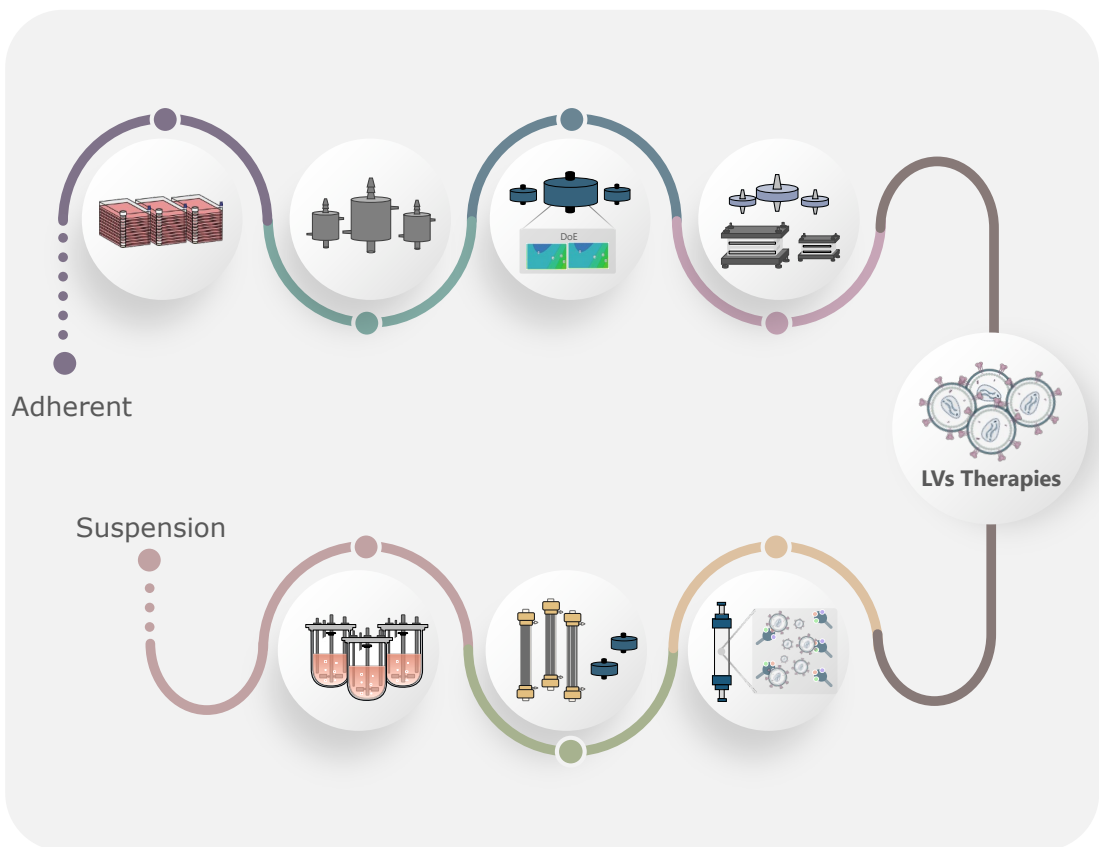


Accelerating Lentivirus Manufacturing

An engineering-driven approach

Ana Sofia da Costa Moreira



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

PhD Program in Bioengineering – MIT Portugal

Oeiras, May 2023

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The work developed in this thesis was supervised by:

- **Doctor Cristina Peixoto**, Instituto de Biologia Experimental e Tecnológica (iBET) (supervisor)
- **Doctor Ana Sofia Coroadinha**, Instituto de Biologia Experimental e Tecnológica (iBET) and Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa (ITQB NOVA) (co-supervisor)

Accelerating Lentivirus Manufacturing: An engineering-driven approach

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Universidade Nova de Lisboa



From left to right: Prof. Dr. Paulo Mota; Dr. Rui Nobre; Dr. Carol Knevelman; Dr. Mercedes Segura Gally; Dr. Sofia Moreira; Dr. Cristina Peixoto; Dr. Ana Sofia Coroadinha and Dr. Pedro Matias

Supervisors:

- **Cristina Peixoto**, PhD, Head of Downstream Lab Head of Downstream Lab, Animal Cell Technology Unit, Instituto de Biologia Experimental e Tecnológica (iBET) (supervisor)
- **Ana Sofia Coroadinha** PhD, Head Cell Line Development and Molecular Virology Lab, Animal Cell Technology Unit, Instituto de Biologia Experimental e Tecnológica (iBET) (supervisor)

President of the Jury

- **Doctor Pedro Matias**, PhD, Principal Investigador of Instituto de Tecnologia Química e Biológica António Xavier da Universidade NOVA de Lisboa

Jury

- **Mercedes Segura Gally**, PhD, Vice President at ElevateBio, Cambridge, Massachusetts, USA
- **Carol Knevelman**, PhD, Vice President and Head of Process Research & Development at Oxford BioMedica, United Kingdom
- **Paulo Mota**, PhD, Full professor of Chemical and Biochemical Engineering, NOVA School of Science and Technology, Lisboa
- **Rui Nobre**, PhD, Operational Manager at ViraVector (UC) and Post-Doctoral Researcher at Center for Neuroscience and Cell Biology, Coimbra

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Foreword

The presented thesis dissertation is the result of four years of research at Animal Cell Technology Unit of ITQB-NOVA/iBET (Oeiras, Portugal) under supervision of Dr. Cristina Peixoto and Dr. Ana Sofia Coroadinha.

*“Passion is the fuel that drives the engine of success.”- Gary
Vaynerchuk*

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Abstract

The journey of Advanced Therapy Medicinal Products (ATMPs) has come a long way in the last few decades, as is reflected in the increasing number of clinical trials conducted every year. Particularly, in the field of gene therapy, they have achieved promising results and almost 40 cell and gene therapy products already in the market. In 2019, the approval of Kymriah, a chimeric antigen receptor (CAR-T) for the treatment of B-cell acute lymphoblastic leukemia, sparked the attention to Lentiviral vectors (LVs). In the following years, LVs have become one of the workhorses for the treatment of monogenic disorders and adoptive cell therapies with seven LVs-based products launched on the market. In fact, LVs are a remarkable subclass of the *Retroviridae* family, with the ability to integrate the genome of proliferating and non-proliferating cells and presenting lower insertional mutagenesis when compared with γ -retrovirus.

LVs bioprocessing shares common downstream challenges with other retroviral vectors that include short half-lives, sensitivity to pH variations, high ionic strength, and shear stress. The existing purification methods consist of multiple units which lead to prolonged processing times, reduced yields, and loss of vector functionality. Over the last decade, these processes have improved considerably, however, enhanced purification schemes still are needed before lentiviruses can sustain the current industry's demand. Therefore, this thesis aimed to provide transformative solutions to accelerate the manufacturing of LVs compatible with current Good Manufacturing Practices requirements. The major goals were to enhance scalability, increase recovery yields, and purity while reducing processing time.

In **Chapter 1**, a comprehensive literature review on LVs bioprocessing is performed. Initially, a brief introduction to gene therapy, lentiviral vector's biology, its applications, and general production methodologies are

presented. The most relevant purification operation units are extensively described including novel applications and technological developments that are currently thriving. Moreover, the importance of analytical technologies is also highlighted, reinforcing the need for faster and more precise methods to efficiently support process development.

At the laboratory scale, adherent cell cultures can be a resourceful and practical method to produce LVs. However, they became limited at larger scales. Scaling-out, multi-tray technologies, or fixed-bed bioreactors can be applied to alleviate the scalability constraints. However, these approaches often lead to high volumes and complex purification processes. Consequently, the extended running time results in loss of viral activity. **Chapter 2** presents the optimization and the establishment of a four-step purification scheme for LVs produced with adherent cell lines. This process is composed of clarification, anion-exchange chromatography (AEX), ultrafiltration, and sterilizing filtration. For each unit, several membrane materials were evaluated to maximize viral recovery, purity and decrease processing time. Particularly, Design of Experiments approach was used to perform a deeper study on the AEX step to determine the best operating conditions, including ionic strength in the elution step, pH, and the chromatography membrane. When compared with existing methodologies, we were able to implement a 5-hour cGMP-compliant process with an overall yield of 45% of transducing units (TU).

The continuous progression of manufacturing technologies has contributed to decreasing product insufficiency of supply, increasing product quality and titer, and, consequently, reducing production costs. For instance, in the last couple of years, LVs production efficiency has improved significantly with the industry transition to suspension platforms. Hence, serum-free production systems are being implemented which lead to the reduction of batch-to-batch variation. Despite the scalability offered by this

transition, new challenges were expected in the purification of LVs since the main properties of the initial feedstock had changed. In **Chapter 3**, the impact of LVs produced in suspension conditions on the downstream process is evaluated. LVs produced in a stirred tank bioreactor were used to evaluate different tangential flow microfiltration devices by comparing TU recovery yield, membrane throughput, and turbidity reduction. The best performer achieved 90% viral recovery, a maximum throughput of 300 L/m² and a final turbidity on the clarified stock below 10 NTU. Afterward, since the absence of animal serum affects the production medium composition, a set of experiments using lipid supplementation was designed to explore the impact of cell metabolism on virus phenotype. Later, a methodical evaluation of multiple AEX membranes was conducted and the most critical process parameters were optimized. Surprisingly, it was observed a shift in the virus elution profile. The highest percentage of viral particles eluted using lower ionic strength when compared with the adherent system. Overall, the recovery yields using LVs produced in suspension is now 56% eluting 1.5 M of NaCl. The quality of purified LVs from suspension cultures was assessed and compared to the results obtained for LVs from adherent cultures.

Several chromatographic modalities have already been exploited and applied to LVs manufacturing. Affinity Chromatography (AC) can be used to simplify the LVs purification process and generate clinical-grade products with high titer and purity. However, one of the major challenges in this field has been the development of an affinity ligand that can target the viral envelope and simultaneously allow the preservation of its biological activity during the elution step. **Chapter 4** presents, for the first time, the implementation of an AC resin for the purification of vesicular stomatitis virus G protein pseudotyped LVs. At a laboratory scale, several ligand candidates were investigated, and different process parameters were assessed and optimized, including residence time, dynamic binding capacity, functional

particle's recovery, and impurity clearance. The best candidate showed a dynamic binding capacity of 1×10^{11} total particles/mL with a recovery yield of 45% at 2 minutes of residence time. The established AC robustness was confirmed by the performance of an intermediate scale with a 10 mL column which demonstrated the scalability and reproducibility of the AC process. An extensive viral characterization was also performed reaching a 1.5 ± 0.2 μ g of host cell proteins for a viral dose of 10^9 TU. Overall, the results achieved in this chapter can significantly contribute to increasing downstream process efficiency and reducing time-to-market of LVs-based products.

In **Chapter 5**, the main achievements of the previous chapters are summarized and discussed. The strategies herein explored allowed us to gain insights into the different impurity profiles between suspension and adherent culture systems; and compare two chromatographic strategies AEX and AC. Hence, we envision that this work can contribute to accelerate the LVs bioprocessing field fulfilling current industry trends and needs.

Resumo

O crescimento acentuado dos Medicamentos de Terapia Avançada (ATMPs) nas últimas décadas deu origem a um aumento significativo do número de ensaios clínicos realizados anualmente. Em particular, na área de terapia génica, os ATMPs têm alcançado resultados promissores, com quase 40 produtos de terapia celular/génica lançados no mercado. Em 2019, a aprovação do recetor antigénio quimérico (CAR-T) *Kymriah* para o tratamento de leucemia linfoblástica aguda de tipo B, acentuou o interesse em lentivírus recombinantes (LVs). Nos anos subsequentes, os LVs tornaram-se uma das principais ferramentas para o tratamento de doenças monogénicas e terapias celulares adoptivas, sendo parte integrante de sete produtos já comercializados. Os LVs são uma subclasse excepcional da família *Retroviridae*, com capacidade de integrar o genoma de células proliferativas e quiescentes, apresentando uma mutagénese insercional baixa, em comparação com os γ -retrovírus.

Nos processos de produção dos LVs conseguimos encontrar vários desafios em comum com os processos de purificação de outros vetores retrovirais, nomeadamente: tempos de meia-vida curtos, sensibilidade a variações de pH, concentrações de sal elevadas e tensão de atrito. Os métodos de purificação estabelecidos consistem num conjunto de múltiplas unidades, que resultam em tempos de processamento prologados, baixos rendimentos e perda de funcionalidade dos vetores. Na última década, estes processos têm melhorado substancialmente, contudo, ainda é insuficiente para que possam sustentar as necessidades da indústria. Desta forma, a seguinte tese propõe trazer soluções transformativas com a capacidade de acelerar o processo de produção e purificação de LVs compatíveis com o Sistema de Boas Práticas de Fabricação (GMP). Os principais objetivos deste trabalho foram melhorar a escalabilidade do processo, os rendimentos de produção e purificação, a pureza e reduzir o tempo de processo.

No **Capítulo 1**, é apresentada uma revisão detalhada da literatura sobre os bioprocessos de LVs, incluindo, inicialmente, uma introdução sobre terapia génica, biologia de vetores lentivirais, respetivas aplicações e metodologias gerais de produção. As unidades de purificação mais relevantes são abordadas, incluindo novas aplicações e os recentes desenvolvimentos tecnológicos. Adicionalmente, é salientada a importância das tecnologias analíticas, reforçando a necessidade de métodos mais rápidos e precisos, para sustentar o desenvolvimento de novos processos.

À escala laboratorial, as culturas de células aderentes permitem a produção de LVs de forma rápida e prática. No entanto, em grandes escalas torna-se um método limitado. Soluções como repetição de unidades em pequena escala, tecnologias 2D/planares, ou reatores de leito fixo podem ser aplicadas para evitar algumas limitações de escalabilidade, contudo, resultam frequentemente em volumes elevados e processos de purificação complexos. Consequentemente, os tempos de corrida extensos podem resultar em perda de funcionalidade dos LVs. O **Capítulo 2** apresenta a otimização e implementação de um método de purificação composto por quatro passos para LVs produzidos em linhas celulares aderentes. Este processo é constituído por um passo de clarificação, uma cromatografia de troca aniónica (AEX), uma ultrafiltração e uma esterilização do produto. Em cada unidade, várias membranas de diferentes materiais foram avaliadas de forma a maximizar o rendimento e pureza dos LVs, e diminuir o tempo de processo. Nomeadamente, a Metodologia de Experiências (DoE) foi utilizada para desenvolver um estudo mais aprofundado do passo de AEX, de forma a determinar as melhores condições de operação, incluindo a força iónica na eluição, o pH e a membrana cromatográfica. No final, foi efetuada a comparação com tecnologias existentes, salientando-se a implementação de um processo com apenas 5 horas, compatível com condições GMP e com rendimento global de 45% de vírus funcionais (TU).

O progresso das tecnologias de produção promoveu uma melhor resposta às necessidades da indústria, aumentando assim a sua qualidade e quantidade produzida, e consequentemente, reduzindo os custos de produção. Um exemplo dessa evolução foi a melhoria significativa na produção com a transição da indústria para plataformas em suspensão. Desta forma, estão a ser implementados sistemas de produção sem soro, contribuindo para a redução de variações entre os diferentes lotes. Apesar da escalabilidade que as plataformas em suspensão trouxeram, são esperados novos desafios na purificação de LVs, uma vez que as propriedades físicas e químicas do material produzido foram alteradas. No **Capítulo 3**, foi estudado o impacto da produção de LVs em condições de suspensão nos processos de *downstream*. Para isso, foram utilizados LVs produzidos em reator de tanque agitado para avaliar diferentes aparelhos de microfiltração de fluxo tangencial, comparando o seu rendimento em TU, a sua taxa de transferência de membrana e a redução da turbidez. O resultado mais promissor atingiu 90% de recuperação de vírus, uma taxa de transferência máxima de 300 L/m² e uma turbidez final do clarificado abaixo de 10 NTU. Posteriormente, tendo em conta que a ausência de soro afeta também a composição do meio, foi desenhado um conjunto de experiências para explorar o impacto da suplementação lipídica no fenótipo viral. De seguida, foi realizada uma avaliação de diferentes membranas de AEX e os parâmetros de processo mais críticos foram otimizados. Surpreendentemente, foram observadas alterações no perfil de eluição do vírus. Grande parte dos LVs eluiu com uma força iónica mais baixa quando comparados com o processo anteriormente estabelecido. No global, o rendimento com LVs produzidos em suspensão passou para 56% com 1.5 M de NaCl. A qualidade dos LVs purificados a partir de culturas em suspensão foi avaliada e comparada com os resultados obtidos em LVs de culturas aderentes.

Vários métodos cromatográficos foram explorados e aplicados na purificação de LVs. A Cromatografia de Afinidade (AC) pode ser usada para simplificar o processo de purificação de LVs e gerar produtos de grau clínico com título e pureza elevados. Contudo, o desenvolvimento de um ligando de afinidade que possa ligar ao envelope viral e, simultaneamente permitir a preservação da sua atividade biológica durante o passo de eluição, tem sido um dos maiores desafios desta metodologia. Assim, o **Capítulo 4** apresenta a implementação pela primeira vez de uma resina de AC para a purificação de LVs funcionalizados com a proteína G de vírus de estomatite vesicular. Inicialmente, os diferentes ligandos gerados foram avaliados assim como os vários parâmetros envolvidos no processo de purificação tais como: o tempo de residência, a capacidade dinâmica de ligação, a recuperação de partículas funcionais e a remoção de impurezas. A resina escolhida demonstrou uma capacidade dinâmica de ligação de 1×10^{11} partículas/mL com um rendimento de 45% para 2 minutos de tempo de residência. A robustez da AC foi também confirmada através de uma corrida numa escala intermédia (coluna de 10 mL), demonstrando assim a escalabilidade e reprodutibilidade do processo. Por fim, foi realizada uma caracterização extensa, obtendo a concentração de 1.5 ± 0.2 µg de proteínas da célula hospedeira (HCP) para uma dose viral de 10^9 TU. Em suma, os resultados obtidos neste capítulo podem contribuir significativamente para uma melhor eficiência no processo de purificação, reduzindo também o tempo de comercialização de produtos derivados de LVs.

No **Capítulo 5**, são resumidos e discutidos os principais resultados dos capítulos anteriores. As estratégias exploradas permitiram: ganhar conhecimento sobre os diferentes perfis de impurezas encontrados nos sistemas de suspensão e aderentes e comparar a performance de duas estratégias cromatográficas (AEX e AC) na captura dos LVs. Assim, este trabalho poderá contribuir para o crescimento da área de bioprocessamento de

LVs abrangendo algumas lacunas com que a indústria se debate atualmente.

Thesis Publications

Moreira, A. S., Cavaco, D. G., Faria, T. Q., Alves, P. M., Carrondo, M. J. T., & Peixoto, C. (2021). Advances in Lentivirus Purification. *Biotechnology Journal*, 16(1), 1–11

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Moreira, A. S., Bezemer, S., Faria, T. Q., Detmers, F., Hermans, P., Sierkstra, L., Coroadinha, A. S., & Peixoto, C. (2023). Implementation of novel affinity ligand for Lentiviral vector purification. *International Journal of Molecular Sciences*, 24(4), 3354.

Moreira, A. S., Faria, T. Q., Oliveira, J. G., Peixoto, C. (2023). Unravelling the impact of Lentiviral vector production in suspension cultures on the downstream purification process. Manuscript in preparation

Additional Publications

Moreira, A. S.*, Carvalho, S. B.*, Gomes, J., Carrondo, M. J. T., Thornton, D. J., Alves, P. M., Costa, J., & Peixoto, C. (2018). A detection and quantification label-free tool to speed up downstream processing of model mucins. PLoS ONE, 13(1).

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List of Abbreviations

AAV	Adeno-associated virus
AC	Affinity Chromatography
AEC	Anion exchange chromatography
ATMP	Advanced Therapy Medicinal Products
BaEV	Baboon Endogenous Virus
CA	Capsid protein
CAR-T	Chimeric Antigen Receptor T-Cell
CE	Capillary Electrophoresis
CE-SDS	Sodium Dodecyl Sulfate Capillary Electrophoresis
cGMP	current Good Manufacturing Practices
CPP	Critical Process Parameters
CV	Column Volume
D-PBS	Dulbecco's Phosphate-Buffered Saline
DBC	Dynamic Binding Capacity
DEAE	Diethylaminoethyl
DoE	Design of Experiments
DNA	Deoxyribonucleic Acid
DSP	Dowsntream Processing
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FAC	Flow Assisted Cell Sorting
FBS	Fetal Bovine Serum
FDA	Food and drug administration
FT	Flowthrough
GFP	Green Fluorescence Protein

HCP	Host Cell Protein
HEK	Human Embryonic Kidney
HF	Hollow Fiber
HIC	Hydrophobic Interaction Chromatography
HIV	Human Immunodeficiency Virus
Hpt	Hours post-transfection
HPLC	High performance liquid chromatography
IEC	Ion exchange chromatography
IMAC	Immobilized Metal Affinity Chromatography
IN	Integrase
JSRV	Jaagsiekte Sheep Retrovirus
LIF	Laser-Induced Fluorescence
LMH	Litres/m ² /h
LVs	Lentiviral Vector
MA	Matrix protein
MOI	Multiplicity of infection
MoLV	Moloney Murine Leukemia Virus
MV	Membrane Volume
MW	Molecular Weight
NC	Nucleocapsid protein
NTA	Nanoparticle Tracking Analysis
PCL	Producer Cell Line
PEG	Polyethylene Glycol
PES	Polyethersulfone
PEI	Polyethylenimine
P_{Max}	Presssure max
PR	Protease

PS	Polysulfone
PVDF	Polyvinylidene Fluoride
QA	Quaternary Amine
RC	Regenerated Cellulose
RCL	Replication Competent Lentiviruses
RPM	Rotations per minute
RT	Residence time
RU	Relative units
RV	Retroviral Vector
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEC	Size Exclusion Chromatography
SF	Sterilizing Filtration
SPR	Surface Plasmon Resonance
STR	Stirred Tank Bioreactor
SU	Surface subunit
TEM	Transmission Electron Microscopy
TFF	Tangential Flow Filtration
TFF-MF	Tangential Flow Filtration microfiltration
TMP	Transmembrane Pressure
TP	Total Particles
TU	Transduction Units
UF	Ultrafiltration
USP	Upstream processing
VCF	Volumetric Concentration Factor
V_HH	Recombinant camelid-derived single-domain antibody
VSV-G	Vesicular Stomatitis Virus G protein

1

Introduction

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Author contributions to the chapter:

Ana Sofia Moreira wrote the chapter based on the referred bibliography.

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1. Paving the way into Viral Vectors Therapies

Cell and gene therapy (CGT) is revolutionary and rapidly developing fields that presents groundbreaking solutions to address unmet medical needs [1], [2]. It typically involves transferring genetic material into a patient's cells to correct abnormalities or introduce new traits [3]. Since the beginning of the 20th century with the first attempts using a retrovirus viral for the treatment of ADA-SCID, nearly 40 CGT products are currently on the market as the field moves rapidly towards clinical translation [4]. Advanced Therapy Medicinal Products (ATMPs) have become a viable option for treating patients with cancer, inherited or immune diseases, and other therapeutic areas [5]–[7]. Viral vectors are widely employed as delivery vehicles in CGT, encompassing both *in vivo* and *ex vivo* approaches.

Over the past years, the number of gene therapy clinical trials based on lentiviral vectors (LVs) has grown to attain 10% of the total gene therapy trials in 2019 [8]. Their use in gene therapy for several conditions (**Figure 1.1**) has been reported both *ex vivo* – involving, usually, the transduction of hematopoietic stem cells [9]–[14] or T-cells [3], [15] – and *in vivo* – with gene delivery to the central nervous system [16] and the retina [17]. Several comprehensive reviews of the clinical applications of LVs haven been published recently [18]–[21]. The approval by the FDA, in 2017, of two CAR-T-cell therapies for the treatment of B-cells lymphoma and leukemia evidences the advantages of these viral vectors and is proof of its successful use at a clinical level [22], [23]. For other types of cancer, the use of LVs for immunization remains a promising application [6], [18]. The success of these clinical trials requires improved methods for purification of LVs both quantitatively, increasing process capacity, and qualitatively replacing traditional methods used at small scales that are not economically and/or technically viable for larger scales [24], [25].

LVs are enveloped viruses members of the *Retroviradae* family that have become increasingly relevant for Biopharmaceuticals since they lead to stable integration of the transgene(s) to be expressed in both dividing and non-dividing cells [26], [27]. Additionally, their integration patterns seem less risky than γ -retroviral vectors [26], [28] justifying their widespread usage from functional genomics to cell engineering studies, as well as recombinant protein production and clinical gene therapy [29]. The most known and studied member of the subclass is Human Immunodeficiency Virus (HIV). Other members include the Simian Immunodeficiency Virus (SIV), Equine Anaemia Virus (EAV), Feline Immunodeficiency Virus (FIV), or Bovine Immunodeficiency Virus (BIV) [30].

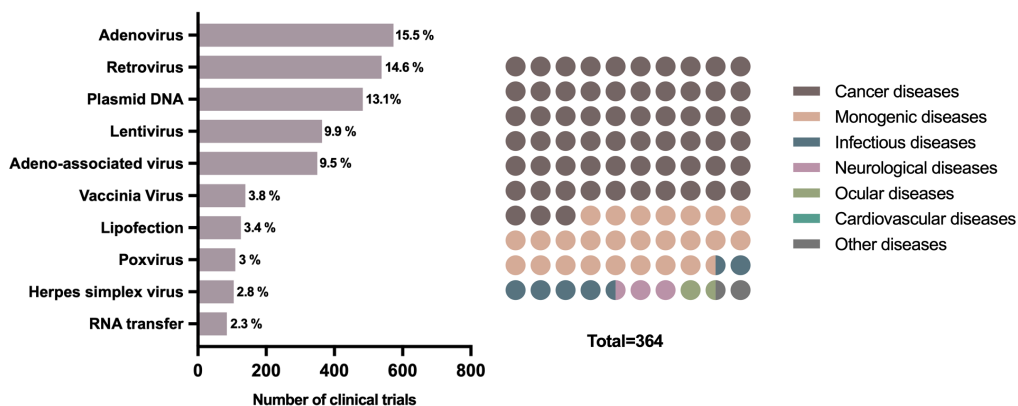


Figure 1.1. Approved gene therapy clinical trials. a) Number of clinical trials per year for different viral vectors; b) Distribution of clinical trials for the different applications. Adapted from [31].

The current production technologies commonly result in low functional titers, ranging from 10^6 to 10^8 transducing units per mL (TU/mL), depending on the production system, LV pseudotype, harvest conditions, or even the

titration technique [32]. Additionally, impurities vary with the production system. For example, the serum is present when LVs are produced by adherent cell lines, and the plasmid DNA is used for transient LVs production.

Therefore, when developing a downstream process (DSP), concentration of LVs is pursued along with increasing LVs purity, that must consider the impurities profile of the production broth. Just like any other biological product, the degree of concentration and purification needed is fundamentally connected to what the LVs are intended for. Research/small scale purposes do not generally require a very pure product [33]. However, when the product is intended for clinical use, the safety of the drug is a concern. The standard depends essentially on the type of administration. For *ex vivo* applications the purity level of the drug product is more flexible [34]. Additionally, the multiplicity of infection (MOI) must be kept as low as possible to reduce the tumorigenic potential and cytotoxicity [24], [35]. For an *in vivo* approach doses of, at least, 10^{11} - 10^{12} per patient are needed, which will also vary according to target tissue [29], [36]. In this case, stricter control of the purity to reduce an immunogenic response by the patients is needed [34], [37]–[39].

