besides, an evaluation of DF in continuous mode on concentration step, allowing this train to be transferred to GMP manufacturing, should be performed. Also, it is necessary to overcome the challenge of the existence of rBVs on the final product. For that, several studies can be performed, as the development of an affinity chromatography specific to rBVs. These steps could be implemented on the downstream train for influenza VLPs or could be changed if better results on HA recovery and impurities removal were achieved compared to the strategies/step already design on this downstream train.

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