DSP must also consider the low stability of the LVs functional particles, which is affected by temperature [40], ionic strength [41], [42], pH [40], [43] and shear stress [44], [45]. Beyond environmental conditions, the stability is highly dependent on the particle itself, mainly on the envelope protein (pseudotype) but also on the viral core [41], [45], [46]. VSV-G is the most used LV pseudotype due to its broad tropism and robustness throughout the purification processes. Nevertheless, DSP schemes for other protein envelopes (e.g., BaEV, RD114-TR, GaLV) are emerging since they present higher tropism for specific target tissues/cells [35], [41], [47].

2. Lentiviral vectors

2.1. Lentiviral vector design and expression

LVs derived mostly from the HIV-1 and for that reason share many of its features like the spherical shape at around 80-120nm in diameter, the capsid core and the functional enzymes. The genome of the LVs is constituted by a single-strand positive-sense RNA ((+) ssRNA) of 7 to 12 kb in length, which is packed into a capsid enclosed by a membranous envelope. In addition to a pair of copies of (+) ssRNA, complexed with nucleocapsid protein (NC), the enzymes reverse transcriptase (RT), integrase (IN) and protease (PR) are also contained inside a capsid. These last three enzymes are codified by the genomic region named as pol. The NC, capsid protein (CA), and matrix protein (MA) are part of a group of structural proteins codified by the genomic region named as gag (an acronym for group-specific antigen). CA forms the capsid, the shell that delimits the viral core, and MA forms the layer outside the core and interacts with a cellular-derived lipid envelope. Viral envelope glycoprotein, codified by the genomic region env, is responsible for the interaction with specific host cell receptors and is incorporated in that membrane (**Figure 1.2**). Native HIV-1 envelope protein comprises two subunits– a transmembrane subunit (TM) and a surface subunit (SU) [29], [30]. In addition to gag, pol, and env, lentivirus also contain regulatory genes rev and tat, and accessory genes - vpu, nef, vif, and vpr [4]. Throughout the development and improvement of LVs design, four generations of expression systems can be identified. They use a split genome approach, and their difference remains mainly on the amount of viral genetic information expressed and biosafety level [29], [48]. Third generation LVs are produced by expression of gag, pol, rev, and envelope protein-encoding gene, which are split in three plasmids or cassettes. It has a high safety level and results

in relatively high titers, being the most widely used generation in R&D or clinical purposes [29], [49].

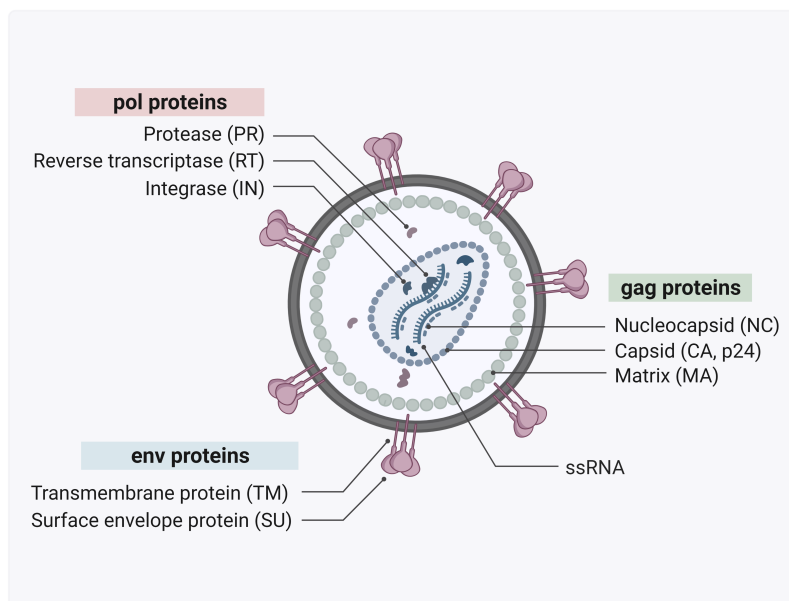


Figure 1.2. LVs particle structure. LVs particles are composed of a lipid bilayer derived from the host cell, an RNA genome and proteins mainly derived from the proteolytic cleavage of precursor polypeptides (gag, gag pol and env) encoded by three viral genes. Envelope may be replaced by the envelope protein of another virus, typically the VSV-G. Adapted from [29], [50]. Image created using BioRender.com.

2.2. Virus life cycle and pseudotyping

Viral vectors used in gene therapy exploit the natural capacity of a virus to package genetic information and deliver it to target cells (**Figure 1.3**) [51]. The lentivirus infection starts with the binding of the viral envelope glycoprotein with a surface receptor followed by fusion with the cell membrane (receptor binding and fusion), and the viral core is then released

into the cytoplasm [4], [18], [29]. The binding step is crucial for viral infection, and the HIV-1 envelope glycoprotein only presents specificity towards CD4+ cells, which limits its tropism [35], [52]. For that reason, a major difference between LVs and lentivirus is the envelope protein. The substitution of its native envelope glycoprotein with that of another virus is called pseudotyping and changes the viral particle tropism [35], [53]. This modification highly impacts the LV's stability and cytotoxicity [35], [41]. Currently, the most used pseudotype, either in clinical applications or development research, is the G protein of vesicular stomatitis virus (VSV-G), given its broad tropism and relatively high stability [29], [32]. After the entering of the virion, the viral RNA is reversed transcribed into double-stranded DNA, within the viral capsid. The complexation of DNA with several viral proteins facilitates its nuclear importation (**Figure 1.3**).

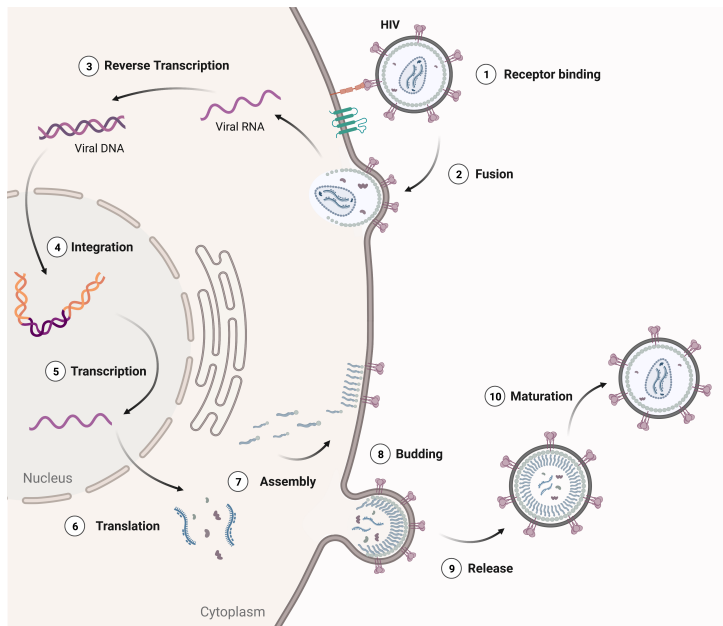


Figure 1.3. HIV-1 replication cycle. Simplified representation of the HIV replication cycle. Adapted from [4], [50], [54]. Image created using BioRender.com.

The provirus is integrated into the host genome by IN cooperating with a cellular protein. After integration, using the cellular machinery, genes are transcribed and/or spliced. The full-length genomic RNA (gRNA) and messenger RNA (mRNA) are transported to the cytoplasm where the latter is translated (gene expression). Thereon assembly occurs and the immature particles bud from the cell membrane [4], [18], [29]. The LV envelope composition is similar to the host cell lipid membrane (or producer cell for LVs) [55], [56], which also can impact its stability [41]. Afterward, the LVs are matured through the digestion of the polyproteins by viral proteases [4], [18], [29].

2.3. Considerations on LVs stability

It is accepted that some parameters in the bioreaction can impact the stability of these viral vectors [29], although only a few studies address such issues for LV. Holic et al. found a stability increase at physiologic temperature (37°C) and against multiple freeze-and-thaw cycles for GaLV-TR and VSVG pseudotyped LV produced in mildly acidic conditions [57]. Moreover, some studies have identified to impact of temperature, envelope's cholesterol levels and production medium osmolarity on others retrovirus lability [42], [58]–[60].

One of the hurdles in LVs purification is its relatively small half-life time. The loss of RT activity has been pointed out as an important mechanism through which LVs loses its functionality [61], [62], although, it also may be associated with the envelope protein, as different inactivation kinetics have been described to different pseudotypes under similar conditions [63], [64]. Different half-life times for VSV-G pseudotyped LV have been described, with values ranging from 3 to around 43 hours at 37°C [30], [40], [57], [63]. Those differences have been attributed to the conditions in which the stability tests are performed, mainly the used buffers and the purification procedures

[63]. However, LV's stability can be significantly increased at lower temperatures [40].

High NaCl concentrations result in a relatively quick inactivation of LV [29], [41]. Although no specific study addressed the kinetics of inactivation for this kind of viral vector, Segura et. al, reported an activity loss of 50% and membrane damage, due to osmotic pressure increase, after a 1-hour exposure of Moloney murine Leukemia Virus (MoLV) – a γ -retrovirus – to 1M NaCl [65]. The loss of functional titer is described in the literature as one of the consequences of freezing-and thawing LV preparations. Particularly, Higashikawa et. al studied the stability of VSV-G pseudotyped LVs upon several cycles consisting in the rapid freeze of LV containing samples and storage at -80°C, followed by thawing at 37°C. The authors described a biphasic kinetic with a quick and pronounced loss in functional titers in the first 5 cycles of freeze-and-thaw, followed by a softer decrease in the following cycles. The number of cycles to which the functional titer was reduced by half was calculated as 11.8 cycles under those conditions [40]. The authors hypothesized two possible explanations for the experimental data: in the first hypothesis, there are two populations of vector particles, one more labile than the other, and in the second hypothesis, the vectors contained two different degradation sites or components, with one being more fragile than the other. Moreover, the authors attributed the biphasic kinetic to the envelope protein binding with the surface receptor of the target cells, as a similar pattern was found for HIV with native envelope glycoprotein [40], [66]. Ruscic, et al. found that while a slower thawing on ice resulted in a recovery of 20% in functional titer, a quicker thawing in a water bath at 37°C resulted in virtually no losses [46]. Different losses have been reported by several authors. For instance, Bandeira et al. described an 84% loss in a thawing process on ice and in the absence of stabilizers. However, the recovery of this step was increased to 74% with the addition of stabilizer

substances [42]. In addition to functional titer losses, the loss of physical particles, measured by Nanoparticle Tracking Analysis (NTA), was reported after multiple freeze-and-thaw cycles, being more expressive for viral preparations in the absence of stabilizers [67].

pH and shear stress are other environmental conditions impacting the functional particle stability [40], [45]. The optimal pH ranges typically between 6 and 8 [40]. It is known that the envelope proteins may be affected by pH shift, and although such environmental change results often in reversible conformation modifications, they can also result in aggregation and precipitation [68]. Regarding shear stress, the use of VSV-G as envelope protein enables relative higher resistance, making this pseudotype more resistant throughout the purification train, especially if it contains ultracentrifugation steps [69].

Notwithstanding the advantages of VSV-G regarding its stability throughout the purification train, in recent years, production and purification schemes for other envelope proteins are being published. Those proteins are derived, for instance, from BaEV (baboon endogenous virus) [70], RD114 (an endogenous feline virus) [46], JSRV (jaagsiekte sheep retrovirus) [71] or GaLV (gibbon ape leukemia virus) [41]. Although still a relatively incipient trend, it might have several reasons. On the one hand, the use of constitutive packaging cell lines implies the use of other pseudotypes than VSV-G, due to its cytotoxicity to producer cells [32]. On the other hand, there are safety concerns regarding insertional mutagenesis. The use of GaLV [72], [73] or BaEV [74], given its high tropism against specific cells, makes possible the reduction of the multiplicity of infection (MOI, e.g., the number of vector copies per cell), which reduces the tumorigenic potential alongside with cytotoxicity to target cells [24]. Additionally, for in vivo delivery, VSV-G LVs is inactivated by human recombinant serum [30], [75] and its broad tropism may imply the transduction of unwanted cells [35].

3. Upstream processing of LVs

Current methods for LVs production use HEK 293 or HEK 293T cells [26], mainly by transient transfection [4]. In fact, it is an easy and efficient strategy [30], with the higher titers being obtained between 24 to 72 hours *post*-transfection (hpt) and followed by a quick and pronounced decrease in productivity due to cell death caused by LVs cytotoxicity [69]. For third generation of LVs, gag, pol, rev, and envelope protein-expressing genes, contained in three different plasmids are introduced in producer cell lines and transiently expressed - two packaging plasmids, one containing gag and pol genes, and the other containing rev gene, an envelope plasmid which contains the genetic information that encodes for the envelope protein [18], [48]. Additionally, the transgene, which is the only genetic material transferred to the target cells, is contained in a transfer plasmid, and flanked by elements necessary for encapsidation, reverse transcription, and integration [32].

Several agents can be used to promote transfection, like calcium-phosphate (CaPO_4), polyethylenimine (PEI), or lipid-based transfection reagents [76]. Other transfection approaches have been described, such as baculovirus infection [33] or electroporation [77]. Nonetheless, the use of inducible producer cell lines (PCL) or constitutive PCL, hereinafter simply referred to as stable PCL, is pointed out as the best option for gene therapy [32]. For inducible PCL, LVs production starts after the addition/removal of an induction agent [78]. One of the major advantages of using inducible PCL becomes apparent when conjugated with continuous media removal, which can increase the duration of production process [79], although only for a short period post-induction [32]. On the other side, constitutive PCL allows the continued LVs production without cytotoxic limitations [49], using other viral envelope proteins different than VSV-G [23]. Compared with transient

transfection, typically, lower titers are obtained with stable PCL, ranging from 10^6 to 10^7 transduction units per milliliter (TU/mL) [32]. Regarding the anchorage-dependence of the cells, monolayer adherent cell culture is the most common approach [34], [80], [81], although suspension cell culture can be used as well [79]. Adherent systems used in large-scale LVs production include multi tray devices [34], [82], [83] that are constituted by multiple layers to which the cells attach.

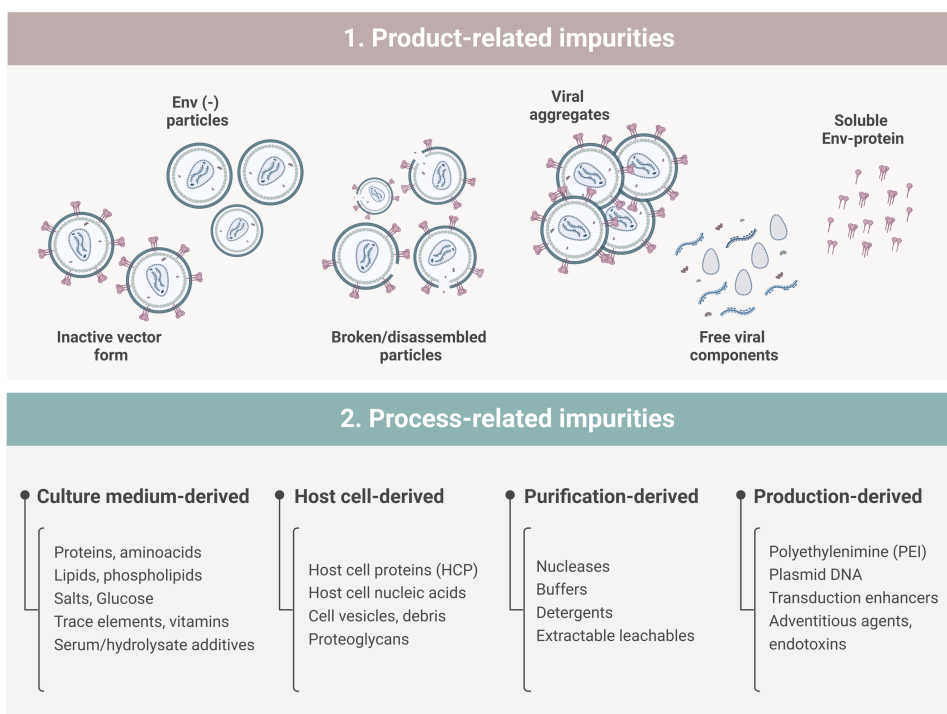


Figure 1.4. Impurity profile of LVs manufacturing. Commonly found impurities: (1) Product-related and (2) Process-related impurities. Adapted from [29]. Image created using BioRender.com.

This strategy has been employed by several companies/ institutions in the past decades, such as Généthon [34], MolMed [73], Oxford Biomedica [84], Beckman Research Institute [83]. Despite its broad use, multi tray devices have some disadvantages. An increase in the volume of production is achieved by scale-out and often do not enable control of culture conditions [85]. Additionally, those technologies result in large footprints and are labor-intensive, resulting in high production costs and contamination risks [86]. Another option reported for LVs production using adherently grown cells is PALL's fixed-bed bioreactor iCELLis 500 [80], [87], [88], which has been gaining ground, hence it enables for a considerable scaled up production (available surface up to 500 m²) and perfusion operation, under a controlled and closed environment [89].

The use of suspension cell culture is growing, being pointed as the most appropriate approach for industrial manufacturing. The easy scale up of this strategy is an advantage, as it allows for larger lots, greater lot homogeneity and easier process control, compared to multi tray approaches [32]. Another advantage of suspension cell culture is the lack of need of adding Fetal Bovine Serum (FBS) to promote cell adhesion [76], [78]. This is a critical parameter since serum proteins account for a high portion of total contaminating proteins [90] and its use brings a risk of contamination with adventitious agents [32]. Companies such as Oxford Biomedica [91] and Genethon [92] are incorporating suspension systems in their manufacturing trains [89]. Some of the previously mentioned systems enable perfusion operation, with continuous fresh medium addition and supernatant removal, but biomass retention in the bioreactor [93]–[96]. Perfusion operation is commonly used in animal cell culture, as it allows higher specific growth rates and enables the overcoming of the high sensitivity of cells to accumulated metabolites (such as lactate and ammonia) through their continuous removal [96], [97]. Being LVs a cytotoxic bioproduct this is of special interest. Several

articles have reported LVs production using perfusion mode, either for suspension [79], [96], [98] or adherent cell culture [87]. An increase of the volumetric productivities can be achieved, when compared to batch [79], [96], [98]. Consequences include the necessity to downstream process larger volumes of LVs containing supernatant [76] and for suspension cell culture, and the need for a retention device. Despite transient transfection of adherent cells cultured in batch mode being still widely used, the emerging methodologies for LVs production are being increasingly reported. This paradigm shift carries changes in the manufacturing train that will likely impact the downstream processing bringing new challenges with which it needs to cope [89] (**Figure 1.4**).

4. Downstream processing of LVs

4.1. Traditional LV purification and concentration methods

The traditional purification and concentration techniques are mostly used if the needs of LVs can be satisfied by small-scale production, for instance, scientific research. The centrifugation-based methods can be used as concentration and/or purification techniques. Viral pelleting and resuspension are often used as a concentration method [81], [99], [100]. The use of density gradient centrifugation or ultracentrifugation using sucrose [71], [101] or iodixanol [102] have been reported and can achieve some degree of purification along with reasonable concentration [100], [103]. Another technique for small-scale purification schemes uses polyethylene glycol (PEG). This polymer sterically excludes viral particles from the solution leading to their precipitation. Viral particles are recovered by centrifugation and high yields can be achieved [71], [103].

Flaws of traditional methods include concerns with scalability and the concentration of impurities in the product [32]. Although a high degree of purification can be obtained, the use of ultracentrifugation reduces the infectiousness given the stress conditions to which LVs are subject. Thus, the field is gradually moving away to more versatile technologies.

4.2. Scalable methods for LVs downstream processing

The development and use of DSP with chromatography steps and membrane-based separation operations have been described over the past years [104]. The processes can be divided into three main phases: i) harvest and clarification; ii) intermediate purification, with one or more concentration and purification steps; and iii) polishing, formulation, and sterilization. The nucleic acid digestion is usually required and can take place in different phases of the process (**Figure 1.5**). Currently, overall recovery yields for large scale DSP range from 20 to 40 % [32].

4.2.1. Harvest and clarification

LV are extracellular bioproducts released by budding from the producer cell. The majority of large-scale LV production processes use transient transfection of HEK293 or HEK293T adherent cells in batch mode and the harvest occurs generally once or twice at 48 to 72 hours *post-transfection*. Although, more recently, the industry has been also transitioning into suspension cultures [70], [77], [79], [92], [105]–[107].

The harvest quality is highly dependent on cell viability which impacts the amount of host cell derived impurities. Thus, the choice of the time of harvest must consider not only the productivity but also the quality of the supernatant [104], [108].

The clarification's main goal is to remove cells and cell debris from the LVs containing media. This step generally comprises depth filtration [25], [42], normal flow microfiltration [41], [109], low-speed centrifugation, or a combination of these operations. The use of centrifugation followed by microfiltration [46], [71], [100] is a common strategy to reduce filter clogging and minimize fouling at small volumes. With similar goals, but for larger scales, a membrane cascade is commonly used [41], [70], [109], the final pore size being 0.45 μm [34].

The recoveries for these early steps are commonly not reported, although, after optimization, small losses in functional titer can be achieved [25], [41], [42]. Valkama et al. recently reported functional recoveries close to 100% using a depth filter for clarification of large supernatant volumes containing VSV-G pseudotyped LVs [25]. However, the success of this operation can be highly dependent on the pseudotype. Using a GaLV-TR pseudotyped LVs, Boudeffa et al. obtained very low recoveries in the clarification step latter improved by supernatant dilution and stabilization [41].

Nonetheless, to meet the LV's increasing demand, recent developments in upstream processing (USP) are emerging and the integration of the early steps of DSP in USP can be highly advantageous [70], [79], [110]. Recently, Oxford Biomedica reported the use of Tangential Flow Depth Filtration (TFDF™) to harvest and clarify LVs produced in suspension culture [95], [96], [111] resulting in high recoveries and potentiating multiple harvests. Moreover, for suspension cell cultures operating under perfusion, retention devices, like the Virus Harvest Unit (VHU™), allow the harvest of clarified viral stocks [79], [112], [113]. Regarding adherent cell cultures, several bioreactor configurations can be used for continuous LV production [80], [87], [88], [114]. TFF-microfiltration [115], [116] or continuous flow disk-stack centrifugation [116] are also continuous techniques, although never reported for LVs clarification.

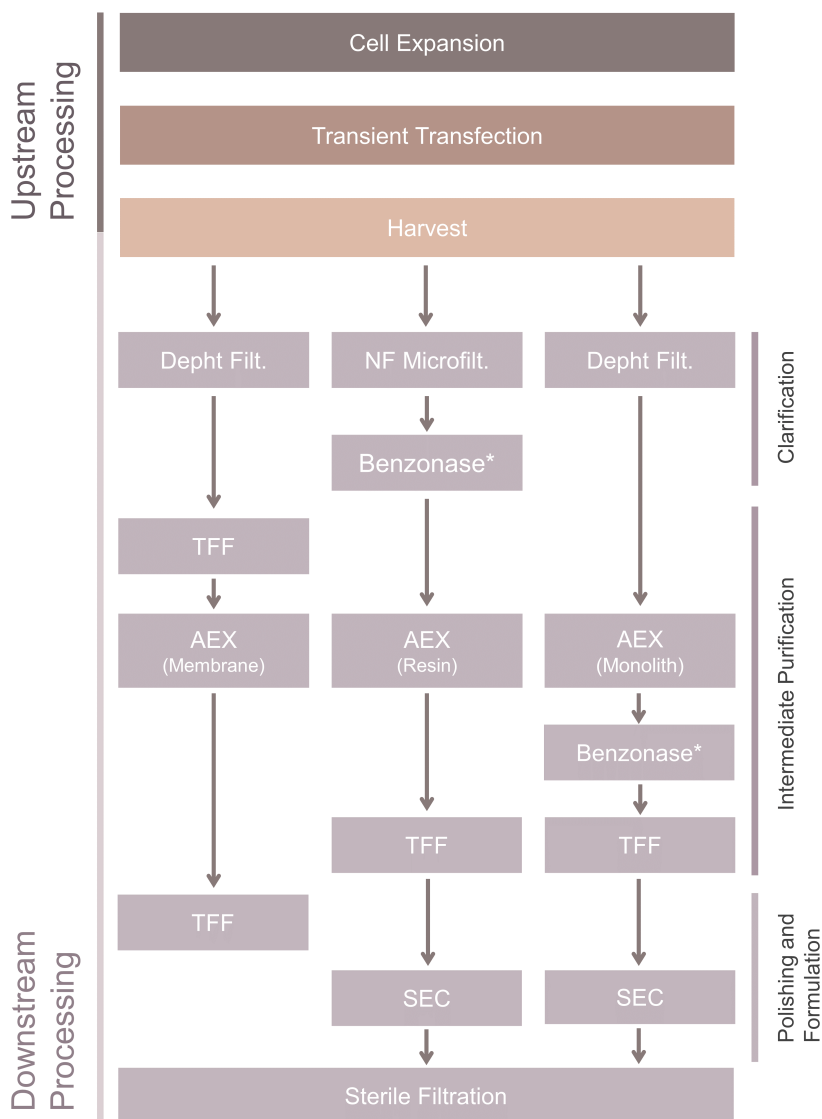


Figure 1.5. Downstream processing workflow for Lentiviral vectors purification. This image illustrates three different sequences of unit operations described for Lentiviral vector processing[25], [34], [42]. The nuclease used in the represented processes was Benzonase® that can be introduced in distinct stages of the process. For Valkama, et al. the nuclease step is part of the upstream harvest. NF – Normal Flow.

4.2.2. Nucleic acid cleavage

To increase the overall biosafety of the final product [24] and comply with the regulatory standards [117] a nucleic acid cleavage step must occur. The use of Benzonase® or Denarase® reduces DNA size and decreases bulk viscosity [100], [104]. These enzymes require magnesium ions as a co-factor and show optimum activity at 37 °C however different experimental conditions (for example, amount of enzyme, temperature, incubation time, salt concentration, pH, and the presence of enzyme inhibitors) are reported regarding nucleic acid cleavage step. Recently, Oxford Biomedica developed a system (SecNuc™) that avoids the addition of nucleases that are co-produced with LVs [118].

Many times, nuclease step immediately precedes [25], [119] or succeeds clarification [34], [83]. In other processes, it is included in the USP [41], [83] or later DSP phases after some volume reduction [120]–[122]. The early location of this step results in higher nuclease consumption increasing the manufacturing costs which can be counterweighted by easier subsequent DNA clearance and also nuclease removal which must be accomplished if LVs are intended for clinical use [29].

4.2.3. Intermediate purification

Following clarification, scalable purification steps for LVs use chromatography and/or Tangential Flow Filtration (TFF), both techniques achieving some degree of concentration of the bulk product. Chromatography is a key technology in purification protocols for the main biotherapeutic products offering scalability, purity, high recovery rates, and reproducibility [123]. The classic resin media were originally designed for protein purification; thus, the large hydrodynamic radius of the LVs raised some technical constraints. The use of beads often restricts access to the

binding sites, resulting in small dynamic binding capacities [124], [125]. Additionally, the high column backpressure also leads to limited flow rates. The mass transfer in this kind of stationary phase is predominantly limited by diffusion, whereas in membrane adsorbers and monoliths it is mostly convective [123], [126]. This allows a quicker processing and larger throughput, making this supports ideal for virus capture. Anion-exchange and affinity chromatography have been used in the early purification steps of LVs (**Table 1.1**).

4.2.3.1. Anion-exchange chromatography

Anion-exchange chromatography (AEX) is widely used for capture in large-scale bioprocesses. Briefly, the purification of these viruses is performed in a bind-elute mode where the LVs particles, having a negative surface charge at working pH, are adsorbed by the positively charged matrix [100], [108]. The elution is performed with buffer's ionic strengths between 0.5 – 1 M NaCl [29], [110]. As LVs are labile, high salt concentration can lead to virus inactivation [26], [53]. A loss of 50% of LVs biological activity after one hour of exposure to 1M NaCl has been reported [65]. To reduce losses during the AEX step it is often necessary to dilute the viral vector right after the elution step [127].

Membranes and monoliths stationary phases, as with conventional chromatography resins, can be functionalized with strong or weak ion ligands [100], [128], [129]. Strong ion exchangers with quaternary amines as ligands have been used to produce high-quality virus stocks [130], in particular with Sartobind® Q and Mustang® Q commercial membranes [25], [41]. Conversely, weak exchangers, for example, CIM® Monolith with DEAE (diethylaminoethanol) ligand, have also been reported in LVs purification with high recovery yields [42]. In general, this technique results in functional particle recovery yields ranging from 30 to 80% (**Table 1.1**).

Since binding is driven by electrostatic interactions, AEX matrices are not selective for LVs, and negatively charged nucleic acids and proteins bind to the chromatographic media [53], [108]. The high salt concentration required to elute LV leads also to the release of bound impurities. Merten et al. achieved around 99% and 86% of protein and DNA removals [34]. However, additional purification steps are usually required to obtain clinical-grade virus stocks [130].

Novel stationary phases are continually being developed. Ruscic et al. reported LV purification using regenerated cellulose nanofibers derivatized with a quaternary amine and produced by electrospinning. The authors claimed to achieve a volumetric concentration factor of 100, with a recovery yield of 85% [46]. Moreover, Labisch (2022) et. al has described the use of steric exclusion with hydrophilic cellulose membranes for the purification of LVs [131]. Although interesting results have been achieved, a deeper study is necessary to enable the scale-up of these devices [132].

4.2.3.2. Affinity chromatography

The affinity chromatography (AC) is used to separate particles from the broth based on a highly specific interaction between them and a selected immobilized ligand [29], [133]. Unlike AEX, AC presents a high selectivity, which potentially leads to higher purity and concentration outcomes. Therefore, its incorporation in a DSP can lead to a reduction in the number of unit operations required and consequently greater overall recoveries [29].

One of the first reported studies used the immobilized metal affinity chromatography (IMAC). This technique explores the binding between an immobilized metal ion and a molecule capable of sharing electrons with it. For LV purification this would involve the modification of the envelope protein with the introduction of a histidine tag. Such insertion can result in lower

expression or compromise the protein's function, with loss in particle infectivity[134]–[136]. Furthermore, the desorption step requires the use of imidazole resulting in LVs inactivation. [29], [135]. Finally, another major drawback of IMAC for LVs purification is the possible adverse effects promoted by His-tag in clinical trials, thus ensuring its current rejection [53].

The use of heparin AC in LVs purification enables an elution under mild conditions [105]. However, its low selectivity can result in a binding competition of host cell proteins and DNA with LVs and consequently co-elution [26], [29], [105]. Given the animal origin of heparin, its use will be avoided in industrial applications [32].

Another strategy involves LV labelling with desthiobiotin [137] or biotin mimics [136], that bind streptavidin columns (or magnetic beads) with low affinity. LVs desorption is made with biotin. Chen et al. [137] modified a cell line to generate desthiobiotin-labelled LVs further purified in monomeric avidin-coated chromatography columns. More recently, Mekkaoui et al.[136] reported the purification of an RDPro pseudotyped LV labelled with cTag8. This biotin mimic was genetically encoded, and the technique is envelope-protein independent. Despite the encouraging results at a lab-scale for LVs purification using AC, much has to be improved to translate this technique into an industrial setting. Particularly, modifications on the LVs envelope may compromise the viral biologic activity, by vector inactivation or tropism alteration.

Table 1.1. Anion Exchange and Affinity chromatography methods used for the purification of LVs.

Column/Matrix	PT	Load	BV	Des. reagent	Buffer	pH	Rec. (%)	Ref.
Anion Exchange Chromatography								
Resin								
HiTrap Q HP	VSV-G	1 mL	1 mL	LG: up to 1 M NaCl	100 mM Tris	7.5	89.6	[100]
PEG-modulated DEAE Sepharose FF	-	-	-	SW: 0.65 to 1 M NaCl	-	-	95	[138]
DEAE resin	VSV-G	48 L	-	0.75 M NaCl	PBS	-	-	[34]
Poros DM/50	GaLV-TR	45/29 mL	8 mL	SW: elution at 0.7 M NaCl	20 mM Bis-Tris + 5 % sucrose + 2 mM MgCl ₂	6.0	61.5	[41]
Poros D 50	GaLV-TR	20 mL	4 mL	0.65 M NaCl	20 mM Bis-Tris	5.5	100	[73]
Membrane								
Sartobind Q	VSV-G	11 kg	400 mL	SW: 0.3 to 1.50 M NaCl	50 mM HEPES	7.5	33	[25]
Mustang Q XT5	BaEV-R-less VSV-G	1 L	5 mL	2 M NaCl	50 mM Tris	8.0	>33 ¹ >55 ¹	[70]
Nanofiber (RC w/Q)	RDPro	400 mL	0.1 mL	LG: elution at 0.6–0.9 M NaCl	PBS + Tween 20	7.45	86	[46]
Mustang Q	VSV-G	6 L	60 mL	1.2 M NaCl	25 mM Tris–HCl	8	-	[109]
Mustang Q XT5	VSV-G	1.5 L	5 mL	1.5 M NaCl	PBS	7.2	-	[81]
Mustang Q Acrodiscs	MVG	540 mL	0.8 mL	LG: elution at 0.2–0.4 M NaCl	25 mM Tris-HCl	7.4	65	[139]

Table 1.1 Continued

Column/Matrix	PT	Load	BV	Des. reagent	Buffer	pH	Rec. (%)	Ref.
Sartobind Q75	VSV-G	-	75 mL	SW: elution at 1.2 NaCl	DMEM (or TSSM)	-	-	[140]
Mustang Q capsule	VSV-G	-	-	1.5 M NaCl	50 mM Tris-HCl	8	-	[141]
Microcapillary film (EVOH w/Q)	VSV-G	15 mL	1.5 mL	1.3 M NaCl	25 mM Tris HCl	8.0	11/10 ²	[142]
LentiSELECT 40/500/1000	VSV-G	40 mL 500 mL 1000 mL	-	-	-	-	52 38 37	[127]
Mustang Q	VSV-G	36-52 L	60 mL	1.5 M NaCl	-	7.5	>60	[122]
Monolith								
CIM DEAE	VSV-G	-	-	SW: elution at 0.65 M NaCl	10 mM Tris	8.0	80	[42]
Affinity Chromatography								
CIMac	RDPro	50 mL	0.1 mL	X-Vivo15 + 15 mM biotin + BSA	PBS	-	20	[136]
Pierce monomeric avidin coated column	VSV-G	12 mL	2 mL	2 mM biotin	PBS	-	68	[137]
Fractogel EMD Heparin ³	VSV-G	35 mL	1 mL	0.35 M NaCl	20 mM Tris-HCl +0.150M NaCl	7.5	53	[105]
CaptureSelect™ Lenti VSVG	VSV-G	25 mL 250 mL	1 mL 10 mL	0.8 M Arginine	50 mM HEPES +0.150 M NaCl	7.5	53	[143]

PT – Pseudotyping; **BV** – Bed Volume; **Des. reagent** – Desorbing reagent; **Rec.** – Recovery of functional particles; **SW** – Stepwise elution; **LG** – Linear gradient elution. ¹. Recovery of more than one unit operation, including chromatography ². Capture of clarified LV/Capture of unclarified LV. ³. Fractogel EMD Heparin had been discontinued

4.2.4. Tangential Flow Filtration

Ultrafiltration/Diafiltration is the gold standard for virus large-scale concentration and buffer exchange since it is a fast and robust technique, easily scaled-up and, transferred to good manufacturing practices (GMP) [29]. Briefly, TFF is used to concentrate viral particles in the retentate, whereas small impurities such as proteins and DNA fragments are removed throughout the pores into the permeate [104]. Membrane fouling can be a major limitation leading to permeate flow rate decrease and transmembranar pressure (TMP) increase during the concentration process. TFF can be applied at different stages of the downstream process. First, it can be used for concentration of the feed stream, after virus clarification, reducing the feed volume for the later steps and if diafiltration is performed, exchanging the buffer to an appropriate composition for subsequent operation units [53]. Secondly, it is also commonly employed in the downstream polishing steps for concentration and buffer exchange or formulation [39].

As previously mentioned, one of the major challenges in LVs purification is the maintenance of their biological activity. Pressure variations, high shear forces, and foaming can lead to loss of infectivity or even envelope disruption [25]. Membrane chemistry and pore size are selected by experimental screening followed by optimization of the critical process parameters such as flow rate, TMP, membrane throughput, and process time to assure a smooth operation. Flat sheet cassettes [25], [137] or hollow fibers [34], [41], [122] are used for LVs concentration and different materials are available – polysulfone (PS), polyethersulfone (PES), polyvinylidene fluoride (PVDF), or regenerated cellulose (RC) [104], [110]. The selected molecular weight cut-offs (MWCO) range between 100 and 750 kDa. A higher impurity clearance and decrease of process time can be achieved for devices with larger pore size; however, this can lead to viral loss, as observed by Valkama et al. [25].

These non-recovered LVs can leave the system in the permeate or be entrapped in the membrane. **Table 1.2** lists the high recovery yields that have been reported by different groups using LVs pseudotyped with VSV-G. This technique started to be employed for more envelope types as described by different research teams [41], [70], [71]. The maximum concentration factor achieved and the number of diafiltration volumes should be established considering the final clinical use of the viruses.

4.2.5. Polishing, Formulation and sterilization

Polishing and formulation of LV preparations can be achieved either by using chromatographic methods (based on size exclusion) or by TFF. Some large-scale processes explore the difference in the size of the LVs compared to remaining impurities for the polishing step [32], [34], [120], [122]. In the size exclusion chromatography (SEC), viral vectors are excluded from the internal pores of the packing material while other biomolecules migrate slowly as they are more likely retained in the pores [29], [123]

A maximum MWCO of 750 kDa (equivalent to 50 nm) is commonly used to prevent titer loss [32]. This method enables the efficient removal of small-sized particles with high reported recoveries, from 50 to 80% [29], [41], [42], [122]. However, low flow rate and sample size limitations make this technique not ideal for the processing of large volumes [144], [145]. Besides, sample dilution by 2- to 4- fold hampers the production of highly concentrated viral stocks [39]. Nevertheless, a scalable process for purification of GaLV-TR pseudotyped LV was reported adopting SEC for polishing [41].

Alternatively, multimodal chromatography, like Capto™ Core 700, can be used for polishing [41], [73], [100], [104]. This kind of flow-through chromatography combines the properties of SEC and AEX. The chromatographic media is composed of particles with a functionalized core,

to which larger particles cannot access, and a non-functionalized outer layer [146]. Boudeffa et al. reported a recovery of 86% in TU for an LVs pseudotyped with GaLV-TR [73].

As mentioned above, TFF is frequently used to concentrate and formulate LVs [25], [84], [109]. A proper virus formulation is crucial to provide maximum stability and guarantee therapeutic success [41]. The LVs must remain biologically active after freeze-thaw or lyophilization [29], [39] and during its clinical use [110], [120]. For *ex vivo* applications, there is some flexibility in the formulation that leads to the use of protein-containing media such as Lonza's X-VIVO™ and CellGenix's CellGro® media [11], [34], [41], [70]. Traditionally, the stabilization of LV has been accomplished with the use of different buffers systems – PIPES [67], [120], HEPES [25], HIS-HCl [147], Tris-HCl [119] or PBS [41], [148] – coupled with stabilizer adjuvants. The more common excipients include sugars (sucrose [25], [41], [120] or trehalose [147], [149]), or proteins such as recombinant human albumin and gelatin [147], [150], [151].

Finally, sterilization of the drug substance is achieved by filtration through a 0.22 µm filter, before vialing. This is a standard regulatory requirement [32] and a critical step regarding vector losses. Valkama et al. reported losses between 30-50% in the last step of their large-scale DSP [25]. The recoveries on this step are dependent on the membrane material [29] and the LVs titer. Oxford BioMedica observed that sterilization after the final concentration step resulted in a low LVs functional recovery. Therefore, in their DSP protocol, the sterile filtration is placed prior to the final TFF which is done under aseptic conditions [84]. Sterilization can be skipped if the process is performed aseptically in a semi-closed system [32], [83]. The final product is then stored at - 80°C to protect the virus from thermal inactivation [3], [29] or lyophilized [119].

Table 1.2. Tangential-flow Filtration for concentration and/or diafiltration of LVs.

		PT	MWCO	Pressure data	Area	Feed	CF	Rec. (%)	Ref.
Cassette									
5× Sartocon Hydrosart (Sartorius)	Cellulose	VSV-G	100 kDa	TMP ≤ 0.1 bar	3.0 m ²	126.8 kg	13	70-80	[25]
2× Sartocon Hydrosart + 1× Sartocube Hydrosart (Sartorius)				TMP ≤ 0.2 bar	4.2 m ²	178.4 kg	18	-	
Sartocon Slice Hydrosart (Sartorius)				TMP ≤ 0.2 bar	0.1 m ²	855 mL [§]	1.5	~60	
Vivaflow 50 (Sartorius)	-	VSV-G BaEV R-less	100 kDa	-	-	-	-	>55 >33	[70]
Centramate Cassette (PALL)	Omega PES	VSV-G	50 kDa 100 kDa 300 kDa	$\Delta P = 0.3$ bar $P_{\text{Feed}} = 1$ bar ¹	-	300 mL	11- 12	100 91.4 81.3	[71]
		Δ GP Jenv	50 kDa			184 mL	7.4	97.9	
Omega T series (PALL)	Omega PES	VSV-G ^I VSV-G ^{NJ} Cocal	100 kDa	-	0.1 m ²	-	40	-	[119]
Pellicon-2 mini filter (Merck Millipore)	PVDF	VSV-G	500 kDa	-	0.1 m ²	-	10	-	[141]
Vivaflow (Sartorius)	-	VSV-G	100 kDa	$P_{\text{Feed}} = 1$ bar	-	-	-	72	[42]
Omega Screen channel-cassette (PALL)	Omega PES	VSV-G	100 kDa 300 kDa	$P_{\text{Feed}} = 1$ bar	-	-	67	100	[152]

Table 1.2 Continued

		PT	MWCO	Pressure data	Area	Feed	CF	Rec. (%)	Ref.
Hollow fiber									
(Spectrum Labs)	mPES	RDPro	500 kDa	TMP = 0.075 bar	-	-	-	~20	[46]
(Spectrum Labs)	-	VSV-G	500 kDa	TMP = 0.35 bar	190 cm ²	1.2 L	8	-	[109]
(Spectrum Labs)	mPES	GalV-TR	500 kDa	P _{Feed} = 0.5 bar	115 cm ²	-	-	40 ^{2,3}	[41], [73]
(GE Healthcare)	PES		750 kDa		110 cm ² 410 cm ²	-	11.2 36.6	64 - 74 ² 86 ²	
(GE Healthcare)	PES	VSV-G	750 kDa		110 cm ²	-	11.2	100 ²	
FlexStand (GE Healthcare)	PS	VSV-G	100 kDa	TMP = 0.5 bar	-	-	-	-	[121]
(GE Healthcare)	-	VSV-G	500 kDa	-	-	-	100	-	[120]
(GE Healthcare)	-	VSV-G	500 kDa	-	- -	-	20	-	[83]
(GE Healthcare)	-	VSV-G	750 kDa	-	- -	20L	29	-	[34]
Tandem TFF ⁴ (Spectrum Labs)	-	VSV-G	500 kDa	P _{Feed} FP1: < 0.45 bar FP2: < 0.6 bar	615 + 40 cm ²	5.5L	2000	94-100	[153]

PT – Pseudotyping; **MWCO** – Molecular weight cut-off; **CF** – Concentration factor; ¹. The authors consistently reported a recovery increase when operating at higher feed and retentate pressures, maintaining the differential pressure constant. ². pH of the DF buffer have a considerable impact on vector recovery. ³. Before optimization. ⁴. Uses a system with two sequential and integrated HF filters, FP1 and FP2.

5. Analytical approaches and regulatory requirements

From the impure LVs containing supernatant to a final product, an increase of the vector titer and a decrease of the total amount of impurities is expected (**Figure 1.5**) [39], [110]. Those impurities are commonly distinguished between process-related and product-related that were extensively reviewed by Segura et al. [29].

Analytical methods play a key role in process development and operation, as well as in the characterization of the produced lots [80]. This characterization is required in GMP manufacturing and to enter clinical trials [29]. However, like for other advanced therapeutic medicinal products, there are still no strict guidelines for LVs manufacturing [110], [154]. Efforts in standardization have been made resulting in a standard for LV analytics [155] and an LVs reference material that will be released soon [25], [156]. Each lot must be tested for identity, purity, potency, and safety [26], [29]. Some examples of assays used for LV release are depicted in **Table 1.3**.

The identity can be confirmed using Western Blot for gag proteins, vector sequencing, restriction enzyme mapping, reverse phase HPLC, or SDS-PAGE [155].

Regarding purity, both process-related and product-related impurities should be well characterized in the final product. The quantification of total proteins can be acquired [157] using colorimetric assays, like Bradford or BCA [3], [29], [32]. Other impurities such as host cell proteins (HCP), nuclease, and bovine serum albumin can be quantified by ELISA [29]. The DNA must be present at a concentration below 10 ng/dose and the DNA size under 200 base pairs [157]. Their quantification can involve spectrofluorimetry and agarose gel electrophoresis [26], [29], [32], [83]. Since HEK293T has a tumorigenic phenotype, the LVs produced with these

cell lines must be analyzed to quantify E1A and SV40 LTA DNA sequences. If the production process involves transient transfection, the number of copies of plasmid sequences (vsv-g and gag-pol) must also be assessed, using qPCR or Southern Blot [29], [32], [83], [157].

A good indication of vector quality is the ratio between total and active particles, correlated with its potency. If this parameter is too high, the lot must be rejected [26], [29]. No reference value is defined by current guidelines but ratio values in the 10^3 order of magnitude are reported [34], [83]. The number of total particles is commonly inferred by p24 titration using ELISA kits [29], [110], [155]. Other analytical methods for quantification of total particles may include the assessment of reverse transcriptase activity and determination of genomic RNA [29], [158]. Physical methods are commercially available and enable a quicker quantification of total particles and characterization of size distribution.

Those methods include field-flow fractionation multiple-angle laser light scattering (FFF-MALLS), nanoparticle tracking analysis (NTA), flow cytometry, and tunable resistive pulse sensing (TRPS) [110], [159], [160]. The number of active particles is estimated by a gene transfer or gene expression assay in a suitable cell line [29]. For research purposes, when using GFP as the transgene, the functional titer is often estimated by flow cytometry of the fluorescent cells [110], [158]. However, for clinical purposes, it should be quantified by the number of reverse-transcribed viral genomes integrated into target cells using droplet digital PCR (ddPCR) or quantitative PCR (qPCR) via classical TaqMan of genomic DNA [155] or by another method suitable for the used transgene [80].

Table 1.3. Assays for quality assessment of LV preparations.

Target	Assay(s)	Ref.
<i>Identity</i>		
Confirm identity	Western Blot, Vector sequencing, Restriction enzyme mapping, Reverse phase HPLC, SDS-PAGE	[155]
<i>Purity</i>		
Total Protein	Bradford, BCA protein assay	[34], [89]
Host Cell Protein	ELISA	[34], [89]
Residual Endonuclease	ELISA	[34], [89]
Residual BSA	ELISA	[34], [89]
Total DNA	Fluorimetry (PicoGreen)	[34], [89]
Host Cell DNA	qPCR, Fluorimetry	[161]
DNA size distribution	Agarose Gel Eletrophoresis, Capillary electrophoresis	[83], [162], [163]
E1A and SV40 LTA sequences in the vector	qPCR, Southern Blot	[34]
Transfer of plasmid (vsv-g and gag-pol), and host-cell (E1A and SV40 LTA) sequences to target cells	qPCR, Southern Blot	[29], [164]

Table 1.2 Continued

Target	Assay(s)	Ref.
Potency		
Total viral particles	p24 ELISA, PERT, RT qPCR	[34], [113], [155]
Functional viral particles (analysis of transduced cells)	Flow Cytometry, qPCR, ddPCR	[34], [113], [155]
Safety		
Recombinant Competent Lentiviruses (RCL)	RCL amplification in suitable cell line by serial passages followed by quantitation assay (e.g., PCR, p24 ELISA)	[29], [34]
Adventitious agents	<i>In vitro</i> testing, PCR-based methods	[29], [83]
Mycoplasma	Culture-based methods, PCR-based methods	[29]
Endotoxins/Pyrogens	LAL test, Rabbit pyrogen test	[29]
Sterility	Bacterial and fungal sterility	[29]

BCA – biconchonic acid; **ELISA** – enzyme-linked immunosorbent assay; **PCR** – polimerase chain peaction; **qPCR** – quantitative polimerase chain peaction; **PERT** – product-enhanced reverse transcriptase; **RT qPCR** – quantitative reverse transcription polimerase chain reaction; **ddPCR** – droplet digital polimerase chain reaction; **LAL** – limulus amebocyte lysate

Also, each vector batch must be evaluated regarding several safety parameters, mainly analyzing for recombinant competent lentiviruses (RCL), endotoxins, mycoplasma, adventitious agents, and sterility [29], [165]. Physicochemical parameters like pH, osmolarity, and appearance complement product characterization [29].

6. Aim and outline of the thesis

ATMPs have evolved over the last three decades, turning the use of viral vectors for cancer and monogenic disorders treatment into a popular strategy in personalized medicine. Furthermore, the recent approval of several LV-based therapies has increased the industry's interest and demand for more efficient manufacturing processes. The standard LVs purification methods currently adopted are composed of numerous operation units resulting in extended processing times, low recovery yields, and loss of vector functionality. Undoubtedly, LVs' sensitivity to most critical process parameters has been hampering the development of efficient and robust bioprocesses. Despite the significant efforts being made over the last few years, the established manufacturing processes are far from reaching their maximum potential and are unlikely to fulfill the industry's needs. Therefore, this thesis aims to explore strategies to accelerate the manufacturing of LVs compatible with GMP requirements. By tackling both adherent and suspension systems, it is intended to improve scalability, TU recovery yields and purity, and decrease processing time.

Part of the preclinical and clinical research involving LVs still relies on anchorage-dependent cell lines which are often associated with low titer production and limited scalability. As a result of an inefficient production platform, high feedstock volumes are needed to achieve the desired viral

dose, leading to complex and long purification schemes. Hence, in the first part of this thesis (**Chapter 2**), a scalable protocol that combines clarification, anion-exchange chromatography (AEX), and ultrafiltration membrane technologies is implemented. The main goals of this work are the maximization of infectious virus recovery and reducing processing time. Design of Experiments methodologies are also used to perform a deeper study in the capture step.

To mitigate some of the constraints associated with adherent cell lines, the industry is moving fast towards the use of suspension systems. This is a natural transition since they contribute to improving scale-up flexibility and the achievement of a serum-free production, allowing the reduction of lot-to-lot variation. Since changes in the initial feedstock's main properties were expected, **Chapter 3** is focused on the development of a novel clarification step. Aiming to provide solutions compatible with the industry's trends, continuous processing and process intensification, different microfiltration devices are evaluated regarding the TU recovery yield, membrane throughput, and turbidity reduction. Then, the impact of the LVs produced under suspension conditions on the capture step is evaluated in an exploratory study. Preliminary culture media supplementation with lipid cocktails is studied to investigate how cell metabolism could influence virus surface phenotype. Later, a systematic comparison of several AEX membranes is performed, where different process parameters are assessed.

Following the focus in the chromatography step, a novel capture strategy is presented in **Chapter 4**. AC is a powerful technique that allows process simplification and represents an appealing solution for large-scale LVs purification. This work demonstrates the development of an AC resin able to specifically target VSV-G pseudotyped LVs for the first time. A set of affinity ligands is generated and optimized to maximize viral recovery yield. The

resin scalability is also assessed, and an extensive characterization of the final product is also presented.

In the last **Chapter 5**, the major findings are summarized and discussed highlighting their contribution to the field of LVs manufacturing processes.

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2

Process optimization for Lentiviral vectors from adherent productions

Chapter adapted from:

Ana Sofia Moreira, Tiago Q. Faria, Joana G. Oliveira, Aydin Kavara, Mark Schofield, Todd Sanderson, Mike Collins, René Gantier, Paula M. Alves, Manuel J.T. Carrondo, Cristina Peixoto. **Enhancing the purification of Lentiviral vectors for clinical applications**. Separation and Purification Technology. 2021, 274: 118598.

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Ana Sofia Moreira designed and performed the experiments, analyzed the data, and wrote the chapter.

Abstract

Lentiviral vectors have been increasingly used as a tool for gene and cell therapies since they can stably integrate the genome in dividing and nondividing cells. The development of efficient, fast, and robust downstream processes that cope with the low stability of this virus is slowing down the clinical-to-market transition.

A purification process comprised by four-unit operation was developed and improved. Several clarification filters with different materials were evaluated for virus recovery and throughput. Anion exchange chromatography was optimized by tuning the critical operation conditions for Mustang™ Q and Sartobind™ Q membrane chromatography devices using Design of Experiments methodology. Mustang Q demonstrated better performance with recovery yields close to 90%. Good scalability and robustness were found running Mustang Q at different scales up to 5 mL capsule. Flat sheet cassettes and hollow fiber with different molecular weight cut-offs were evaluated for concentration and formulation and recovery yields above 60 % were obtained for both supports operated under different experimental conditions. The sterile filtration step was successfully performed and allowed a global virus recovery up to 45%.

This work unravels strategies to enhance lentivirus vector purification process which may accelerate the development of therapeutic products based on lentiviral vectors.

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1. Introduction

Lentiviral vectors (LV) based on human immunodeficiency virus (HIV), are increasingly gaining space as a tool for gene and cell therapy due to their valuable features [1]. This 80-120 nm vector presents the ability to integrate a large payload (up to 9 kb), to transduce dividing and non-dividing cells and has lower insertional mutagenesis compared, for example, to Murine leukemia virus [2], [3]. LV is the vector of choice for stable and sustained long-term gene expression [4] and is currently mostly used for delivery therapeutic genes to primary cells, especially *ex vivo*, to address infectious diseases or genetic disorders [5]–[7].

Different envelopes are available, being the vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped LV the most studied. Besides presenting a broad tissue tropism via VSV-G receptors, allowing an efficient transfection of different cell types, this envelope also presents high stability in comparison with their counterparts [8], [9]. The number of clinical trials using LV has grown to over 10% (<http://www.abedia.com/wiley/vectors.php>) with the approval of Kymriah® [10] and Yescarta® [11] therapy products expanding their applications. The viral dose can vary between 10^{11} – 10^{12} transduction units (TU) per patient [12]–[14]. LV are typically produced by transient transfection in the Human embryonic kidney 293 (HEK 293) cell lines resulting in titers ranging from 10^6 – 10^8 TU/mL [15]. To reach the expanding manufacturing demands, production processes based on suspension cell cultures and improved packaging cell lines are being optimized as well as bioreactors operation in perfusion mode are becoming available. Furthermore, improved downstream processes ensure increased final titers and appropriate impurity clearance.

GMP-compatible downstream purification processes usually start with nucleic acid digestion and clarification steps to decrease DNA content and

size and to remove cells and cellular debris; typically, the final DSP operation is a sterile filtration. The intermediate purification and concentration steps include anion exchange chromatography (AEX) and tangential flow filtration (TFF) which are critical due to LV sensitivity to shear stress and high conductivities [16], [17]. Therefore, DSP improvements must focus on these operation units and particularly on AEX as this step presents the lowest recovery yield [18]. The use of Design of Experiments (DoE) approach allows for a comprehensive investigation of AEX step and has proven very useful in the optimization of purification processes of biopharmaceuticals by identifying and optimizing critical process parameters (CPP) and lastly build predictive models [19]–[23]. This multivariate statistical methodology also allows to determination of the interaction of CPP's on a selected response [24], [25].

The LV formulation buffer must be carefully selected to provide maximum stabilization, considering also the virus final application [26], [27]. Different stabilizing agents have been used being the most frequent non-reducing carbohydrates (e.g sucrose, trehalose, lactose), fetal bovine serum, or proteins such as recombinant human albumin and gelatin [28]–[30]. Among them, sucrose [18], [31], [32] or trehalose [29], [33] up to 15% w/v are repeatedly used in formulation buffers, as well as salts such as MgCl_2 [18], [31] or NaCl [32].

In this work, different challenges that are hampering more efficient LV purification processes are address. Initially, a set of clarification filters containing distinct materials and configurations are evaluated for LV recovery, throughput and turbidity reduction to prepare the virus for the chromatographic step. Afterwards, two membrane devices with a quaternary amine ligand are used to study the impact of the virus loaded, buffer pH and NaCl concentration during the elution step using DoE methodology. The results obtained from the 96-well plates assays are scaled-up and the effect

of sucrose on AEX is also investigated. Finally, concentration and formulation by TFF are assessed using flat sheet cassettes and hollow fibers with different molecular weight cut-offs. The best materials and operation conditions were used to perform an entire GMP compatible purification process.

2. Materials and methods

2.1. Cell line, production, harvest and benzonase treatment

HEK293T cells (ATCC® CRL-3216™) obtained from the American Type Culture Collection (ATCC, Manassas) were cultured in Dulbecco's modified Eagle's medium (DMEM) (ThermoFisher Scientific, USA) supplemented with 10% (v/v) Fetal Bovine Serum (ThermoFisher Scientific), 4 mM of GlutaMAX™ (ThermoFisher Scientific) and 1 mM Sodium Pyruvate (ThermoFisher Scientific) and maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO₂ in air. Cell concentration and viability were assessed by the trypan blue exclusion method. LV were transiently produced by transfecting at the seeding of HEK293T cells using a four-plasmid system (pVSV-G, pGag-Pro-Pol, pRev and LV plasmid expressing green fluorescent protein (GFP), kindly provided by Dr. Didier Trono through Addgene plasmid repository) as described elsewhere [34]. Briefly, 1.5×10^5 cells/cm² were transfected in a tissue flask using linear 25 kDa polyethyleneimine, PEIpro®, (Polyplus transfection) at a mass ratio of 1:1 (DNA:PEI). Medium was exchanged 24 hours after transfection and LV were harvested 24 and 48 hours after the medium exchange (48- and 72-hours post transfection (hpt), respectively). The DNA digestion was performed immediately after harvest using 50 U/mL of Benzonase® (Merck Millipore) and 2 mM magnesium chloride. The digestion proceeded for 30 minutes at room temperature.

2.2. Clarification

Several single layer filters with 0.45 μm retention rate were assayed with different membrane materials: Polyethersulfone (PES) (Supor®, Pall Corporation), polyvinylidene fluoride (PVDF) (Fluorodyne® II, Pall Corporation), nylon (Ultipor(R)Q, Pall Corporation) and glass fiber (GF) (UB Preflow™, Pall Corporation). In addition, a 0.45 μm polypropylene (PP) depth filter (Sartopure PP3, Sartorius Stedim) and single layered 0.65 – 0.2 μm PES filter (Supor EAV, Pall Corporation) were also evaluated. The effect of a GF prefilter on the performance of PVDF, nylon and PES filters was assessed. Prior to use, all filters were washed with 20 L/m² of Milli-Q® water and equilibrated with 20 L/m² of 10 mM Histidine, 0.15 mM NaCl, pH 7.5 buffer. Filter throughput was determined at a constant feed pressure of 0.5 bar from the decrease in the filtrate flow over time, using a Sartorius Sartoflow® Slice 200 benchtop pump equipped with a Tandem 1081 head. Turbidity before and after clarification was measured using a portable turbidimeter (Hach). The 48 hpt clarified supernatant was stored overnight at 4°C and pooled with the clarified 72 hpt medium before proceeding to the chromatographic step.

2.3. Design of experiments

A full factorial Design of Experiments (DoE) with three mid points was performed to evaluate the impact of virus load (from 50 to 200 Membrane Volume, MV), buffer pH (from 7 to 8) and NaCl concentration for elution (from 0.7 to 1.3 M) on LV transducing and total physical particles recovery. AcroPrep™ Advance 96-Well Filter Plates with Mustang Q and Sartobind Q 96-well plate were operated with a centrifuge swing-out multi-well plate rotor according to the manufacturer's instructions. Plates were equilibrated with 50 MVs of 10 mM Histidine, 0.15 mM NaCl, 3% Sucrose, pH 7 or 8.

Benzonase treated and PP clarified harvest was loaded onto the membranes which, afterwards, were washed with 15 MV of equilibration buffer. A single step elution was performed with 15 MV of elution buffer (with 0.7, 1.0 or 1.3 M NaCl at pH 7 or 8) and eluate was immediately diluted with 30 MV of equilibration buffer. Experimental data were analyzed in the MODDE software (Umetrics®, Sartorius Stedim). Multi linear regression was used to fit experimental data to an interaction model and, for each response, statistically insignificant factors were removed, and the model refitted. Statistical significance was tested by ANOVA at 95% confidence interval.

2.4. Chromatography

Virus purification by chromatography was performed in an Äkta avant 150 equipment (Cytiva) controlled by Unicorn 6.3 version at room temperature (20 – 22 °C). An initial screening of chromatographic media was carried out with Capto DEAE resin (Cytiva), Mustang Q membrane (Pall Corporation), Sartobind Q membrane (Sartorius Stedim) and Natriflo® HD-Q membrane (Merck KGaA). All membranes were prepared according to the manufacturer's instructions. Approximately 40 mL of Capto DEAE resin was packed in a XK 26 column (Cytiva) according to the instructions and asymmetry factors between 1.0 and 1.1 and reduced plate heights between 2.7 and 2.9 were obtained for the packed columns. Devices were equilibrated with 10 mM Histidine, 0.15 M NaCl, 17% Sucrose, pH 7 buffer; after clarification, Benzonase treatment, loading and washing, bound LV were eluted with a 0.7 M NaCl step. LV transducing particles recovery and volumetric concentration factor were assessed to compare the performance of the different chromatography media. A deeper investigation was carried out to compare the performance of LV purification using Mustang Q and Sartobind Q membranes at different scales from 96-well plates (DoE) to 0.86- or 1-mL membrane volume (MV) devices, respectively. Small-scale devices

were run at a flow rate of 10 MV/min and pH 7; after loading, unbound material was washed with 60 MV of equilibration buffer and elution was performed with three NaCl concentrations (0.4, 1.0 and 1.5 M) steps. The effect of sucrose concentration on Mustang Q and Sartobind Q membranes was assessed by carrying out experiments without sucrose and with 3% (m/v) and 17% (m/v) sucrose in chromatography buffers. Dynamic binding capacity of Mustang Q membrane was determined with a 0.86 mL device using a flow rate of 10 MV/min and evaluating total particles concentration in the membrane's flow through up to 1600 MV loading. For Mustang Q 5 mL capsule, elution was carried out with two NaCl concentrations (0.4 and 1.5 M). In all chromatography experiments, immediately after elution from chromatography membranes, LV fractions were diluted 2 to 3 times with equilibration buffer to minimize viruses' exposure to high salt concentrations.

2.5. Tangential flow filtration

After chromatography, eluted and diluted LV were further concentrated and diafiltered by TFF. Several cassettes were used: Cadence™ Single-use Omega 100 kDa, 93 cm² (Pall Corporation); Omega Centramate™ T-Series 300 kDa, 200 cm² (Pall Corporation); Pellicon® XL50 Biomax® 300 kDa Membrane, C screen, 50 cm² (Merck Millipore). Hollow fibers with a molecular weight cut-off (MWCO) of 750 kDa and areas of 16, 26 and 50 cm² (Cytiva) were also evaluated. All cassettes were made of polyethersulfone while hollow fibers were made of polysulfone. Before use, normalized water permeability of the membranes was determined according to the manufacturers' instructions and then devices were equilibrated with 0.15 M NaCl buffer (10 mM Histidine, 0.15 M NaCl, 3% Sucrose, pH 7 buffer). According to the manufacturers' instructions, TFF devices were operated at constant feed flux between 300 and 540 L/m²/h (LMH) for cassettes and between 900 and 2250 LMH for hollow fibers. A valve in the retentate line

was partially closed to achieve a feed pressure of approximately 1 bar and a transmembrane pressure between 0.6 and 1 bar. After concentration, LV were diafiltered with 1 to 3 volumes of buffer. In the end, TFF devices were operated in full recirculation with the retentate valve fully opened and at a lower feed flow rate so that TMP was inferior to 0.2 bar to ensure a narrow polarization layer and thus increase LV recovery.

2.6. Sterile Filtration

Sterilizing grade filtration of concentrated and diafiltered LV was performed with Supor® EKV Mini Kleenpak™ syringe filters (Pall Corporation) washed with Milli Q water and equilibrated with buffer (minimum of 20 L/m² of each) before use. Filtration was performed manually.

2.7. LV total physical particles quantification

Total particles (TP) concentration was calculated by determining the amount of p24 protein using the ELISA kit Innostest® HIV Antigen mAb (Fujirebio, Diagnostics, Malvern, PA, USA) according to the manufacturer instructions. A ratio of 1.25×10^7 TP/ng of p24 was used to calculate the total particles titer.

2.8. LV transducing particles quantification

The functional LV titers were evaluated by transducing HEK293T cells with the produced LV supernatants. The GFP expression was analyzed by flow cytometry as described elsewhere [34]. Briefly, HEK293T cells in 24 well plates were transduced with LV at different dilutions and, 48 hours post transduction, infected cells were sorted by fluorescence microscopy (Leica) to select the wells with approximately 5 -20 % of positive GFP containing cells. After trypsinization of the cells in those wells, the percentage of GFP

positive cells were quantified by flow cytometry (FACSCelesta™, BD Biosciences). The concentration of LV Transducing particles (TU/mL) was calculated using the following equation:

$$\text{Titer} \left(\frac{\text{TU}}{\text{mL}} \right) = \frac{\% \text{ of GFP positive cells} \div 100}{\text{volume of transduction}} \times \text{dilution factor} \times \text{number of cells transduced} \quad (2.1)$$

2.9. Total protein, host cell protein and DNA quantification

Total protein was assessed with Pierce™ BCA assay kit (ThermoFisher) and total DNA was quantified with Quant-iT™ PicoGreen dsDNA assay kit (ThermoFisher) used according to the manufacturer's instructions. Host cell protein was determined with HEK 293 HCP ELISA Kit (Cygnus Technologies).

3. Results

3.1. Nuclease treatment and clarification

In the initial clarification step, cells, cellular debris, and other contaminants are (partially) removed to facilitate the following purification steps. Firstly, the Benzonase treatment of harvest medium lead to $76 \pm 9 \%$ reduction on DNA content and no LV transducing particles were lost neither during Benzonase treatment nor during the overnight 4°C holding of the 48 hpt clarified harvest. Afterwards, the harvests were clarified using: 0.45 µm pore size single layered filters of several materials; a depth filter with also 0.45 µm pore size; and a dual layer PES filter with a nominal rating of 0.2 µm.

The effect of GF 0.45 μm nominal rating as a prefilter for PES, PVDF and Nylon 0.45 μm filters was evaluated (**Table 2.1**). LV transducing particles recovery from all filters tested was above 70 % and, on average, a reduction in solution turbidity of 46 % was observed. In the case of GF filters, it was found that throughput was dependent on feed stream turbidity with values between 3440 and 1620 L/m^2 for solution with turbidity ranging from 2.5 to 7.1 NTU, respectively.

Table 2.1. Filters throughput for LV harvest and Benzonase treated clarification (n=3).

Filter material	Rating (μm)	Throughput (L/m^2)	Turbidity of feed solution (NTU ¹)	Turbidity reduction (%)
PES	0.45	235 $\pm$ 39	4.1 – 7.1	50 – 62
PVDF	0.45	380	6.7	55
Nylon	0.45	400	6.7	50
PP	0.45	2150 $\pm$ 313	4.1 – 5.3	27 – 59
GF	0.45 (nominal)	1845 – 3180 ²	2.5 – 6.7	35 – 37
GF + PES	0.45 (nominal)+ 0.45	1820	4.3	46
GF + Nylon	0.45 (nominal)+ 0.45	1535 $\pm$ 21	4.3	18
GF + PVDF	0.45 (nominal)+ 0.45	2220 $\pm$ 150	4.3	25 – 76
PES	0.65 – 0.2 (nominal)	549 $\pm$ 34	2.6 – 7.9	37 – 68

1 – NTU: Nephelometric turbidity units; 2 – The performance of GF filter is strongly dependent on the turbidity of the feed stream (see text).

3.2. Chromatographic screening

Initial evaluation of the chromatography step was performed with Capto DEAE resin and Mustang Q, Sartobind Q, and Natriflo HD-Q membranes.

Transducing particles recovery yield and volumetric concentration factor (VCF) were the parameters evaluated upon elution with 0.7 M NaCl (**Figure 2.1**). Similar recovery yields were observed for all the evaluated chromatographic media; however, the VCF was different across the tested media. The highest VCF values obtained were 100 and 30 for Mustang Q and Sartobind Q membranes, respectively.

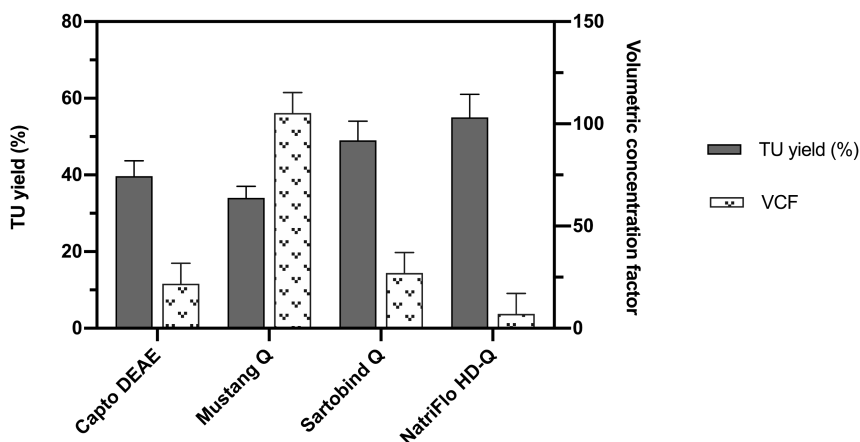


Figure 2.1. Recovery of transducing units (TU) and Volumetric Concentration Factor (VCF) for LV elution from different anion exchange chromatographic media.

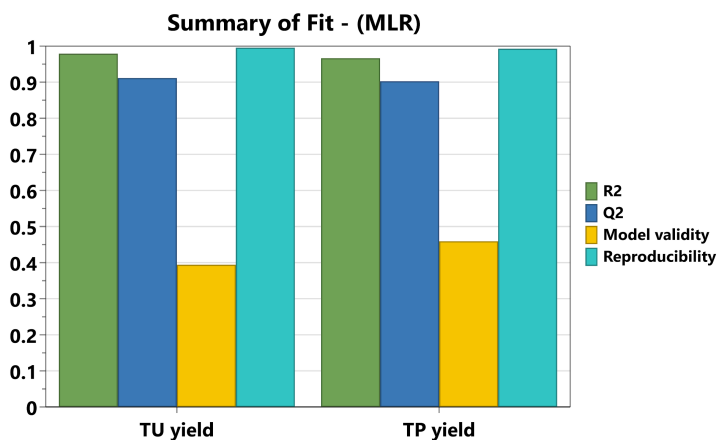


Figure 2.2. Summary of Fit plot using Multiple Linear Regression Analysis (MLR) of both responses Transducing Units (TU) and Total Particles (TP) recovery yields.

The chromatographic optimization proceeded by performing a DoE in 96-well plates of Mustang Q and Sartobind Q, assessing three factors (amount of virus load, elution pH and NaCl concentration for elution). The variation observed on the results of the three middle points replicates was much smaller than the variation observed for the entire experiment set (data not shown), hence raw data error does not impair data analysis and regression model fit. From the summary of fit plot (**Figure 2.2**), it is observed that R^2 and Q^2 values for both responses are close to 1 and that the $R^2 - Q^2$ difference is smaller than 0.2 (0.07 and 0.06 for TU and TP recovery, respectively). For both responses, model validity values higher than 0.25 and reproducibility values higher than 0.5 were obtained.

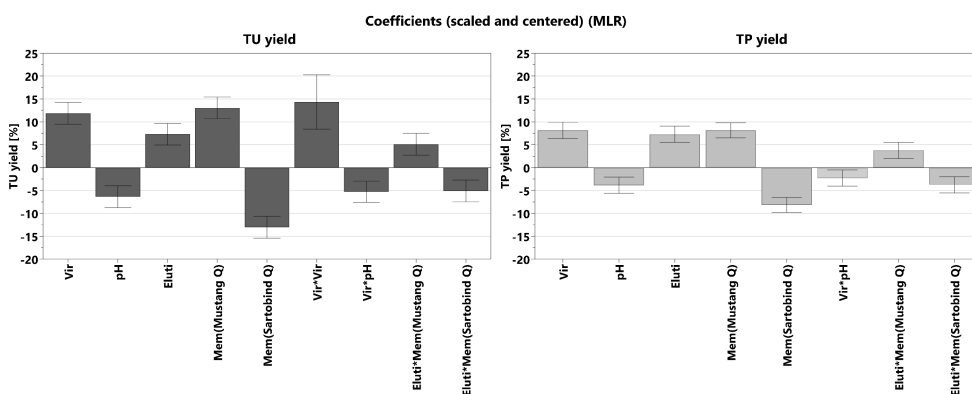


Figure 2.3. Scaled and centered coefficients for Transducing Units (TU) and Total Particles (TP) recovery yields. The coefficient plots represent the influence of the significant factors depending on the response evaluated.

From coefficient (**Figure 2.3**) and contour (**Figure 2.4**) plots, it is observed that higher recoveries are obtained using Mustang Q membrane with high virus load and elution with higher NaCl concentrations. Although less relevant, low pH values also lead to higher TU and TP recoveries. Regarding Sartobind Q membrane, DoE results reveal that not only

recoveries are lower than with Mustang Q but also that the effect of NaCl concentration was much smaller being the recoveries almost identical for the three concentrations assayed.

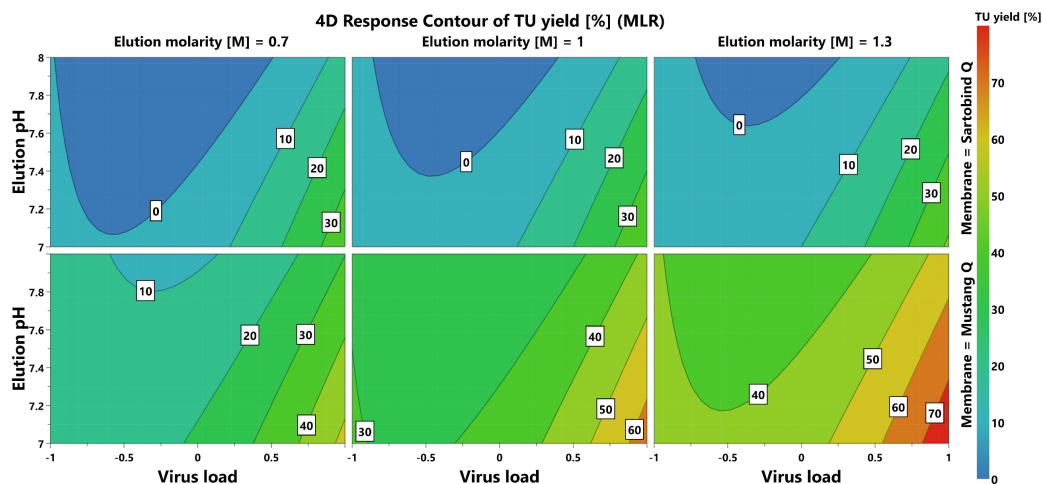


Figure 2.4. Contour plots of interaction of the factors involved in the recovery of transducing particles. These contour plots reflect the influence of the elution molarity, the elution pH and virus load on Sartobind Q and Mustang Q membranes.

3.3. Dynamic binding capacity

Mustang Q dynamic binding capacity (DBC) was determined from the ratio of total particles concentration in the flow through relative to the concentration in the feed solution at different time points during loading of 1600 MV of clarified harvest (**Figure 2.5**). The curve shows the typical sigmoidal shape but, from the beginning of flow through, the concentration of total particles is around 8 % of the feed concentration. This may be explained by the presence of p24 protein not associated with LV in the clarified harvest loaded onto the membrane. From the results obtained, a DBC_{10%} of approximately 600 MV was obtained which corresponds to 1×10^{12}

TP/mL of Mustang Q. Kutner et al. reported a DBC value for LV ranging from 3×10^9 and 9×10^{10} TU/mL for Mustang Q [35]. Although an objective comparison of DBC values is precluded because no total to transducing particles ratio is referred, if one assumes a TP/TU ratio of 500, the reported DBC values range from 1.5×10^{12} and 4.5×10^{13} TP/mL of Mustang Q, which is in the same order of magnitude of reported values.

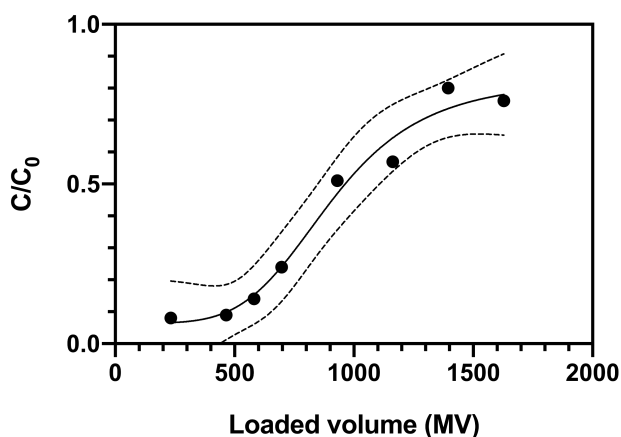


Figure 2.5. Breakthrough curve for the Mustang Q membrane. The load flow velocity was 10MV/min, where C is the virus concentration of flow-through samples and C₀ is the virus concentration of injected feed material. Physical particles concentration was determined by p24 LV protein detection.

3.4. Chromatography – Small-scale devices

To validate DoE results, experiments were performed with small-scale devices: 0.18- and 0.86-mL Mustang Q membranes and 1 mL Sartobind Q membrane. Considering DoE and DBC results, membranes were loaded with 600 MV of clarified harvest and elution was performed with 0.4, 1.0 and 1.5 M NaCl steps. For 0.18 mL Mustang Q and 1 mL Sartobind Q membranes, the mass balance of LV transducing particles was not complete, i.e., the total

amount of LV eluted from the membranes was 63 % and 82 % of the amount loaded onto the membrane, respectively. For 0.86 mL Mustang Q, 100 % of the loaded LV were found in the eluted fractions. Normalizing recovery data to the total eluted viruses allows for a comparison of the performance of the different devices (**Table 2.2**).

In Mustang Q membranes, approximately 20 to 30 % of LV elute with 0.4 M NaCl, 35 % in the 1.0 M NaCl fraction and between 25 to 30 % in the 1.5 M NaCl fraction. In Sartobind Q, approximately 40 % of LV are found in the 0.4 M NaCl fraction and 30 % in the 1.0 M NaCl fraction. In comparison to Mustang Q, a lower recovery is observed in the 1.5 M NaCl fraction (6 % vs. 25-30 % in Mustang Q membranes). Around 10 % of viruses are lost in Mustang Q flowthrough (FT) and twice of that value was observed for Sartobind Q. A large amount of DNA elutes in FT (more than 60 %) and 30 – 40 % is also present in the 1.0 M NaCl fraction. Slightly higher removal values were observed for Mustang Q in comparison with Sartobind Q membrane.

Table 2.2. LV transducing particles recovery yield (%) from small scale membranes.

Elution fraction	Mustang Q 0.18 mL	Mustang Q 0.86 mL	Sartobind Q 1 mL	Mustang Q 5 mL
FT	12 ± 5	10 ± 4	24 ± 2	7 ± 0.3
Fr 0.4 M NaCl	21 ± 4	31 ± 6	36 ± 6	37 ± 9
Fr 1.0 M NaCl	35 ± 11	35 ± 6	33 ± 4	-
Fr 1.5 M NaCl	32	24 ± 5	6 ± 2	56 ± 11

Regarding protein clearance, almost all total protein is found in the FT, approximately 2% of the loaded amount elutes in the 0.4 M NaCl step and only minor amounts are present in 1.0 and 1.5 M NaCl fractions.

Considering these results, Mustang Q was selected for scale-up experiments (see below).

Since sucrose is known to be a stabilizer of biological products, the effect of this sugar on LV chromatography step was evaluated. After loading, membrane washing, and elution was performed without sucrose or with 3% or 17% sucrose. For all devices, LV recovery profile was similar across the different sucrose concentrations; for example, in Mustang Q 0.86 mL membrane, approximately 9 % of viruses can be found in FT, 34 % elute with 0.4 M and 1.0 M NaCl fractions and 24 % elute in the 1.5 M NaCl fraction (**Figure 2.6**). Regarding contaminants, no differences were observed on protein removal (almost all protein is present in the membranes' FT) but DNA removal in the 1.0 M NaCl fraction increased with sucrose concentration (57, 61 and 71% for 0, 3 and 17% respectively).

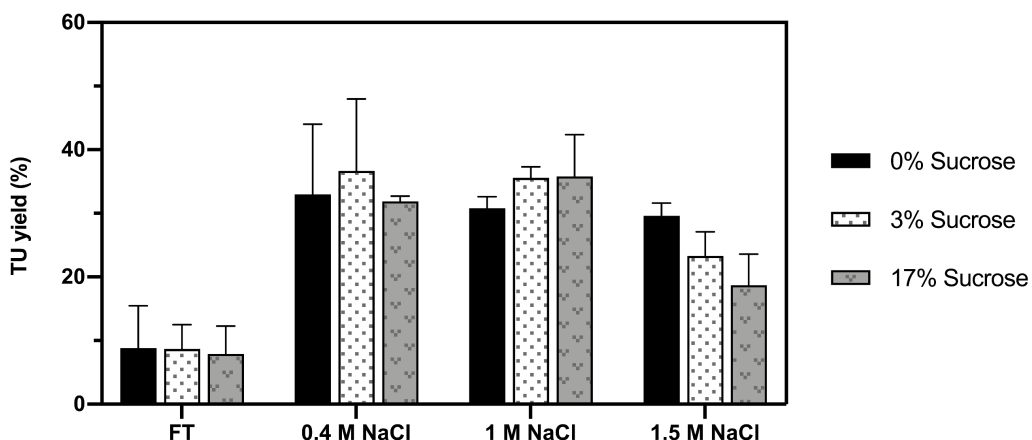


Figure 2.6. Evaluation of sucrose percentage during the elution step. The concentration of sucrose (3 or 17%) was evaluated using a 0.86mL Mustang Q membrane where the transducing units (TU) recovery in each elution step (0.4, 1 and 1.5 M NaCl) was assessed.

3.5. Chromatography – Lab-scale Mustang Q capsule

Two larger scale runs were performed with 5 mL Mustang Q capsules to confirm the results obtained from small-scale experiments. Considering the elution profile of LV and impurities, two NaCl steps with 0.4 and 1.5 M NaCl were performed. The results obtained are in accordance with those from small-scale experiments: transducing particles elution was 37 and 56 % in the 0.4 M NaCl and 1.5 M NaCl fractions, respectively and 7 % of LV were found in the membranes' FT (**Table 2.2**). Regarding impurities clearance, 5 mL Mustang Q capsules performance was better than small-scale devices. DNA removal higher than 70% removal was observed in the 1.5 M NaCl fraction. Almost all proteins eluted in the FT and 1 % was found in the 0.4 M NaCl fraction. To increase the purity of the final product, the 0.4 M NaCl fraction was not used for further processing.

3.6. Tangential flow filtration and sterile filtration

LV eluted with the 1.0 and 1.5 M NaCl chromatographic steps were diluted with buffer containing 0.15 M NaCl and loaded onto different TFF devices to concentrate and formulate. Cassettes and hollow fibers with different molecular weight cut-offs were evaluated for LV recovery and impurities removal. High TU yields were obtained being the average 75 ± 12 and 75 ± 19 % for cassettes and hollow fibers, respectively (**Figure 2.7**). Results obtained with different MWCO membranes and TMP values ranging from 0.55 to 1 bar showed no correlation with virus recovery yield. Permeate fluxes of 80 LMH were observed for both devices regardless of feed flux and MWCO.

Regarding impurities clearance, cassettes perform slightly better but without statistical significance. Overall protein removal was 74 ± 15 % and DNA removal was 56 ± 8 %. Upon formulation, the product sterility was achieved with a sterile filtration step using the Supor EKV filter. For all the experiments, the transducing particles recovery was higher than 85%.

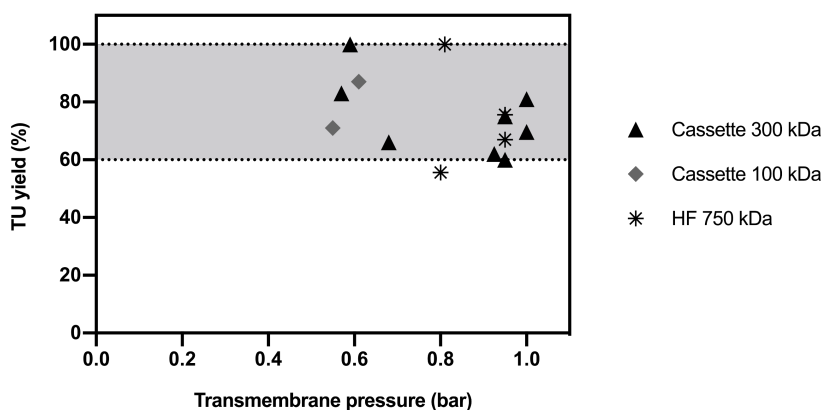


Figure 2.7. Transducing units' recovery from cassettes with 100kDa and 300kDa cut-off and 750 kDa hollow fibers (HF) as a function of transmembrane pressure (TMP).

3.7. Quality assessment of purified LV

The ratio between total and transducing particles is an indicator of LV quality preparations, sometimes presented as infectivity which is defined as the ratio between transducing particles (TU/mL) and p24 (in ng/mL) concentrations. LV purified with Mustang Q followed by TFF (100 or 300 kDa cassettes) and sterile filtration presented infectivity values of 2.1×10^4 TU/ng of p24 which corresponds to a TP/TU ratio of approximately 580.

The total protein and total DNA were quantified in each purification step. It was observed that almost all proteins and DNA were found in the

membranes' flow through. No differences were found between protein removal in Mustang Q or Sartobind Q with removal values always higher than 94 %. From the remaining protein upon the chromatographic step, it was also possible to remove 55 to 87 % in the TFF step and no differences were observed between cassettes or hollow fiber devices, or even varying membranes' MWCO. Regarding total DNA, the amount found in Mustang Q flow through varied between 70 to 80 % of the amount loaded onto the membrane and slightly lower values were found for Sartobind Q membrane. The residual amount of DNA that binds to the membranes was eluted in the 1.0 M NaCl fraction. During TFF, DNA removal ranged from 47 to 66 %.

The removal of host cell proteins (HCP) was also evaluated in the crucial steps of the LV purification process. For the experiments performed in the small-scale devices using Mustang Q and Sartobind Q membranes, the HCP removal was greater than 97%. In addition, on the TFF step the removal of residual HCP was around 60 %. An amount of $5.9 \pm 0.2 \mu\text{g}$ of HCP per 10^9 TU was obtained in the purified LV stock.

4. Discussion

Currently, LV have become a fundamental tool for gene and cell therapy [36]. However, the large-scale manufacturing of these viruses represents a major bottleneck in the success of clinical trials. Consequently, there is a need for fast, simple, robust and GMP-compatible purification methods that can efficiently process larger volumes of viral vector in order to achieve preparations with high titers and high quality [37]–[39].

Initially, a set of different material filter with 0.45 to 0.2 μm pore range were investigated by evaluating turbidity reduction, membrane throughput and TU recovery (**Table 1.1**). From the analysis of the clarification filters' results, it is observed that LV transducing particles recovery for all the filters

was above 80%, an outcome supported by other published results where filters of several materials, single or dual layered, and also depth filters were used and proved to allow high LV recoveries [40]–[42]. Due to the small area and hold-up volume of the filters evaluated, no buffer flushing was performed. However, larger filter devices are usually in the format of capsules and flushing to increase virus recovery should be considered. Regarding GF filters, despite reaching the highest throughput value, a dependence on feed stream turbidity was observed, showing better performance for lower turbidity. The use of glass fiber filter upstream of Nylon, PVDF and PES filters leads to an increase in the throughput of 4, 6, 8-fold, respectively. A capacity of approximately 2150 L/m² was achieved for the PP filter with LV transducing particles recovery greater than 85% and with no turbidity dependence. Therefore, the PP filter was selected for the clarification step, this has already been used in purification processes for LV and other virus particles [21], [43]. It is interesting to notice that, even though 0.2 µm filters are not commonly used for the clarification of LV harvests, very high TU recoveries were obtained; however, the impact of using this pore size filters on subsequent purification steps is not clear and thus not selected for further studies. The throughput of filters for clarification of LV harvest media is rarely reported in the literature. This is one of the key characteristics to consider for the selection of clarification filters, but other factors like economics and the availability in ready-to-use formats with GMP compliant certificates must also be taken into account when selecting filters for large scale manufacturing processes.

To optimize the AEX step, a DoE was used to assess the effect of virus load, elution buffer pH and NaCl concentration for elution on LV recovery from Mustang Q and Sartobind Q membranes. The multi linear regression model obtained from experimental data analysis was significant and, Mustang Q, high virus load and high NaCl concentration were the most

significant factors to maximize virus recovery. Surprisingly, for Sartobind Q, NaCl concentration was shown to have a small effect upon LV recovery (**Figures 2.3 and 2.4**).

To validate these conclusions, experiments with small-scale membranes were carried out using three NaCl steps (0.4, 1.0 and 1.5 M) for elution. The total LV recovery from both membranes was higher than that predicted from DoE experiments. In the case of Mustang Q, a maximal recovery of 70 % was estimated from DoE experiments but a recovery of 90 % was observed for 0.86 mL membrane. For 1 mL Sartobind Q device, a recovery of 63 % of the loaded LV transducing particles was observed while from DoE assays, a value of 40 % was determined. Regarding the LV elution profile, and as indicated from DoE results, higher NaCl concentrations lead to higher recovery values for Mustang Q whereas for Sartobind Q only a small amount was found in the 1.5 M NaCl fraction (**Table 2.2**). This difference may arise from the higher ligand density of Mustang Q in comparison to Sartobind Q (bovine serum albumin capacity per mL of membrane of 60 and 29 mg, respectively) in combination with a narrower pore size (0.8 and > 3 μm , respectively). The chromatographic step was scaled-up using Mustang Q 5 mL capsules with a two NaCl steps elution. Results are in very good agreement with those obtained with small-scale devices showing that Mustang Q performance is reproducible and scalable. TU recovery yield of 60% for Mustang Q are reported in the literature [35], [44]–[46], but it is worthy pointing out that the optimization described in this work led to much higher recovery values (~90%).

Better performance of Mustang Q in comparison with Sartobind Q membrane is already reported in other publications [18], [47]. Other chromatographic media, like DEAE resin or monolith, LentiSELECT membrane or HiTrap Q resin are also showed to have an inferior performance than Mustang Q [16], [48]–[50]. The use of weak DEAE ligands

is described to be a preferable choice as chromatographic medium since lower NaCl concentrations are required for elution [31], [48].

To overcome this issue, immediate dilution of eluate into a low NaCl concentration buffer was performed and this is proved to be effective as no significant losses were found. Several publications report that different additives like non-reducing carbohydrates (sucrose, trehalose, lactose), polyols (glycerol, mannitol, polyethyleneglycol) or proteins (bovine or human serum albumin) among others may be beneficial for LV stability [28], [29], [32], [33]. In this work, the effect of sucrose on the chromatography step was assessed but no significant differences on LV recovery were observed nor on the elution profile of LV from both membranes (**Figure 2.6**). However, sucrose was shown to improve recovery yields of gibbon ape leukemia virus pseudotyped LV (GaLV-TR-LV) or vesicular stomatitis virus (VSV) enveloped viruses from anion exchange chromatography using Poros D resin or Mustang Q membrane, respectively [31]. In the case of rhabdovirus VSV, an increase from 19 to 32 % was obtained by the addition of 2 % sucrose to the chromatography buffers used in Mustang Q membrane [51]. Additives were also proved beneficial to some LV preparations or for freezing or lyophilization procedures [29], [52], [53].

Following chromatography, TFF was used to further concentrate and formulate LV. Both cassettes and hollow fibers with different cut-offs were evaluated and both devices showed a 75% recovery yield. No correlation between TMP, or membrane MWCO, and recovery yield were found (**Figure 2.7**). Additionally, similar permeate fluxes were obtained for the different devices tested and thus, identical processing times were found. The use of cassettes or hollow fibers to concentrate and formulate LV is described in the literature and, although details on operational parameters are scarce, very high recovery yields are usually found [18], [48], [54]. Higher impurities clearance for larger cut-off devices are occasionally referred however, no

sound results put in evidence the better performance of those devices. Curiously, upon comparison of 100 and 300 kDa cassettes, Valkama et al. selected the 100 kDa device to further optimize LV purification processes [18].

To produce clinical grade LV, a final sterilizing filtration is required. This step is critical and LV losses up to half of the purified viruses are reported which has a great impact on the overall yield and cost of the LV manufacture process [18], [40]. However, the use of Supor EKV filter led to a very satisfactory result with an almost complete recovery of LV. Purified samples presented infectivity values of 2.1×10^4 TU/ ng of p24 (corresponding to a TP/TU ratio of 580) which is similar to values reported in the literature that range from 1.4×10^3 and 1.25×10^5 TU/ng of p24 [48], [55], [56]. Optimization of the number of diafiltrations in the TFF step might be required to guarantee in the future impurity content within guidelines for clinical application. It is worth pointing out that the presented process can be easily adapted to purify LV produced by new emerging upstream strategies, like suspension cell cultures in serum free media. In this case, it is foreseen that the most affected operation unit will be the clarification and thus, must be carefully optimized.

LV processing time is a key aspect that must be considered when establishing an improved purification process. In this sense, the purification process time should not exceed the virus half-life, which for LV is approximately between 5 and 8 h at 37 °C [57], [58]. The proposed process can be conducted in a 5-hour timeframe assuring minimal loss of intrinsic LV biological activity with a recovery of 45 % range matching the highest yields described for LV purification [15], [38], [56].

5. Conclusion

A scalable and GMP compatible purification process for LV is proposed and several improvements were described for the different purification steps. Firstly, distinct clarification filters were evaluated. PP filter was selected since it presented the highest throughput with no turbidity dependence, which offers some flexibility to cope with the upstream variability. As was already mentioned, the AEX step is one of the most critical steps during the LV purification and for that reason, an exhaustive optimization was made using DoE methodology. For both membrane devices, Mustang Q and Sartobind Q, it was possible to achieve recovery yields higher than those described in the literature. Mustang Q presented a better performance and was chosen to proceed for process scale-up.

Cassettes and HFs, with different cut-offs, were evaluated for virus concentration and formulation. No correlation was observed in the recovery yield with the MWCO and TMP variations, and different devices were proven suitable for LV concentration and formulation. Afterwards, the Supor EKV filter allowed the sterilization of the final product without compromising the recovery yield. Finally, a scale-up of the enhanced purification process was performed, as a proof-of-concept, with a total recovery yield of 45%. The established purification process is composed by four operation units allowing a total processing time of approximately 5 hours which is suitable for labile LV vectors.

LV purification is not a one-fits-all process will be also dependent on the target envelope and production system, however this work built up knowledge to successfully and cost-efficiently manufacture LV and contributes to fulfil the increasing market demands.

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3

Impact of adherent to suspension transition on LVs downstream process

Chapter adapted from:

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Abstract

Lentiviral vectors (LVs) have emerged as a powerful tool for cell and gene therapy applications. Over the last few decades, the industry's interest and demand for more efficient manufacturing processes have grown, supported by the recent approval of LV-based therapies.

Currently, most of these processes rely on multi-tray technologies or fixed-bed bioreactors for LVs production. Biopharma industry is quickly migrating to suspension cultures that allow the reduction of batch-to-batch variation and the establishment of serum-free production platforms. This transition can somehow improve the process scalability but simultaneously introduces different challenges to the downstream processing of LVs. To evaluate the impact on the downstream process, LVs produced in suspension cell lines were used. In this work, it was established an alternative clarification step using tangential flow microfiltration. A competitive filtrate throughput of 300 L/m² was achieved with an overall TU viral recovery of 90% and final turbidity on the clarified stock below 10 NTU. The impact of this new system was also evaluated in the intermediate purification step. LVs produced in adherent and suspension were purified by anion exchange chromatography. The elution profile obtained for the suspension system was different from previous reports, but after the elution step fine-tuning, a TU yield of 56 % was achieved.

Overall, it was possible to maintain the identity, purity, and potency of the LVs and create a comprehensive comparison of both manufacturing processes that will help to prompt the development of simpler, easily scalable, and more efficient processes.

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1. Introduction

Lentiviral vectors (LVs) are growing as valuable delivering tools in the Cell and Gene Therapy field [1], [2]. Their ability to stably integrate the genome of dividing and non-dividing cells [3] and provide a lower frequency of insertional mutagenesis [4] led LVs to be one of the most used vectors in clinical trials [5]. This enveloped virus has been typically used for the treatment of genetic disorders and infectious diseases [6], [7]. Nevertheless, in recent years they achieved remarkable results in the generation of chimeric antigen receptor (CAR)-T cells for cancer immunotherapies [8].

Currently, there are already 7 available LV-based therapies that demonstrate the potential of this technology and increase the pressure for more scalable and efficient manufacturing processes [1], [9], [10]. The therapeutic modality (e.g., *ex vivo* application or *in vivo* administration) and medical disorder will dictate the required viral dose, although most authors have been reporting doses between 10^{10} and 10^{12} per patient [11]–[13]. LVs are traditionally produced in mammalian cell lines, usually HEK 293 or HEK 293T, mainly by transient transfection in the presence of animal serum [14], [15]. Anchored-dependent cell lines have been able to provide enough viral material to sustain clinical trial development, however, they exhibit evident limitations in terms of process scalability when there is a need for high dosages or high demand [16], [17].

As the interest in LVs for multiple applications continues to grow, an imperative improvement in viral manufacturing was necessary. For that reason, the biopharmaceutical industry has been transitioning to suspension cell cultures that can offer in-line monitoring (e.g., pH, pO₂, pCO₂); are less labor-intensive; eases scale-up; and allows new modes of operation (e.g., perfusion) [17]–[19]. In recent years, there has been a notable development in the production of LVs using suspension cell lines. Currently, viral titers

reported in serum-free media are already comparable to traditional production methods, ranging from 10^7 transducing units (TU)/mL to 10^8 TU/mL [11], [13], [19]. Undoubtedly, implementing serum-free production platforms can reduce batch-to-batch variability and decrease the risk of contamination with adventitious agents, thereby improving safety and facilitating approval by regulatory agencies [20], [21]. However, new challenges may arise when removing the animal serum, since critical components such as lipids and growth factors must be replaced [20], [22]. LVs being extracellular particles are released by budding and retain in the host cell membrane enriched with viral proteins that constitute the viral envelope [2], [10], [23]. In fact, it has been described that a significant fraction, 30 to 40 %, of viral particles are constituted by lipids derived from the producer cell membrane [24], [25]. Moreover, membrane fluidization has also been related to infectivity and stability being affected by temperature, and the amount of cholesterol and lipids [25], [26]. Therefore, serum depletion can influence viral biological activity and stability [22], [27], [28]. Currently, efforts are being made to improve and create new chemically defined media to mitigate some of these constraints [28]–[31].

The adoption of suspension cell lines may also introduce modifications to the current downstream process. This can be particularly evident in the clarification step since is highly dependent on the upstream process. The expression system could vary in terms of cell density and viability at the harvest, product release mechanism (e.g., intracellular, or extracellular), and consequently the level of contaminants to be removed [32]–[34]. An efficient clarification step should be able to remove cell debris and large aggregates (product or process-related impurities), maximizing yield and preserving LVs integrity and biological activity [15], [35], [36]. At a laboratory scale, low-speed centrifugation is traditionally used [35]. Currently, filtration technologies that offer efficiency, scalability, and disposable options

available are commonly applied in the LVs clarification step [17], [29]. To avoid pore-clogging, loss of viral activity, or envelope damage, a filter cascade can be designed with membrane pore size progressively decreasing until 0.45 μm [37], [38]. Different filtration materials and/or configurations can be applied to LVs clarification: tangential flow filtration (TFF) and normal flow filtration (NFF) which includes depth, surface, or membrane filters [35], [39]–[43]. Tangential flow filtration microfiltration (TFF-MF) application on LVs clarification has not been thoroughly investigated, however, the crossflow improves mass transport minimizing membrane fouling and achieving significantly higher filtrate flux [42]. Nevertheless, process parameters must be carefully selected to minimize the shear rate and loss of biological activity [32], [44].

Following clarification, LVs intermediate purification is mostly accomplished by anion exchange chromatography (AEX). This step may also be affected by the transition to suspension cell cultures since the binding mechanism of AEX relies on electrostatic interactions [14], [29], [35]. Thus, any changes in the viral envelope caused by animal serum depletion can impact the operation efficiency. Additionally, the virus impurities level (nucleic acid and proteins) can impact virus interactions with the chromatographic media. Consequently, changing from adherent to suspension productions can affect AEX devices' capacity and optimal elution conditions. In this work, different TFF-MF devices were evaluated for LVs clarification. After, the selected filter critical process parameters such as transmembrane pressure, filtrate flux, maximum capacity, turbidity reduction, and viral yield, were determined. Lastly, the impact of transitioning to suspension cell cultures in the chromatography step was assessed followed by viral analytical characterization.

2. Materials and Methods

2.1. Lentiviral vector production

2.1.1. Adherent cell maintenance, LVs production, harvest and nuclease treatment

HEK 293T cells (ATCC® CRL-3216™) obtained from the American Type Culture Collection (ATCC, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (ThermoFisher Scientific™, USA) supplemented with 10% (v/v) Fetal Bovine Serum (ThermoFisher Scientific™, USA), 4 mM of GlutaMAX (Thermo Fisher Scientific™, USA) and 1 mM Sodium Pyruvate (Thermo Fisher Scientific™, USA), and maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO₂ in the air. Cell concentration and viability were assessed by the trypan blue exclusion method. LVs were transiently produced by transfecting at seeding using a four-plasmid system (pVSV-G, pGag-Pro-Pol, pRev, and LV plasmid expressing green fluorescent protein (GFP), kindly provided by Dr. Didier Trono through Addgene plasmid repository) as described elsewhere [45]. Briefly, 1.5×10^5 cells/cm² were transfected in a tissue flask using linear 25 kDa polyethyleneimine, PEIpro®, (Polyplus transfection®, France) at a mass ratio of 1:1 (DNA:PEI). The medium was exchanged 24 hours after transfection and LVs were harvested 24 and 48 hours after the medium exchange (48- and 72-hours *post-transfection*, hpt, respectively). For DNA digestion after harvest, 50 U/mL of Benzonase® endonuclease (Merck, Germany) and 2 mM magnesium chloride were used. The digestion proceeded for 30 minutes at room temperature.

2.1.2. Suspension cell line maintenance and LVs production in shake flask

HEK 293T cells adapted to grow in suspension [46] were cultivated in BalanCD® HEK293 medium (FUJIFILM IrvineScientific, USA) supplemented with 4 mM GlutaMAX™ (Thermo Fisher Scientific™) and maintained at 37 °C in a shaking incubator at 80 rpm (50 mm throw) with a humidified atmosphere of 8% CO₂ in the air. Cell concentration and viability were assessed by the trypan blue exclusion method. LVs were produced by transient transfection using pALD-Lenti System (Aldevron®) including the pALD-VSV-G-K, pALD-GagPol-K, pALD-Rev-K, and pALD-Lenti-EGFP-K plasmids. Briefly, the cells were transfected at 2.5×10^6 cells/mL using linear 25 kDa polyethyleneimine, PEIpro® Transfection Reagent (Polyplus-transfection®, France) at a mass ratio of 1:3 (DNA:PEI) and 1 µg of total DNA per 1×10^6 cells. At 48 hpt, LVs were harvested at a cell density of $4\text{--}6 \times 10^6$ cells/mL.

2.1.3. LVs production in stirred tank bioreactor

LVs production was performed in batch mode by computer controlled 2 L stirred-tank bioreactors (BIOSTAT® DCU-3, Sartorius, Germany). Cells were inoculated at 0.3×10^6 cells/mL in BalanCD® HEK293 medium supplemented with 4 mM GlutaMAX™ at a working volume of 1.5 L. The set points for the bioreactor were 37°C for temperature, 7.2 for pH, and pO₂ at 40% of air saturation maintained by varying the agitation between 70 - 250 rpm and the percentage of O₂ in the gas mixture between 0 and 100%. HEK 293T cells were transfected at a concentration between $2 - 2.5 \times 10^6$ cells/mL and LVs were harvested 48 hpt at a cell density of $4\text{--}6 \times 10^6$ cells/mL and a viability between 90 and 100%. After harvesting the bioreactor, nucleic acids digestion was performed with Benzonase® endonuclease (Merck, Germany)

at a final concentration of 100 U/mL in the presence of 2 mM magnesium chloride. The digestion proceeded for 30 min at room temperature.

2.2. Baseline purification process of adherent produced LVs

After nuclease treatment, LVs produced under adherent conditions were purified as described elsewhere [47]. Briefly, a 0.45 μm Polypropylene Sartopure PP3 filter (Sartorius, Germany) was used for viral clarification using the Sartorius Smartflow Slice 200 pump equipped with a Tandem 1081 head (Sartorius, Germany). The capture step was performed by anionic exchange chromatography (AEX) in an AKTA Avant 150 equipment (Cytiva, USA) controlled by Unicorn 7.6 version at room temperature. Mustang® Q XT5 membrane (Pall Corporation, USA) was equilibrated using 50 mM HEPES, 150 mM NaCl, and pH 7.5 at a flow rate of 10 MV/min. After viral loading (600 MV/mL_{membrane}), the unbound material was washed with the equilibration buffer and elution was achieved with two NaCl concentrations (400 and 1500 mM) steps. An immediate dilution with equilibration buffer was performed to minimize viruses' exposure to high ionic strength. Afterward, LVs were concentrated and diafiltrated using Omega Centramate T-Series 300 kDa, 200 cm² (Pall Corporation, USA). The TFF device was operated at a feed flux between 300 and 540 L/m²/h (LMH) maintaining a feed pressure of approximately 1 bar and a transmembrane pressure between 0.6 and 1 bar. After concentration, LVs were diafiltered with 1 to 3 volumes of equilibration buffer. Sterilizing filtration was carried out with EKV Mini Kleenpak syringe filters (Pall Corporation, USA) washed with Milli-Q® water and equilibrated with buffer. Filtration was performed manually. The collected samples (load, flowthrough, and elution steps) were stored at -80 °C for further analysis.

2.3. Suspension Lentiviral Vectors Purification

2.3.1. Clarification using tangential flow microfiltration

To evaluate different microfiltration devices, a Sartorius Smartflow Slice 200 pump equipped with a Tandem 1081 head and connected to SciPres luer pressure sensors (Scilog, USA) was used. All filtration modules were previously flushed and conditioned according to the manufacturers' recommendations with Milli-Q® water and working buffer (50 mM HEPES, 150 mM NaCl, pH 7.5), BioOptimal™ MF-SL, HF1, (Asahi Kasei, Japan) and two MidGee microfiltration hollow fiber cartridges (Cytiva, USA), HF2 and HF3, were investigated (**Table 3.1**). The devices were evaluated using a shear rate of 3000 s^{-1} by maintain a constant feed flux. Data pressure was monitored and recorded at the feed inlet, retentate outlet, and permeate outlet. The feed/retentate and the permeate volumes were monitored using a technical scale (Sartorius, USA). Filters' capacities were determined from the permeate flux decay over time. The cumulative weigh was recorded as a function of time. The acquired data was plotted as "Time/Filtrate Weight" vs. "Time" and the slope where a linear variation is observed was determined. Filters' throughput was given by the inverse of the calculated slope [48], [49]. The turbidity of the initial and clarified samples was measured using a Turbidimeter (2100 Qis Portable HACH®, Colorado, USA) and is given in nephelometric turbidity units (NTU).

Table 3.1: Specifications of tangential flow microfiltration devices used in clarification experiments.

Filter name	Membrane Chemistry	Nominal pore size (μm)	Surface area (cm^2)	Inner Diameter (nm)
HF1 - BioOptimal™ MF-S	Polysulfone	0.45	50	1.4
HF2 - MidGee™ MF	Polysulfone	0.45	16	1
HF3 - MidGee™ MF	Polysulfone	0.65	24	0.75

2.3.2. Anion exchange chromatography optimization

An AKTA Avant 150 chromatography system (Cytiva, USA) equipped with conductivity, UV, and pH detectors was used to perform small-scale studies and the intermediate scale-up at room temperature. System control and data analysis were done through UNICORN™ 7.6 software. Mustang® Q XT Acrodisc Unit 0.86 mL and Sartobind® Q Nano 1mL (Sartorius, USA) small-scale devices were evaluated for LVs purification. Membranes were equilibrated with 50 mM HEPES, 150 mM NaCl at pH 7.5 at 10 MV/mL, and after clarified harvest load and unbound material washing viral elution was performed with 1 M and 1.5 M of NaCl steps. The effect of sucrose concentration on Mustang® Q XT Acrodisc Unit performance was assessed by carrying out experiments with 0% and 17% (w/v) sucrose in chromatography buffers. All collected samples (load, flowthrough, and elution) were stored at $-80\text{ }^{\circ}\text{C}$ until analysis.

2.3.3. Culture medium supplementation effects on LVs production

HEK 293T cells growing in BalanCD® HEK293 medium supplemented with 4 mM GlutaMAX™ (defined as basal medium) were used to inoculate 250 mL shake flasks at 0.3×10^6 cells/mL. The effect of 5% (v/v) of Cell Boost 5™ (Cytiva, USA) or 0.4% (v/v) of Cholesterol Lipid Concentrate (250X) basal medium supplementation on LVs production was evaluated in shake flasks (section 2.1.2). To assess the impact of these supplements on transfection, experiments in which transfection was performed in basal medium and supplements added 4 hours later were compared to productions in which transfection was performed in supplemented basal medium. In total 3 culture conditions were investigated: i) control condition; ii) basal media supplemented with 5% (v/v) of Cell Boost™ 5; iii) basal media supplemented with 0.4% (v/v) of Cholesterol Lipid Concentrate (250X). The impact of cholesterol supplementation on LVs phenotype was assessed by loading the viral supernatant on Mustang® Q XT Acrodisc. All collected samples (load, flowthrough and elution) were stored at $-80\text{ }^{\circ}\text{C}$ for further analysis. For writing simplification Cell Boost 5 and Cholesterol Lipid Concentrate (250X) will be called as supplement 1 and supplement 2, respectively.

2.4. Analytics

2.4.1. LVs Transducing particle quantification

The adherent HEK 293T cell line was cultured in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific™, USA), supplemented with 10% fetal bovine serum (Thermo Fisher Scientific™, USA), 4 mM of GlutaMAX™ (Thermo Fisher Scientific™, USA) and 1 mM sodium pyruvate (Thermo Fisher Scientific™, USA). The functional LVs titers were evaluated by

transducing HEK293T cells and assessing GFP expression by flow cytometry as described elsewhere [50]. Briefly, HEK293T cells in 24-well plates were transduced with LVs samples at different dilutions in fresh DMEM with 10% (v/v) FBS and 8 µg/mL of polybrene (MilliporeSigma, Burlington, MA, USA) and incubated at 37 °C. After 2 h, 0.5 mL of fresh DMEM with 10% (v/v) FBS was added to each well, and the plates were incubated at 37 °C. Infected cells were sorted by fluorescence microscopy (Leica) to select the wells with approximately 5–20% of positive GFP-containing cells at 48 hours *post* transduction. After trypsinization of the cells in those wells, the percentage of GFP-positive cells was quantified by flow cytometry in the FACSCelesta™ equipment (BD Biosciences, USA). The concentration of LVs transducing units (TU/mL) was calculated using Equation (3.1):

$$\text{Titer} \left(\frac{\text{TU}}{\text{mL}} \right) = \frac{\% \text{ of GFP positive cells} \div 100}{\text{volume of transduction}} \times \text{dilution factor} \times \text{number of cells transduced} \quad (3.1)$$

2.4.2. Total particle quantification

Total particles (TP) concentration was estimated using the ELISA kit Innostest HIV Antigen mAb (Fujirebio, Malvern, PA, USA). To determine the total amount of p24 protein, a standard curve was established using the positive control with a 150–25 pg/mL concentration range according to the manufacturer's instructions. A ratio of 1.25×10^7 TP/ng of p24 was used to calculate the total particles titer.

2.4.3. Total protein, host cell protein and DNA quantification

Total protein was determined using the Pierce™ BCA assay kit (Thermo Fisher™, USA) and total DNA was quantified with Quant-iT PicoGreen

dsDNA assay kit (ThermoFisher™, USA) used according to the manufacturer's instructions. Host cell protein was determined with a HEK 293 HCP ELISA Kit (Cygnus Technologies, USA). The detection was performed using the Infinite PRO NanoQuant (Tecan, Switzerland) microplate multimode reader. The samples were applied at multiple dilutions.

2.4.4. Nanoparticle tracking analysis

The concentration and size distribution of viral particles were measured using the NanoSight NS300 (Malvern Panalytical, UK). Process samples were diluted in 0.22 µm filtered D-PBS (Gibco®, UK) to be in the instrument linear range of 10^8 - 10^9 TP/mL. The video frames were processed with Nanoparticle Tracking Analysis (NTA) 3.3 Analytical software. The camera level and the screen gain were set to 15 and 2, respectively. For each sample, three measurements were made by 30-second videos each, and the average of the three measurements was considered. For analysis of the LVs profile, it was considered a particle range between 50 and 200 nm.

2.4.5. Transmission electron microscopy

The morphological analysis of LVs was performed using transmission electron microscopy (TEM). Sample preparation consisted of a drop (5 µL) of each sample that was adsorbed onto formvar-coated 150-mesh copper grids from Veco (Science Services, Germany) for 2 minutes. Afterward, the grid was washed 5 times with sterile dH₂O, then soaked in 2% (w/v) uranyl acetate for 2 min and dried in air at room temperature. To analyze the grid, a Hitachi H-7650 120 Kv electron microscope (Hitachi High-Technologies Corporation, Japan) was used.

2.4.6. Data availability statement

The sensitive nature of some of the reagents used in this study (e.g., cell lines, plasmids, and antibodies) means that they are only readily available internally to the author's institution staff for R&D purposes. For external researchers, the approval of reagents' requests may be obtained via email addressed to the corresponding author.

3. Results and discussion

3.1. Evaluation of tangential flow microfiltration for LVs clarification

The clarification step, being heavily dependent on the initial harvest properties, must be adapted to accommodate the increased turbidity on the initial LVs produced in suspension. Filtration has proven to be an efficient and scalable technique to be used in LVs clarification step. In particular, tangential flow microfiltration (TFF-MF) configuration has not been extensively explored although it may serve as an alternative strategy to the commonly used filter cascade. The use of crossflow filtration reduces the cake layer formation and the shear rate control which contributes to prevent loss of biological activity [13], [32], [42]. Additionally, TFF can have multiple applications from upstream to downstream being an attractive operation mode for process integration. Therefore, a set of filter candidates were selected to be evaluated as potential clarification devices.

3.1.1. Tangential flow microfiltration filters screening

To be considered an efficient clarification filter, a few requisites should be met such as efficient turbidity reduction, high viral recovery, and high filter throughput. In this preliminary screen, the performance of three different

pore-size HFs was investigated by determining the filter load capacity, turbidity reduction, and viral recovery yield (**Table 3.1**). Hence, stirrer tank bioreactors were utilized to produce the LVs feedstock further used for filter evaluation. The cells were harvested at 48 hpt with a turbidity of approximately 460 NTU at a cell concentration of 3.9×10^6 cells/mL. Different inner diameters, namely 1.4, 1, and 0.75 mm were tested, all exhibiting pore sizes higher than $0.22 \mu\text{m}$ to prevent viral damage and loss of biological activity [14], [35]. In this work, filters selection was not only based on filtration performance but also on the ability to preserve cell viability and virus integrity. Therefore, the shear rate was set at 3000 s^{-1} for all runs since it was reported to promote a gentle operation compatible with the biological material involved in this bioprocess [51], [52].

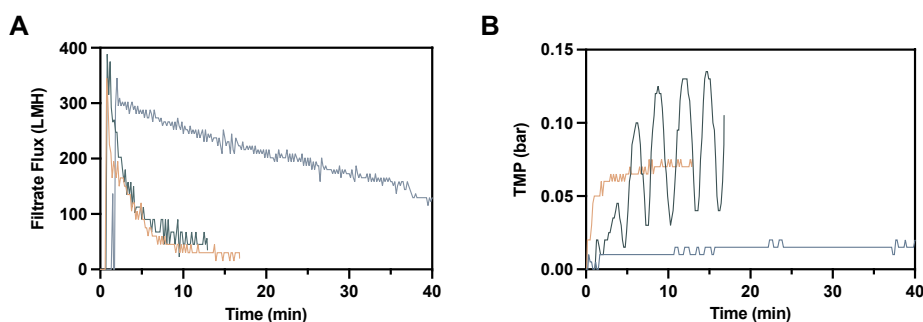


Figure 3.1. Initial scouting of hollow fiber devices. Evaluation of the impact of hollow fiber characteristics (Table 3.1) on LVs clarification step. In these experiments, the filters' capacity was calculated based on permeate flux decay (**A**), and the TMP profile was also studied (**B**). A shear rate of 3000 s^{-1} was kept constant for all experiments. Color code: HF1 corresponds to BioOptimal™ represented by gray color; HF2 corresponds to MidGee™ of $0.45 \mu\text{m}$ represented by green color; HF3 corresponds to MidGee™ of $0.65 \mu\text{m}$ represented by orange color.

The filtrate flux profile as a function of time for the three filters is indicated in **Figure 3.1A**. HF-2 and HF-3 displayed similar performance with an initial flux of 300 LMH, however, a fast permeate flux decay in the following 15 minutes was observed. The filter capacities obtained was 36.5 L/m² and 31.5 L/m² for HF-2 and HF-3, respectively. Additionally, a turbidity reduction of 90% was achieved with a final turbidity value of approximately 50 NTU, for both filters. Regarding viral yield, no significant differences were observed with all devices retrieving more than 90% of TU recovery. The results obtained for HF-1 demonstrated better performance with a filter capacity of 216 L/m². This filter also reduced turbidity by 99%. In fact, the final sample presented a turbidity of 2.2 NTU which was compliant with the GMP requirements (<10 NTU) [32], [33], [53].

The transmembrane pressure (TMP) was also studied (**Figure 3.1B**) and results showed that only for HF-1 no TMP increase was detected during LVs filtration. In HF-2 and HF-3, membrane clogging could be the cause of the rapid TMP augment leading to lower filter capacity. TFF-MF can contribute to the prevention of cake formation although some degree of membrane fouling is inherent to a filtration operation [42], [54]. One could expect that higher pore-size devices (0.65 µm) could lead to improved filter capacity [29]. However, the results obtained showed an outperformance of HF-1 (0.4 µm). It is hypothesized that the larger fiber inner diameter of HF-1 (1.4 mm > 0.75 mm) and the membrane structure contribute to lower pressure and increased permeate flux. Additionally, another factor that can affect the success of LVs manufacturing is the processing time. This enveloped virus has a short half-life of 5 - 8 hours at 37°C. Thus, the higher throughput and permeate flux achieved with HF-1 can result in decreased processing time. Moreover, the improved filter capacity might lead to cost reduction and footprint decrease in the manufacturing setting. These findings demonstrated HF-1's potential for LVs clarification, leading to its selection for further evaluation.

3.1.2. Robustness analysis of the selected filter and scale-up prediction

After identifying the filter candidate, it was necessary to characterize the clarification step in terms of filtration time, process reproducibility, and impurity clearance. Therefore, to assess the consistency of the established filtration step, three independent runs were performed. LVs productions were harvested at cell densities of $4.6 \pm 1.3 \times 10^6$ cells/mL and turbidity of 490 ± 40 NTU. **Figure 3.2** depicts filtrate throughput and TMP as a function of time.

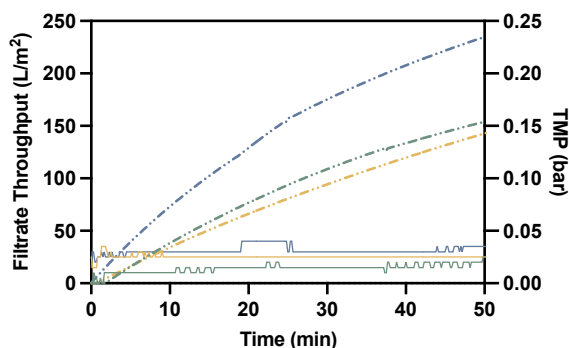


Figure 3.2. Robustness of HF-1 for clarification of LVs feed stocks. Three TFF-MF runs were performed and the filtrate throughput (dashed lines) and TMP (solid lines) were monitored.

No significant differences in the filtrate throughput profile were found across all runs. Some constraints have been raised with the use of polysulfone membranes due to their hydrophobicity which can lead to cake formation [55], [56]. In this work, TMP pressure remained constant with no evidence of significant membrane fouling, which supports the device robustness. Different process parameters were determined and are shown in **Table 3.2**. Results show that a turbidity reduction of 99% was achieved in all trials in just a single step (final turbidity < 10 NTU). Due to the nature of the TFF-MF operation, a fraction of the LVs supernatant remains in the

retentate bottle at the end of the process. According to the manufacturing guidelines, the operation proceeded until a volumetric concentration factor (VCF) of 12 was reached.

Table 3.2. Overview of the evaluated process parameters during HF-1 characterization.

Run #	Cell conc. (cells/mL)	Turbidity reduction (%)	TU recovery yield (%)	TU conc (TU/mL)	Filtrate Throughput (L/m ²)	Filtration Time (h)
1	6.1 x 10 ⁶	99	98	2.4 x 10 ⁷	300	1.3
2	3.8 x 10 ⁶	99	92	2.6 x 10 ⁷	216	1.4
3	4.0 x 10 ⁶	99	97	3.9 x 10 ⁷	246	0.9

Surprisingly, a TU recovery yield higher than 90% was determined and the virus's biological activity was successfully preserved. In the last few decades, different LVs filtration methods have been explored reporting high recovery yields in adherent cultures (> 70%) [35], [39]–[41], [57]. Recently, Labish (2021) et. al reported the use of diatomaceous earth (DE) for LVs filtration. Although this is a promising technology, the turbidity threshold (< 10 NTU) was not achieved with low DE concentrations. Additionally, this system currently presents limited scalability, and the exact TU recovery yields are not mentioned.

HF-1 maximum capacity was calculated and values between 400 and 700 L/m² were determined and, experimentally, clarification of 300 L/m² was easily accomplished with very high turbidity reduction and LVs recovery. HF capacity should be highly dependent on the initial feedstock properties (e.g., cell concentration, viability, viscosity), however, no relation between cell concentration and the filter's capacity was observed. To determine the

existence of any potential correlation between these parameters, a more comprehensive study with increased cell densities may be necessary. As previously mentioned, higher filtrate fluxes may lead to processing time reduction. Particularly, HF-1 device with a surface area of 50 cm² could effectively filter 1.5 L of LVs material in 1.3 hours, with filtration time being proportional to the volume processed. These results allowed a rough scale-up estimation of an LVs clinical batch production. Based on a membrane capacity of 300 L/m², it is estimated that a 1000 L bioreactor clarification using an 8 m² device could be completed in under one hour. Given the short half-life of this enveloped virus, the implementation of this optimized filtration step could positively impact the LVs manufacturing process. Afterward, the impurity clearance was also evaluated through the quantification of total protein with an average removal of 44 ± 2 % which is comparable with other reported values [39], [58]. Lastly, the cell viability in the retentate bottle was quantified. Results showed no loss on cell viability demonstrating that the selected shear rate allowed the preservation of not only the viral particles but also the producer cells. This constitutes an advantage since cell disruption can increase the concentration of product related impurities and impact the following operation units.

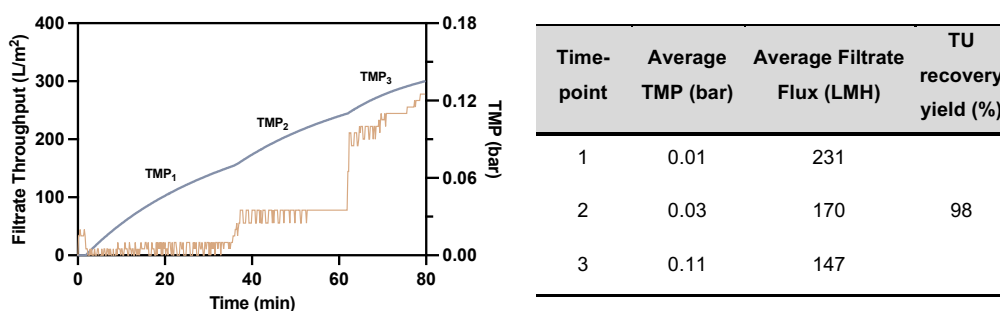


Figure 3.3. Impact of TMP pressure on filtrate throughput and viral recovery yield. Variation of the permeate flux throughout the clarification process by applying

three different TMP values. Color code: TMP is represented by blue color; filtrate throughput is represented by orange color.

The permeate flux is dependent on the difference between hydraulic and osmotic pressure driving forces [59], [60]. Consequently, increasing the TMP pressure leads to a higher permeate flux. Thus, an experimental run was performed to better understand the impact of increasing the TMP pressure on LVs recovery yield. Since no fluctuation in the TMP pressure was observed in previous runs, a restriction valve was placed on the retentate outlet to increase the retentate pressure and consequently achieved a higher TMP pressure. The main results are shown in **Figure 3.3**. During this experiment, TMP was modified three times and at the end of the clarification process a final value of 0.12 bar was achieved with a global TU recovery yield of 98% demonstrating that HF-1 can be operated until a TMP of 0.1 bar without compromising TU recovery yield which is in line with previous reports [13], [61].

The high costs associated with LV-based therapies have been one of the driving forces for the design of more efficient and cost-effective processes [53], [62], [63]. Continuous manufacturing offers several advantages including enhanced product quality, faster processing times, reduced costs, and increased productivity [53]. Therefore, process intensification can be a powerful approach to establish integrated continuous processing that combines continuous harvesting in perfusion mode with downstream capture. The alternative clarification step herein presented has the potential to be applied as a retention device for LVs production in perfusion mode and enable an integration with the chromatography step. In fact, Barbara (2022) et. al have already reported the use of HF-1 device for the continuous production of Gag-HA VLPs at high cell density. Although a reduction of cell viability was observed, this issue could be easily mitigated by replacing the

recirculation pump. Nevertheless, the Gag-HA VLPs production was sustained for 20 days [64]. Tangential Flow Depth Filtration [™] (TFDF), Repligen's new filter technology, has been used to improve LVs production in perfusion and offers several advantages for viral harvesting when compared with the previously Alternating Tangential Flow [™] (ATF) technology [13], [19], [61]. However, since TFDF [™] presents an effective pore size ranging from 2–5 μm , a second stage clarification filter might be needed to avoid pressure increase in the chromatography step.

Given the efficient performance of the HF-1 which retrieves a high TU recovery yield and loading capacity, combined with flexible scalability and final turbidity below 10 NTU, its applicability to LVs process integration should be straightforward.

3.2. Baseline purification process comparison between LVs from adherent and suspension productions

Following clarification, the LVs purification process typically includes a concentration step that can be accomplished by ultrafiltration and/ or chromatography methods. Anion exchange chromatography (AEX) is a workhorse for LVs purification since enables scalable, fast, and cost-effective processes [39], [57], [65]–[68]. Recently, Moreira (2021) et. al. reported an improved purification train for LVs produced in adherent cultures, composed of only 4 steps. In this process, LVs are directly loaded on Mustang Q anion exchange membrane with an overall TU yield of 90%, although not all fractions were used for further processing [47]. In this section, the impact of the expression system (adherent versus suspension) was evaluated by performing the capture step as previously described by Moreira (2021) et. al. [47]. Therefore, LVs were produced by transient transfection in both culture

systems. The virus-containing supernatant was loaded into 0.86 mL Mustang Q membranes. To assess LVs TU recovery, the initial sample, flow-through, and elution fractions were collected and analyzed for transduction efficiency. **Figure 3.4** depicts the TU recovery yield obtained for this initial evaluation.

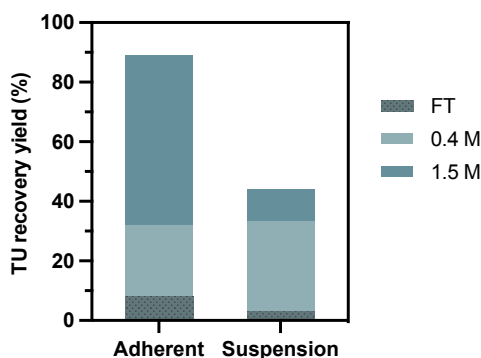


Figure 3.4. LVs baseline chromatography step. Recovery of transducing units (TU) comparison for the purification of LVs produced in adherent and suspension culture systems. For both evaluations, LVs were loaded at 10 MV/mL in a Mustang Q membrane of 0.86 mL and elution.

Results obtained from LVs produced in the adherent system showed to be consistent with previous studies. Only a small percentage of LVs, 8 %, were found in FT and the majority eluted at 0.4 M and 1.5 M of NaCl with 24 % and 57 % respectively. However, unexpected results were obtained for LVs produced in suspension cultures. The overall TU recovery yield decreased by half when compared to the adherent system. Moreover, a modification in the elution profile was also observed. Contrary to what has been published in the literature, it was found that the lowest ionic strength step was sufficient to elute most of the viral vectors, with an approximate recovery yield of 31 %. At 1.5 M of NaCl, only 11% TU recovery yield was obtained, whereas in adherent systems it was the fraction that retrieved the highest viral recovery. While the exact reasons for this unexpected outcome remain unclear, potential strategies were proposed. To mitigate the TU

recovery yield decreased in suspension cultures the impact of adding of sucrose on LV chromatography step and the lipid supplementation during LVs production were assessed.

3.2.1. Strategy I – Impact of lipid supplementation on viral quality

One potential explanation for the observed decrease in viral recovery during the AEX step could be attributed to the new production system. In recent years, efforts toward adopting suspension cultures and establishing serum-free production platforms have been made [29]. However, finding suitable alternatives to replace the crucial components added by the serum and produce new chemically defined media can be a challenging task [22]. Consequently, serum-free production processes may lead to modifications in cell media composition and influence cell metabolism and viral-specific production [22], [28], [69]. Specifically, changes in lipid and cholesterol levels in the cell membrane could affect lipid bilayer fluidization impacting viral quality and infectivity [69]–[71].

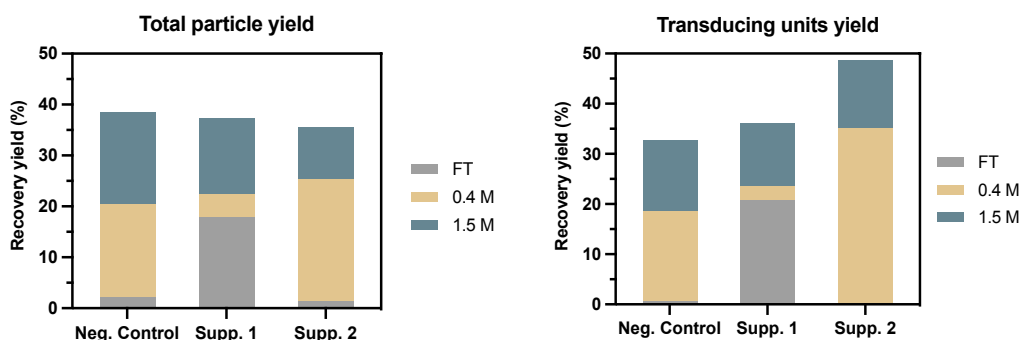


Figure 3.5. Assessment of lipid supplementation in LVs chromatography. The effect of two lipid supplements (Supp.1 and Supp.2) was evaluated on AEX step using a 0.86 mL Mustang Q membrane. TP and TU recovery yield was determined for each chromatographic step (FT, 0.4 M, and 1.5 M NaCl).

Several reports already studied the impact of lipid supplementation on viral production, but to the best of the authors knowledge, the effect on viral purification has not yet been described. Hence, aiming to assess the impact of lipid supplementation in the TU recovery yield in the AEX step, a preliminary study was performed using two lipid cocktails. Lipid supplementation was performed using the lowest concentration value recommended by the manufacturer and was added 4 hours after the transfection to avoid interference with the internalization of DNA complexes [12], [22]. Regarding viral production, it was not observed a significant increase on the volumetric LVs concentration (data not shown) in both media supplements, therefore corroborating the findings of other reported screenings [12], [22]. The results comparing the supplemented conditions and the negative control are represented in **Figure 3.5**. No significant improvements for both conditions were observed in comparison to the negative control. Furthermore, supplement 1 exhibited a distinct elution profile with a 28- fold increase in TU loss in the FT fraction. Additionally, the lowest TU yield was found for the fraction 0.4 M of NaCl, in contrast to what was observed for the negative control. These results suggests that medium supplementation can impact viral surface charge and membrane-virus interaction. Regarding supplement 2, a 2-fold increase was observed in the fraction 0.4M of NaCl. These results suggested that the addition of supplement 2 upon production leads to LVs more resistant and able to cope with the purification process.

Overall, a possible explanation for the different behavior of LVs produced in suspension cultures might be the modification of the lipid source can impact the viral envelope lipidic structure. On the other side, the reduction of viral quality, can decrease viral resistance to process critical parameters (e.g., shear stress, temperature, or ionic strength) leading to lower global TU recovery yields. Despite a possible alteration on envelope protein exposure

can result in reduction of membrane-protein interaction during viral loading, it appears that this effect can be mitigated through a proper lipid supplementation.

3.2.2. Strategy II – Evaluation of sucrose addition on viral stability

LVs sensitivity to most process conditions has been extensively reported, thus it is imperative to ensure a proper viral formulation to cope with the purification process [39], [72], [73]. Usually, albumin and lipid sources contained in fetal bovine serum are associated to increase viral stability [28], [41]. Then, establishing serum-free production processes might lead to the modification of cell media composition and consequently impact viral recovery. During the purification process, different excipients could be added such as recombinant proteins (e.g., human recombinant albumin), and sugars (e.g., sucrose) to enhance vector stability and prevent viral loss [39], [40], [74].

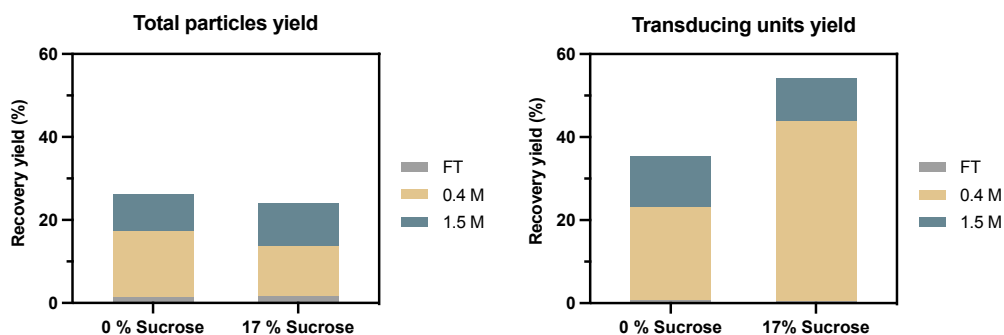


Figure 3.6. Assessment of sucrose concentration in LVs chromatography. The concentration of sucrose (0% and 17%) was evaluated using a 0.86 mL Mustang Q membrane. TP and TU recovery yield was determined for each chromatographic step (FT, 0.4 M, and 1.5 M NaCl).

In this approach, the main goal was to evaluate the impact on TU viral yield by improving viral stabilization during the chromatographic step. Sucrose has been used in different buffer formulations due to its stabilizing properties [72], [75]. Thus, its potential for enhancing overall TU recovery through optimization of processing conditions, was studied. **Figure 3.6** illustrates the TU recovery yield for both 0% and 17% sucrose concentrations across each fraction collected. A 2-fold increase on TU yield was obtained which might indicate beneficial effect, however further studies must be carried out to confirm this preliminary result. Sucrose has been reported to improve recovery yields for enveloped virus (gibbon ape leukemia virus pseudotyped LV or vesicular stomatitis virus) for other anion exchange chromatography devices [40]. This effect may be process-dependent since no significant differences with sucrose addition were previously reported [47]. Lastly, considering that LVs were being partially loss in the 0.4 M fraction, a final purification process was performed using a single isocratic step of 1.5 M of NaCl. In this final run, a TU recovery yield of 56 % was obtained, with neglectable loss on the FT fraction. The characterization and impurity clearance assessment are further discussed.

3.3. Quality assessment of purified LVs produced in suspension cell lines

Impurity clearance is a critical aspect of viral manufacturing [76]. LV-based therapies present potential to address cancer diseases and a variety of genetic disorders. Therefore, an important factor to take into consideration is the therapeutic modality, which may involve *ex vivo* applications or *in vivo* administration [14], [77]. Currently, there are no established guidelines regarding the purity requirements for preclinical materials, although an increase in product purity is expected when transitioning to clinical stages [78]. Thus, a proper viral characterization and the elimination of the

remaining contaminants/impurities is imperative to establish GMP compliant downstream processing workflow and ensure their quality, safety and effectiveness [68], [79], [80].

In this work, the impact of adherent to suspension transition on LVs downstream process was evaluated. For that reason, it was also important to assess the quality of the purified LVs. This evaluation was performed using samples purified with a DSP workflow composed by clarification and an AEX step. A total protein removal higher than 95 % and a DNA removal of approximately 90 % was achieved. Furthermore, the high impurity reduction achieved is consistent with findings from other purification techniques [39], [47], [81]. The inherent features of suspension cell cultures might lead to the increase of cell-based impurities, host cell proteins, and add new challenges to the suspension LVs DSP.

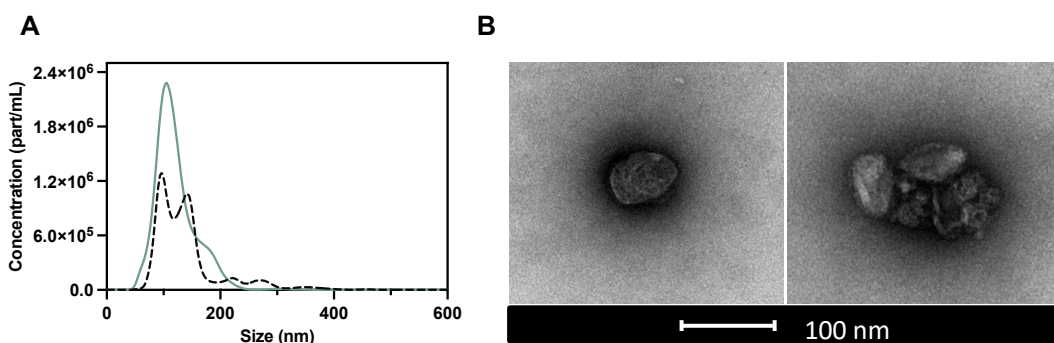


Figure 3.7. (A) Size distribution profile by NTA of 2 process samples (load – initial clarified LVs, dashed line; and elution – fraction at 1.5 M NaCl, green solid line). The represented distributions are an average of three measurements diluted 1:100. **(B)** TEM analysis of LVs sample obtained after the purification process (clarification and capture with AEX step). Scale bar 100 nm.

Therefore, the quantification of the residual host cell proteins derived from the producer cell line (HEK 293T) was performed. Results show the

presence of $5.5 \pm 0.82 \mu\text{g}$ of HCP for a viral dose of 10^9 TU. Unexpectedly, this result was comparable with similar doses obtained after a full downstream process (e.g., $5.5 \pm 0.82 \mu\text{g}$ of HCP / 10^9 TU) [47], although lower values have also been reported using different chromatographic techniques [50], [81]. A possible explanation for this result might be the implementation of a gentler clarification step, since shear rate was carefully selected to promote the preservation of cell viability preventing the release of cell fragments and increase host cell related impurities.

LVs were also characterized by transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA). Initially, NTA technique was used to measure particle size distribution and concentration. **Figure 3.7A** depicts the size distribution obtained for the clarified and after elution samples. This representation shows the broader profile obtained in the initial sample, probably related to presence intrinsic impurities of the production system. After elution, the sample presented only one sharp peak with a mean size of 105 nm and a 2-fold concentration increase. Lastly, TEM analysis was performed to detect the presence and morphology of LVs after the chromatography step (**Figure 3.7B**). Both images, confirm the shape and viral integrity were maintained after the purification.

4. Conclusions

In recent years, there has been an increasing interest in suspension-based upstream processes for lentiviral vector production. However, this transition has put some pressure in establishing a successful and scalable purification process of LVs, since suspension cultures bring new challenges. In this work, the main goal was to assess the impact of LVs produced by transient transfection in HEK 293T suspension cells in the downstream

processing. Initially, an alternative filtration step that could be applied in different production modalities, was evaluated. The selected TFF-MF filter proved to be a gentle step and high throughput option, offering competitive yields and impurity clearance compared to other reported technologies. Specifically, a recovery yield higher than 90 % and final turbidity below 10 NTU were easily accomplished. Furthermore, the effect on chromatography step was also explored. LVs produced in adherent and suspension were purified by AEX, and their performance was compared. Results showed a decrease in overall TU recovery yield as well as a modification in the elution profile. Although these suspension cultures presented additional hurdles, after the elution step fine-tuning, a TU yield of 56 % was achieved.

These findings highlight the potential for large-scale LVs manufacturing, as well as the role that lipid supplementation and buffer formulation can play in enhancing the stability of LVs.

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4

Establishment of affinity chromatography for VSV-G pseudotyped LVs

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Abstract

The use of viral vectors as therapeutic products for multiple applications such as vaccines, cancer treatment, or gene therapies, has continued to grow exponentially. Therefore, improved manufacturing processes are needed to cope with the high number of functional particles required for clinical trials and, eventually, commercialization.

Affinity Chromatography (AC) can be used to simplify purification processes and generate clinical-grade products with high titer and purity. However, one of the major challenges in the purification of Lentiviral vector (LVs) in using AC has been combining a highly specific ligand with gentle elution conditions to cope with viral biological activity. In this work, it is reported for the first time the implementation of an AC resin to specifically purify VSV-G pseudotyped LVs. After ligand screening, different critical process parameters were assessed and optimized, including the residence time, dynamic binding capacity, functional particle recovery, and impurity clearance. A dynamic capacity of 1×10^{11} total particles per mL of resin was determined and an average recovery yield of 45% was found for the small-scaled purification process. The established AC robustness was also confirmed by the performance of an intermediate scale with infectious particles yield of 54%, which demonstrates the scalability and reproducibility of the AC matrix.

Overall, this work contributes to increasing downstream process efficiency by delivering a purification tool that enables high purity, scalability, and process intensification in a single step, then contributing to time-to-market reduction.

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1. Introduction

Lentiviral vectors (LVs), a complex subclass of the *Retroviridae* family, have emerged as one of the most used delivering tools in cell and gene therapy [1]. The increasing interest in LVs is mostly related to their ability to transduce proliferating and non-proliferating cells [2], to integrate and deliver long-term gene expression [3] and to provide a safer integration profile when compared to γ -retroviral vectors [4]. This enveloped virus stands out as a versatile tool since it can be applied for the treatment of infectious diseases or genetic disorders [5], [6], or even in the generation of chimeric antigen receptor (CAR)-T cells for cancer immunotherapies [7], [8].

Of the several envelopes available, the G protein of vesicular stomatitis virus (VSV-G) is the most commonly used, either in development research or in clinical applications, since presents broad tropism and relatively high stability [9], [10]. The replacement of the native envelope protein with a clinically relevant glycoprotein, pseudotyping, is another attribute of the LVs. Remarkably, from the list of more than 20 cell and gene products already on the market, 7 are LV-based therapies demonstrating the success of this viral vector [11]. Even though there is no strict value for therapeutic doses for either *ex vivo* or *in vivo* applications, most authors have been reporting viral doses between 10^{10} and 10^{12} per patient [12], [13].

The growing interest in LVs exposes the current bottlenecks underlying downstream processing (DSP). LVs are known for their biological complexity since viral stability can be affected by temperature [14], [15], ionic strength [16], [17], pH [14], freeze-and-thaw cycles [14], [18], and shear stress [19]. Despite the significant improvements that have been made over the past few years, the production and purification of LVs are far from reaching their maximum potential. Currently, manufacturing platforms cannot fulfill clinical demands, reinforcing the need for robust, scalable, and cost-effective

processes to sustain the commercialization of new LV-based therapies [20]–[22]. The downstream process of LVs comprises several operation units where the primary goal consists of concentration and purification of the virus, while preserving the biological activity [17], [19]. Viral capture is probably one of the most challenging operation steps in the LVs purification process and, for that reason, different chromatography modalities have been investigated as alternatives to overcome low recovery yields [16], [17], [23], [24].

Affinity chromatography (AC) explores highly selective and reversible interactions between the immobilized ligand and the product of interest, offering high purity, high fold concentration, and scalability in a single step [25]–[27]. This robust modality can generate economic benefits since it increases process standardization and simplification by reducing the number of unit operations [28]. Therefore, different approaches are described in the literature for the purification of LVs using affinity chromatography. Immobilized metal affinity chromatography (IMAC), involving envelope protein engineering with a histidine tag (his-tag), presents a low-cost and highly stable solution [29]. However, concerns regarding the harsh desorption agents resulting in viral inactivation (e.g., imidazole or EDTA) or the possible adverse effects in further clinical applications (e.g., proteases, his-tag ligands, metal ions leakage) have been a major drawback [19], [30]. Another envelope affinity tag strategy was the expression of biotin onto the LVs surface for the capture with immobilized streptavidin [31], [32]. Recently, the purification of an RDPro pseudotyped LV labeled with cTag8 was also described by Mekkaoui et al. where a biotin mimic was genetically encoded and pseudotyping independent [33]. Additionally, heparin affinity can be an attractive technology as it presents affordable costs and an elution step at mild conditions contributing to viral stability [1]. Nevertheless, the lack of selectivity leads to DNA and host cell proteins (HCP) co-elution. Furthermore, most commercial heparin ligands have animal origin which

brings some constraints for clinical applications, since the traceability and validation of raw materials can be challenging [16], [34], [35]. Therefore, this highlights the need for an affinity chromatography step that can offer high specificity and scalability using gentle elution conditions compatible with the biological activity of the enveloped viral vectors. Over recent years, the use of recombinant camelid-derived single-domain antibody fragments (V_{HH}) has been reported for several biopharmaceuticals [36], such as different AAV serotypes [27], [37] and, more recently, Moleirinho (2020) et al. used similar technology to develop a negative mode AC for baculovirus removal [38]. Due to its small size, high specificity, mild elution conditions, and free of animal components, V_{HH} ligands can be an alternative to mitigate some of the concerns in affinity approaches.

In this work, the first affinity matrix design to bind specifically to VSV-G pseudotyped LVs was successfully established. Initially, a set of ligands produced by phage display was screened for viral specificity and elution efficiency in mild conditions. The lead candidates were evaluated using functional assays to determine the best performer. In addition, the critical process parameters were assessed and optimized for the lead candidate. Afterward, the full performance of the established Lenti VSV-G Affinity Matrix was determined at different scales. Finally, LVs purified with the novel affinity matrix were characterized in terms of impurity removal and quality attributes.

2. Materials and Methods

2.1. Lentiviral vector production

2.1.1. Cell line maintenance and transfection

HEK293T cells (ATCC® CRL-3216™) obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were adapted to grow in suspension as described in [39]. The cells were cultivated in BalanCD®

HEK293 medium (FUJIFILM IrvineScientific, Santa Ana, USA) supplemented with 4 mM GlutaMAX™ (Thermo Fisher Scientific™, USA) and maintained at 37 °C in an incubator with a humidified atmosphere of 8% CO₂ in the air. Cell concentration and viability were assessed by the trypan blue exclusion method. LVs were produced by transient transfection using pALD-Lenti System (Aldevron®, USA) including the pALD-VSV-G-K, pALD-GagPol-K, pALD-Rev-K, and pALD-Lenti-EGFP-K plasmids. Briefly, the cells were transfected at 2.5×10^6 cells/mL in a shake flask using linear 25 kDa polyethyleneimine, PEIpro® Transfection Reagent (Polyplus-transfection®, France) at a mass ratio of 1:3 (DNA:PEI) and 1 µg of total DNA per 1×10^6 cells. At 48 hour-*post-transfection*, VSV-G-pseudotyped LVs (VSVG-LVs) were harvested at a cell density ranging from $4 - 6 \times 10^6$ cells/mL.

2.1.2. Nuclease treatment and virus clarification

Nucleic acid digestion was performed with Benzonase® endonuclease (Merck, Germany) at a final concentration of 100 U/mL and in the presence of 2 mM MgCl₂. The digestion proceeded for 30 min at room temperature. The VSVG-LVs containing supernatant were clarified by centrifugation at 500 x g for 10 minutes at 18 °C followed by filtration using a Nalgene™ Rapid-Flow™ Sterile Single Use Vacuum Filter Unit with a PES (Polyethersulfone) membrane with 0.45 µm pore size (Thermo Fisher Scientific™, USA).

2.2. Discovery, selection and production of V_HH fragments

Phage display was performed using Thermo Fisher's non-immune V_HH libraries following a screening of V_HH supernatants derived from isolated single clones in a label-free binding assay based on surface plasmon resonance (SPR) as described by Moleirinho (2020) et al. [38]. In short, Maxisorp® flat-bottom, 96-well plates (Thermo Fisher Scientific™, USA)

were pre-coated overnight at 4 °C with a 10 times dilution of a VSVG-LV preparation in PBS, pH 7.4 at 100 μ L/well at a final concentration of $\sim 1 \times 10^{11}$ total particles per mL. The plates were washed, and then subsequently blocked for 1 h at room temperature with 2 % w/v dried skimmed milk (Protifar, Ireland) in PBS at 200 μ L/well (2% P-PBS). V_{HH} phages from the non-immune libraries were diluted 10 times in 1 % PBS, 0.05 % (v/v) Tween-20 (1 % PBST) and incubated for 1 h at room temperature on the plates. After vigorously washing of the wells with PBST the residually bound V_{HH} phages were eluted following a rescue of the phagemids by infection of TG1 cells as described by Adams (2014) et al. [40]. A second round of phage display was performed with the enriched libraries now using a 10 – and 25 times dilution of the V_{HH} phages. After the second round of selection, single TG1 clones from the VSVG-LV enriched libraries were isolated following V_{HH} production in deep well microtiter plates. The crude *E. coli* supernatants with soluble V_{HH} fragments equipped with a C-terminal tag sequence amenable to site-specific enzymatic biotinylation [41] were directly spotted onto a streptavidin biosensor chip (Strep G Senseye, The Netherlands) using a continuous flow microspotter (Carterra®, USA) according to the manufacturer's protocol. The biosensor chip, now functionalized with an array of V_{HH} fragments, was used in a first screen to identify binders against VSVG-LV particles using the IBIS MX96 instrument (IBIS technologies, The Netherlands) that facilitates label-free and real-time binding analysis based on SPR. The sequences encoding V_{HH} s of the positive clones identified in this first screen were elucidated by Sanger sequencing (BaseClear, The Netherlands). In order to obtain sufficient V_{HH} material for further characterization of the positive binders in a second screen, the unique V_{HH} sequences were re-cloned into a yeast expression vector following the integration of the V_{HH} genes into the genome of *Saccharomyces cerevisiae* as previously described [36], [42]. V_{HH} production was carried out at shake-flask scale followed by purification from the extracellular medium using cation

exchange chromatography. The purified V_HH fragments were chemically conjugated to biotin by Thermo Fisher Scientific according to their standard procedures. The resulting biotin conjugates facilitated more detailed characterization of the V_HH fragments on functionality and selectivity in SPR and streptavidin agarose beads-based assay set-ups.

2.3. Surface plasmon resonance (SPR)

2.3.1. Binding profiles

The purified and chemically biotinylated V_HH fragments were diluted in PBST at different concentrations (i.e., 20, 2, 0.2 and 0.02 µg/mL) and subsequently spotted onto a Strep G Senseye biosensor chip as previously described. To determine potential non-specific binding of the VSVG-LVs particles to the biosensor surface, two irrelevant V_HH fragments directed against AAV and baculovirus (Thermo Fisher Scientific™, USA) were included as biotin conjugate using the same concentration series for spotting. The functionalized biosensor was placed into the IBIS MX96 instrument following a standard run for target binding analysis using PBST as running buffer. Binding profiles of the different V_HH fragments were analyzed using the VSVG-LVs preparation diluted 10, 20, 40, 80 and 160 times in running buffer from an initial sample at an estimated concentration of $\sim 1 \times 10^{10}$ total particles per mL. Briefly, after setting a baseline with running buffer for 1 minute, the samples were allowed to bind to the spotted array of V_HH fragments for 5 minutes (association phase) following a washing step with running buffer for 5 minutes (dissociation phase). After each run, the biosensor was regenerated using 100 mM tri-ethylamine (TEA) for 1 minute following a re-equilibration step with running buffer for 30 seconds. For each spotted V_HH fragment of the SPR-array, individual sensorgrams were generated per sample and an overlay of all resulting sensorgrams per spot was obtained using the IBIS MX96 software.

2.3.2. Elution efficiency

To study the release of the bound VSVG-LVs particles under mild elution conditions by SPR, a variety of elution buffers was evaluated using 50 mM Tris, pH 7.5 as buffer system. The elution buffers used were either arginine up to a level of 800 mM, NaCl or CaCl₂ up to a level of 1M and 0.5M respectively. For the arginine buffer, different pH values were tested ranging from pH 6.0 to pH 8.0. For target binding the VSVG-LVs preparation was diluted 80 times in 50 mM Tris, 150 mM NaCl, 0.075% (v/v) Tween-20, pH 7 (running buffer). After binding, the target release efficiency of each elution buffer composition was determined. In short, after setting a baseline with running buffer for 1 minute, the VSVG-LVs sample was allowed to bind to the spotted array for 5 minutes (association phase) following a wash step with running buffer for 1 minute (endpoint set as response signal before elution).

After the wash step an elution buffer composition was incubated for 5 minutes following a wash step with running buffer for 1 minute (endpoint set as response signal after elution). After each run, the biosensor was regenerated using TEA for 1 minute following a re-equilibration step with running buffer for 30 seconds. The difference in response signal before and after the elution step was determined to calculate the target release efficiency for each of the buffer compositions tested using the IBIS MX96 software. If the response signal after the elution step reached baseline, the elution efficiency was valued 100 % whereas in the case that no decrease was observed the elution efficiency was valued at 0 %.

2.4. VSVG-LVs viral capture using streptavidin agarose beads

Selected biotinylated VSVG-LVs binding V_HH fragments were coupled to high-capacity streptavidin agarose beads (ThermoFisher Scientific™, USA)

following the manufacturer's instructions. Beads were washed and equilibrated using 50 mM HEPES, 150 mM NaCl at pH 7.5 as binding buffer. Briefly, the produced VSVG-LVs were incubated for 1 hour with the 50 μ L streptavidin agarose beads under gentle agitation at room temperature. Afterward, the supernatant was collected by centrifugation, followed by washing the beads three times with binding buffer. Viral elution was performed with 50 mM HEPES, 150 mM NaCl, 800 mM Arginine at pH 7.5, and the strip step was performed by boiling the beads in SDS-PAGE sample buffer. The collected samples (flowthrough, elution, and strip) were stored at - 80 °C for further analysis.

2.5. Generation chromatography studies

In order to obtain sufficient V_{HH} material for preparing prototype affinity resins to evaluate performance in chromatography, the selected VSVG-LVs binding V_{HH} fragments were produced at larger scale by running 10 L yeast fermentations following a 2-step purification protocol using ion-exchange chromatography (IEC). This set-up, carried out by Thermo Fisher Scientific, also verifies whether the selected V_{HH} candidates can be manufactured at an economically feasible scale. The purified V_{HH} fragments that passed all scalability requirements were chemically coupled to different types of resin beads and at different V_{HH} (ligand) densities carried out by Thermo Fisher Scientific according to their standard procedures.

2.6. Affinity Chromatography studies

An AKTA Avant 25 chromatography system (Cytiva, USA) equipped with conductivity, UV and pH detectors was used to perform small-scale studies and the intermediate scale-up at room temperature. System control and data analysis were done through UNICORN™ 7.6 software. Omnifit Labware Columns (Kinesis, USA) or XK 16/20 (Cytiva, USA) were packed with

epoxide-activated agarose beads at a bed volume of 1 or 10 mL. The packing quality was evaluated by a pulse injection of 1 M NaCl where asymmetry factors between 1.2 and 1.6 were obtained. Columns were equilibrated with 10 column volumes (CV) of an equilibration buffer containing 50 mM HEPES, 150 mM NaCl at pH 7.5. After virus loading, the columns were washed with 5 CV of equilibration buffer, followed by elution with 3 CV of 50 mM HEPES, 150 mM NaCl, 800 mM Arginine at pH 7.5. The strip step was performed with 5 CV of 50 mM Sodium Phosphate at pH 12. The collected samples (flowthrough, elution, and strip) were stored at -80 °C for further analysis.

2.6.1. Residence time optimization

To evaluate the impact of the residence time on VSVG-LVs recovery yield, a 1 mL column packed with the CaptureSelect™ Lenti VSV G Affinity Matrix [43] was used. These experiments were performed at different residence times (1, 2 and 4 minutes). The columns were equilibrated, washed, and eluted as previously described. After, flowthrough and elution fractions were collected and pooled based on the in-line UV signal at 280 nm and stored at -80 °C, for further analysis.

2.6.2. Dynamic binding capacity determination

To determine the dynamic binding capacity (DBC) for the CaptureSelect™ Lenti VSVG Affinity Matrix, a 1 mL column was used. Clarified VSV-LVs feedstock was loaded onto the column at a flow rate of 0.5 mL/min. The total particle concentration (TP) was determined in the flowthrough fractions. The DBC at 10% was calculated according to Equation 4.1, where C_0 corresponds to initial virus concentration (TP/mL), V_b is the breakthrough volume at which the flow-through stream achieves 10% of the

loaded concentration (mL), V_0 is the void volume (mL), and CV is the total column bed volume (mL). The DBC was determined in TP per mL of resin.

$$DBC_{10\%} = \frac{C_0(V_b - V_0)}{CV} \quad (4.1)$$

2.7. Analytics

2.7.1. VSVG-LVs transducing particles quantification

The HEK293T cells (ATCC ® CTRL-3216 TM) were acquired from the American Type Culture Collection (ATCC®, USA) and cultured in Dulbecco's Modified Eagle's Medium (ThermoFisher Scientific™, USA), supplemented with 10% Fetal Bovine Serum (ThermoFisher Scientific™, USA), 4 mM of GlutaMAX™ (ThermoFisher Scientific™, USA) and 1 mM Sodium Pyruvate (ThermoFisher Scientific™, USA). The functional VSVG-LVs titers were evaluated by transducing HEK293T cells and assessing GFP expression by flow cytometry as described elsewhere [44]. Briefly, HEK293T cells in 24 well plates were transduced with VSVG-LVs at different dilutions in fresh DMEM with 10% (v/v) FBS and 8 µg/mL of polybrene (MilliporeSigma, USA) and incubated at 37 °C. After 2 hours, 0.5 mL of fresh DMEM with 10% (v/v) FBS was added to each well, and the plates incubated at 37 °C. and, 48 hours post transduction, infected cells were sorted by fluorescence microscopy (Leica) to select the wells with approximately 5 -20 % of positive GFP containing cells 48 hours post transduction. After trypsinization of the cells in those wells, the percentage of GFP positive cells was quantified by flow cytometry in the FACSCelesta™ equipment (BD Biosciences, USA). The concentration of VSVG-LVs transducing units (TU/mL) was calculated using the equation 4.2:

$$\text{Titer} \left(\frac{\text{TU}}{\text{mL}} \right) = \frac{\% \text{ of GFP positive cells} \div 100}{\text{volume of transduction}} \times \text{dilution factor} \times \text{number of cells transduced} \quad (4.2)$$

2.7.2. Total particles quantification

Total particles (TP) concentration was estimated using the ELISA kit Innostest HIV Antigen mAb (Fujirebio, USA). To determine the total amount of p24 protein, a standard curve was established using the positive control with a 150 – 25 pg/mL concentration range according to the manufacturer's instructions. A ratio of 1.25×10^7 TP/ng of p24 was used to calculate the total particles titer.

2.7.3. Total protein, host cell protein and DNA quantification

Total protein was determined using the PierceTM BCA assay kit (ThermoFisherTM, USA) and total DNA was quantified with Quant-iT PicoGreen dsDNA assay kit (ThermoFisherTM, USA) used according to the manufacturer's instructions. Host cell protein was determined with HEK 293 HCP ELISA Kit (Cygnus Technologies). The detection was performed using the Infinite PRO NanoQuant (Tecan, Switzerland) microplate multimode reader. The samples were applied at multiple dilutions.

2.7.4. Transmission electron microscopy

The morphological analysis of VSVG-LVs was performed using transmission electron microscopy (TEM). Sample preparation consisted of a drop (5 µL) of each sample that was adsorbed onto a formvar-coated 150

mesh copper grids from Veco (Science Services, Germany) for 2 minutes. Afterwards, the grid was washed 5 times with sterile dH₂O, then soaked in 2% (w/v) uranyl acetate for 2 minutes and dried in air at room temperature. To analyze the grid a Hitachi H-7650 120 Kv electron microscope (Hitachi High-Technologies Corporation, Japan) was used.

2.7.5. Quality analysis of VSV-LVs particles by CE-SDS with LIF detector

VSVG-LVs characterization was performed by sodium dodecyl sulfate capillary electrophoresis (CE-SDS) combined with a laser-induced fluorescence (LIF) detection. Sample preparation and labelling was performed as described elsewhere [44]. All the analyses were carried out in a CESI 8000 Plus system (SCIEX, USA) with a 488 nm laser-induced fluorescence detector module using an emission filter of 600 nm. The separation was performed using a bare fused silica capillary of 50 µm inner diameter, 30.2 cm of total length and 20.2 cm of effective separation length (SCIEX, USA).

2.7.6. Western blot analysis

The pattern of p24 protein was assessed by western blot analysis. After protein denaturation, SDS-PAGE was carried out under reducing conditions in a 4-12% NuPage Bis-Tris protein gel (Thermo Fisher Scientific™, USA) and SeeBlue Plus2 Prestained Standard (Thermo Fisher Scientific™, USA) as molecular weight marker. The proteins were transferred to a PVDF membrane using the iBlot™ 2 system (Thermo Fisher Scientific™, USA).

After blocking with Tris-Buffered Saline (Merck, Germany) with 0.1% (w/v) of Tween-20 (Merck, USA) with 5% (w/v) skim milk (Merck, USA) for 1 h, the membrane was incubated overnight with the anti-HIV-1 p24-Mouse monoclonal antibody (Abcam, USA). Afterward, the membranes were washed and incubated with the secondary antibody, anti-Mouse IgG conjugated with alkaline phosphatase conjugate labelling (Merck, Germany) for 1 h. The protein's detection was performed using NBT/BCIP 1 Step (Thermo Fisher™, USA).

3. Results and Discussion

3.1. Ligand discover and initial scouting

To generate novel affinity ligands that could specifically bind VSV-G pseudotyped LVs (VSVG-LVs), a non-immune V_HH library was used to specifically enrich VSVG-LVs binders by phage display. After isolation of single clones, a set of V_HH ligands showing binding to VSVG-LVs particles in a surface plasmon resonance (SPR) array was selected for further analysis and re-cloned into to a yeast production strain. Based on yeast expression expression results, a set containing lead candidates was composed for the reactivity analysis.

3.1.1. Binding reactivity using biotin-ligand conjugates

The binding reactivity of the yeast produced V_HH ligands was initially screened by surface plasmon resonance (SPR) array. To study the relative response (RU) of the anti-VSVG-LVs V_HH ligands (ligands A to E), different concentrations of VSVG-LVs were injected in the sensors at four concentrations of each ligand (**Figure 4.1** and **Figure S4.1** to **S4.5**). Additionally, two negative controls, BacuClear and AAVX affinity ligands with

specificity for baculovirus and adeno-associated virus respectively, were included in the SPR assay (**Figure S4.6 and S4.7**).

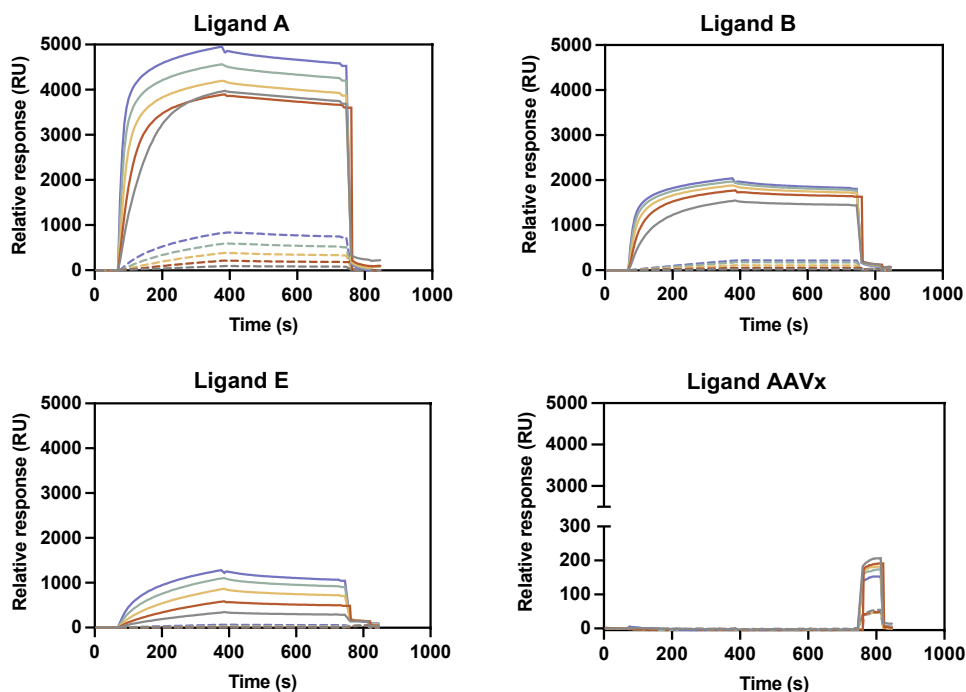


Figure 4.1. LV binding reactivity using biotin ligand conjugates. Representation of the SPR Sensorgrams containing the relative response (RU) versus time (s). The three different interaction profiles observed between the VSVG-LVs and the novel ligands are illustrated by the ligand A, B, E and by the negative control with the AAVX ligand. For each ligand, different binding responses were obtained by varying the dilution of a VSV-G pseudotyped LVs feedstock injected (2-fold dilutions from 10 to 160 times). Color code: blue represents the 10 times dilution, green represents 20 times dilution, yellow represents 40 times dilution, red represents 80 times dilution and gray represents 160 times dilution. Solid lines represent the experiments performed using the maximum biotinylated V_HH fragments concentration (20 μg/mL) spotted onto a Strep G Senseye biosensor chip, whereas dashed lines represent the minimum concentration used (0.02 μg/mL).

The sensorgrams depicted in Figure 1 contain examples of the three different interaction profiles observed between the VSVG-LVs and the novel ligands. All the remaining binding profiles are represented in **Figures S4.1 to S4.7**. In this preliminary screen, the highest binding reactivity was found for the ligands A and D with a value of 5000 RU, followed by the ligands B and C which displayed values around 2000 RU. Regarding ligand E, a moderate binding response was observed, which was 4 times lower when compared with the highest response obtained. For BacuClear and AAVX ligands, no reactivity towards VSVG-LVs particles was observed, supporting the apparent specificity of the generated ligands. According to the SPR results, it was possible to obtain an indication of the most promising candidates that were further evaluated using functional assays.

3.1.2. VSVG-LVs release efficiency using biotin-ligand conjugates

Although ligands should present high affinity towards the viral target, a reversible binding at mild conditions is necessary to establish an efficient purification step for this envelope virus. Thus, the ligand scouting was focused not only on specificity but also on elution efficiency. Different elution buffers were evaluated in this work such as monovalent (sodium chloride) and divalent (calcium chloride) salts and also arginine. Even though the first ones are relatively common, arginine has been used for different applications such as suppressing protein aggregation [45], promoting protein refolding [46], as a stabilizing agent [47] and has also been reported to improve milder elution of antibodies from protein-A affinity resins [48]–[50]. In these experiments, three concentrations for each elution solvent were evaluated using 50 mM Tris as a buffer. This initial assessment was performed at pH 7.5 to avoid viral inactivation. Except for ligand E, in all the remaining ligands

elution efficiency was improved by increasing the elution buffer concentration (**Figure 4.2A, 4.2B, and 4.2C**).

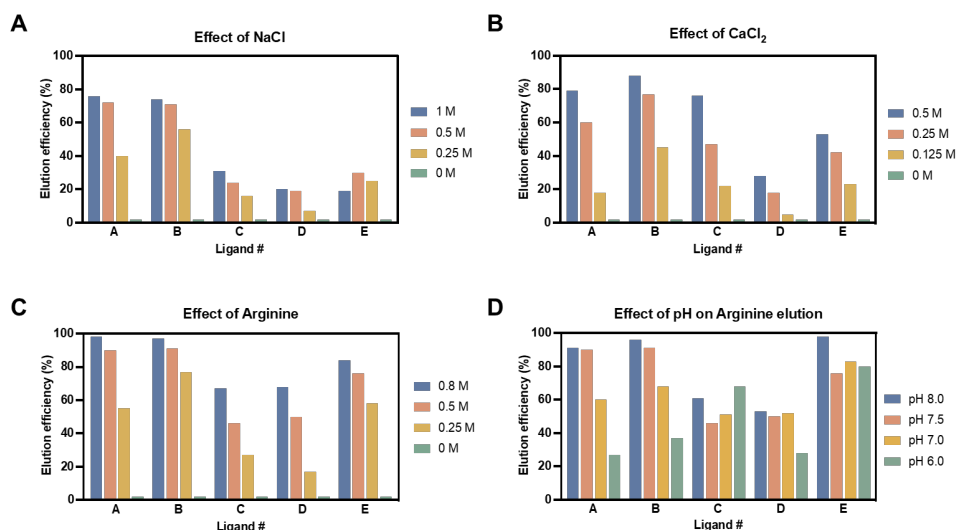


Figure 4.2. LV release efficiency using biotin ligand conjugates. Impact of different desorption buffers sodium chloride (**A**), calcium chloride (**B**) and arginine (**C**) on elution efficiency (%) for the ligand candidates assessed by SPR assay. Three concentrations were included in the buffer elution optimization. (**D**) The influence of the elution pH on elution efficiency (%) was investigated using an intermediate arginine concentration, 0.5 M, at four pH values (8.0, 7.5, 7.0 and 6.0).

The results obtained suggested that release efficiency was ligand-dependent when using sodium chloride (**Figure 4.2A**). For this elution buffer, the highest viral release was obtained for ligands A and B with 1M of salt, where the maximum value of 76 % was achieved for ligand A. However, the same behavior was not observed in ligands C, D, and E, where elution efficiency was lower than 30 % for all conditions. Moreover, the high salt concentration necessary to achieve high elution release was considered a major drawback since it can lead to viral inactivation [51]. Thus, sodium chloride was excluded as an elution buffer in this study. On the other side, calcium chloride (**Figure 4.2B**) and arginine (**Figure 4.2C**) presented similar

tendencies regarding the viral release. Accordingly, since the elution efficiencies obtained for the arginine buffer were higher for all ligands when compared with the calcium chloride, the first one was chosen as the final elution agent. The presence of several charged groups in its structure (positively charged amines and negatively charged C-terminal carboxylic acid) could be the explanation for the good performance of arginine across the different affinity ligands [52]. This strategy has also been applied to other viral vectors: for instance, in the purification of adeno-associated viruses [53].

The effect of the pH on viral release was also investigated for the selected buffer. In this case, the elution efficiency was evaluated using the intermediate arginine concentration at four pH values. **Figure 4.2D** depicts the variation in viral release across the ligand candidates. The results obtained showed that elution efficiency was higher at pH 8 for all ligands except for ligand C where the highest elution value was achieved at pH 6, curiously, the pH value at which worst elution was observed for the other ligands. LVs are sensitive to extreme pH values being its negative impact extensively studied and reported by different authors [14], [54]. Therefore, the selection rationale was to find a compromise between elution efficiency and viral compatibility [18] and so pH 7.5 was the condition selected to proceed for further studies.

3.2. Generation of VSVG-LVs affinity chromatography prototype

3.2.1. VSVG-LVs depletion using streptavidin agarose beads

To assess VSVG-LVs functional recovery yield in a small-scale model, streptavidin agarose beads were functionalized with biotinylated ligands selected by the SPR assays. This model was a versatile tool as it

offered stable immobilization chemistry and high binding capacity. Thus, VSVG-LVs bound - or viral depletion - was estimated by comparing the functional particle's titer in the supernatant before and after capture. Results showed that maximum depletion was observed for ligands B and D with more than 75% viral depletion which confirms the high binding capacity of these ligands. Regarding viral elution, all the ligands presented values below 23%, ligand E being the best performer (**Figure 4.3A**).

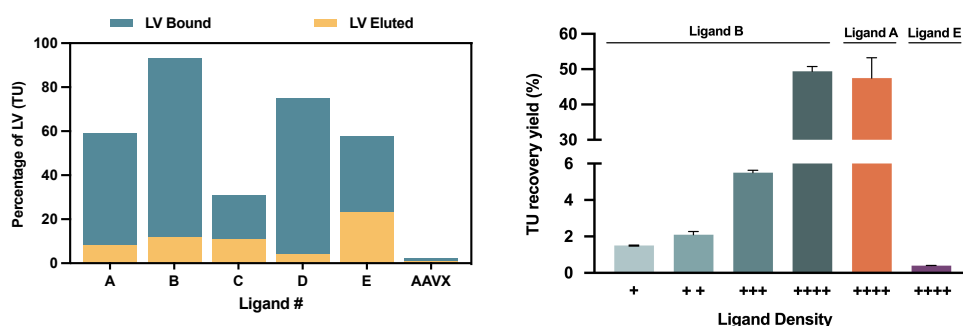


Figure 4.3. Lead affinity ligand candidate selection. (A) Adsorber screening using streptavidin agarose beads functionalized with biotinylated ligands (A to E). Viral depletion (blue bars) and viral elution (yellow bars) comparison between the different ligands. The AAVX ligand was included as a negative control. **(B)** Performance comparison between A, B, and E adsorber prototypes at the highest ligand density (++++). The effect of ligand density was evaluated for the Ligand B as it was shown to be the leading candidate. Ligand densities are ordered from the lowest (+) to the highest (++++). Colour code: the four bars coloured with shades of green correspond to different ligand densities of ligand A, orange correspond to ligand B and purple bar to ligand E.

The low elution yields observed can be explained by the small-scale setting (50 μ L beads) used, and by the fact that the values obtained were near the limit of detection of the FACS equipment. The AAVX resin was also included in this experiment as a negative control with no binding of VSVG-LVs particles being observed thus reinforcing the specificity of the ligands

evaluated. The final three leading candidates were selected based on two criteria: ligand depletion capacity and elution efficiency. Although ligand D presented a viral depletion of 75%, it showed a poor elution yield lower than 4%. This may be caused by a too-strong interaction between the ligand and the viral vector preventing elution at mild conditions [26]. For this reason, ligands A, B, and E were chosen for further evaluation.

3.2.2. Matrix and final ligand evaluation

After selecting the final candidates, it was necessary to decide on an appropriate matrix to assemble the novel affinity prototypes. To be considered an ideal matrix, a few requisites should be met such as homogeneity, stability, residual nonspecific binding, large surface area for ligand attachment, and easy ligand coupling [25], [55]. Therefore, to determine the most suitable resin for functionalization with the VSVG-LVs binding ligands, a set of different matrices were compared (**Table 4.1**).

Table 4.1. Properties of the matrices explored for the establishment of a new affinity resin.

Matrix Type	Coupling method	Selectivity profile	Average particle size (μm)
Matrix 1	Direct	-	50
Matrix 2	Direct	+/-	50
Matrix 3	Direct	+++	65
Matrix 4	Biotin	++	45-165

Except for matrix 4, all the other media presented similar particle size and the same coupling method. Results suggest that matrix 3 was the best performer showing the higher selectivity profile and consequently minimal

nonspecific binding towards the VSVG-LVs particles. Once the matrix was chosen, it was possible to produce adsorber prototypes with the final three ligands. To validate the depletion results obtained using the streptavidin agarose beads, VSVG-LVs capture was determined using packed columns at a 1 mL scale. Since ligand B presented the highest viral depletion, four ligand densities were investigated for this prototype. Regarding the remaining candidates, viral depletion was studied only in the highest ligand density condition (**Figure 4.3B**). Both ligands A and B achieved near 50% recovery yield in the maximum ligand density, with the latter ligand performing slightly better. On the opposite side, the ligand E's performance did not corroborate the previous results showing a residual recovery yield of approximately 1%. Considering all these results, ligand B at the highest ligand density was selected to produce the novel affinity resin prototype that will be referred to as Capture Select Lenti VSVG Affinity Matrix.

3.3. Chromatography optimization for the lead ligand

An affinity chromatography media should offer several prerequisites related to its performance, such as, high binding capacity, high recovery yield, maintenance of product biological activity, high removal of impurities inherent in each system [56]. To discover the full potential of the assembled new matrix and define the best operating conditions, some critical process parameters (CPP) were explored and optimized.

3.3.1. Residence time evaluation

Residence time (RT) usually plays an important role in affinity resins' performance. The optimization of this process parameter can lead to the increase of the dynamic binding capacity and, consequently, process efficiency improvements. In order to assess the influence of residence time on the Lenti VSVG Affinity Matrix, clarified VSVG-LVs were loaded at a linear

velocity of 144 cm/h, 72 cm/h, and 36 cm/h corresponding to residence times of 1, 2, and 4 minutes, respectively. Regarding viral capture, the residence time of 1 minute was the worst condition resulting in the loss of 10 % in the flowthrough fraction combined with the lowest TU recovery yield of 40% after elution (**Figure 4.4A**).

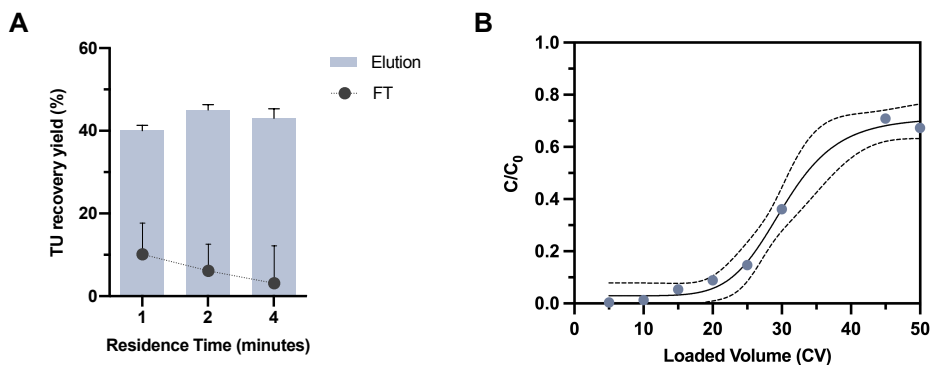


Figure 4.4 Process parameter optimization for the novel affinity adsorber. (A) Comparative VSVG-LV recovery yield results for different residence times (1, 2 and 4 minutes). For each condition, the viral recovery and viral loss was estimated. **(B)** Breakthrough curve representation for the Lenti VSVG Affinity Matrix. The clarified VSVG-LV were loaded at flow velocity of 72 cm/h in a 1 mL packed bed. C is the virus total particles' concentration in the flowthrough samples and C_0 is the virus concentration of feedstock material, the C_0/C ratio is represented by blue dots. Dashed lines represent the 95 % confidence interval. Total particle concentration was estimated by detection of p24 protein.

As expected, the performance of the adsorber improved by increasing the residence time with a maximum recovery of 45% at a residence time of 2 minutes, while the lowest viral loss in the flowthrough was observed for the residence time of 4 minutes. However, at these residence times, no meaningful differences were observed indicating a similar performance of the adsorber for these conditions. In fact, these results are in line with previous

reports where the impact of the residence times on resins' productivity has been studied. Several authors have demonstrated that shorter residence times can result in reduced resin capacity [56]–[58].

To select the most suitable residence time, another parameter must be taken into consideration as it can influence the success of LVs manufacturing: the processing time. One of the most challenging features of this enveloped virus is its short half-life of 5 - 8 hours at 37 °C [14], [59], [60]. This highlights the need to reduce the time of the production process and establish fast, efficient, and simpler purification technologies [1], [61]. Therefore, the 2 minutes residence time was selected aiming to contribute to process time reduction. Notably, the present adsorber exhibited a competitive performance, when compared with existing affinity platforms for viral capture. Most affinity technologies commercially available have been reporting a contact time of 0.5 - 3 minutes, such as POROS™ CaptureSelect™ BacuClear, AAVX, AAV8, and AAV9 [20], [38], [62], [63].

3.3.2. Dynamic binding capacity determination

After determining the residence time that maximized viral recovery and minimized viral loss, the dynamic binding capacity (DBC) of the Lenti VSVG Affinity Matrix was evaluated. A breakthrough curve for the novel matrix was built using a 1 mL packed column, loaded with 50 CV of clarified harvest at 4.0×10^9 TP/mL (**Figure 4.4B**). Samples at different loaded volumes were collected to determine the total particle concentration. A DBC_{10%} of approximately 1.0×10^{11} TP/mL of resin was determined from the breakthrough curve that displayed a typical sigmoid shape. When compared with the 1.3×10^{10} TP/mL binding capacity reported by Segura (2005) et al. [16] for the well-known Heparin affinity resin, the Lenti VSVG Affinity Matrix

adsorber achieved a 10-fold improvement, which can have a positive impact in terms of process scalability. Considering other affinity resins based on CaptureSelect™ technology, it can be stated that the performance achieved is in line with the existing adsorbers. The superior binding capacity found for the different AAVs serotypes resins, which ranged from 10^{13} to 10^{14} genome containing particles/mL of resin [37], may be related to the viral particle size. The size of an AAV vector is approximately 20 nm, whereas for LVs it is six times larger. This can lead to a reduction in the surface area available and result in lower binding capacities [28].

Undeniably, different parameters can influence the capacity of affinity supports. Not only by resin physical properties (e.g. bead and pore size, ligand density, and conformation, surface chemistry) but also by the critical process parameters (e.g. pH, buffer system, temperature, linear flow rate, particle size) [64]–[66]. Additionally, all the inherent impurities associated with each system, for instance, the presence of free VSV-G proteins, can behave as competitors for the affinity ligand and impact resin capacity.

3.3.3. Affinity chromatography performance and scalability evaluation

Considering all the previous results, the performance of the VSVG-LV affinity matrix was assessed. A 1 mL packed column was used to load 25 CV of VSVG-LVs containing supernatant with a residence time of 2 minutes and viral elution was performed in a single isocratic step of 0.8 M arginine at pH 7.5. To determine the LVs recovery yield, the initial sample, flowthrough, and elution fractions were collected and analyzed for total particle concentration and transduction efficiency. Column stripping was also collected and analyzed. A standard chromatographic profile for the LVs purification using the novel affinity adsorber is shown in **Figure 4.5A**.

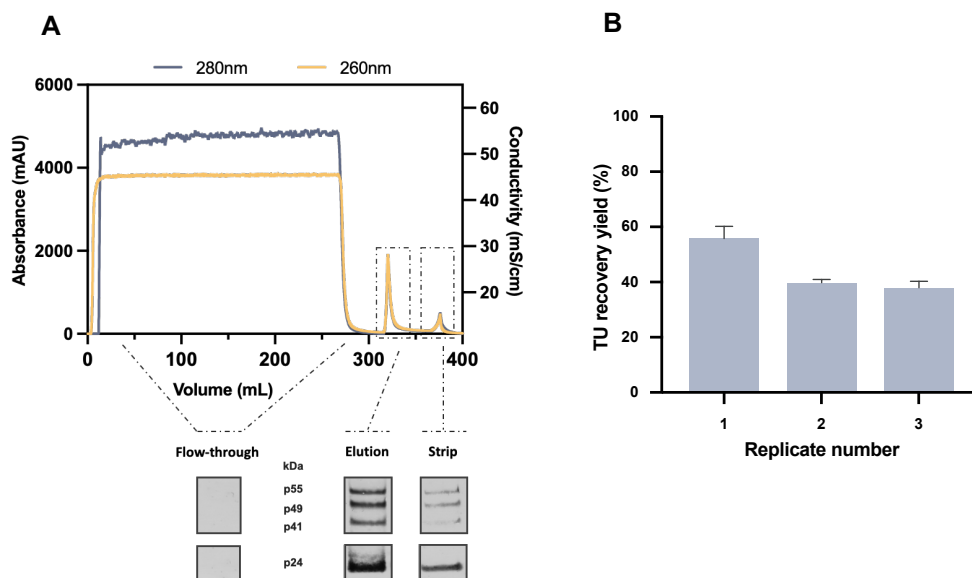


Figure 4.5. Evaluation of a novel Affinity chromatography for lentiviral vector purification. (A) Chromatogram of VSVG-LV purification using Lenti VSVG Affinity Matrix at 1 mL scale and 2 minutes residence time (top) and Western blot signal profile for flowthrough, elution and strip fractions using anti-p24 antibody (bottom). Blue and yellow lines represent absorbance at 280 nm and 260 nm, respectively. (B) Purification performance of the VSVG affinity resin at a 1 mL scale. TU recovery yield (%) is represented for each replicate (n=3). Error bars are the standard deviation obtained from the titration method.

On the bottom, it is represented the Western blot analysis using the Gag p24 protein antibody in all process samples. In the flowthrough, no signal was detected showing that the Lenti VSVG Affinity Matrix could successfully capture the VSVG-LVs particles without resin saturation. In contrast, a strong signal was found in the elution fraction which exhibited the characteristic LVs p24 pattern [67], [68], supporting that an efficient viral elution occurred. For the stripping sample, a subtle signal was detected, although was stronger for the p24 protein. This can indicate that only p24 free protein was eluted after column strip and a viral quality improvement is expected.

To verify the reproducibility of the affinity adsorber, three similar runs at 1 mL scale were performed. Clarified VSVG-LVs at an average concentration of $5.1 \pm 2.5 \times 10^7$ TU/mL were loaded in a total of 25mL, and the results are presented in the **Figure 4.5B**. The elution step retrieved an average of 45 ± 10 % of the loaded virus, while 6 ± 3 % were lost in the flowthrough. These results show the reproducibility and high elution efficiency of this adsorber. Importantly, viral activity was retained demonstrating good compatibility between LVs and mild elution conditions. Additionally, in just one purification step a volumetric concentration factor (VCF) of 6 was achieved together with a 3-fold increase in the TU concentration. After a successful purification at a small scale, it was necessary to evaluate the scalability of the novel affinity resin. Therefore, an intermediate scale up of 10 mL resin was performed. In this case, 250 mL of clarified VSVG-LVs at an average concentration of $7.3 \pm 2.1 \times 10^7$ TU/mL (**Table 4.2**) were loaded, and the linear velocity adjusted to 150 cm/h in order to maintain the residence time at 2 minutes.

Table 4.2. LVs quality assessment for the intermediate scale (10 mL resin) purification process in terms of TP/TU ratio using the novel Lenti VSVG Affinity Matrix. (n=3)

Fraction	Transduction Units (TU/mL)	Volume (mL)	Total particles (TP/mL)	TP/TU ratio	Residual HCP (%)
Load	$7.3 \pm 2.1 \times 10^7$	250	$1.0 \pm 1.5 \times 10^{10}$	143 ± 23	-
Flowthrough	$8.1 \pm 8.9 \times 10^6$	258	$1.7 \pm 6.8 \times 10^9$	270 ± 107	98.5 ± 1.3
Elution	$3.7 \pm 1.5 \times 10^8$	22.5	$3.3 \pm 1.0 \times 10^{10}$	97 ± 40	1.5 ± 1.2

The results obtained showed a slight improvement in comparison with 1 mL scale by displaying an average recovery yield of $54 \pm 4\%$, and only $4 \pm 4\%$ was lost in the flowthrough (**Figure 4.6A**). A 5-fold increase in TU

concentration and a VCF of 12 were observed. These results reinforce the high selectivity, reproducibility, and scalability of this new affinity resin. Over recent decades, different LVs purification methodologies have been reported from AC to AEX. For instance, Segura et al. showed a recovery yield of 53%, although resin scalability was not addressed [16], [35]. The LVs purification using Mustang Q membrane has also been studied, where a cumulative recovery yield of 90% was demonstrated by performing the elution with two NaCl steps [23]. However, LVs eluted in each step presented different purity profiles being the virus eluted with 0.4 M NaCl (comprising 37 % of VSVG-LVs) more contaminated with residual proteins and fractions eluted with 1.5 M NaCl (containing 56 % of the viral vectors) contaminated with DNA.

Additionally, due to the high salt concentration used for viral elution, a dilution step was necessary for viral stability and, eventually, followed by a final concentration and formulation step. Other chromatographic media have also been explored for LVs capture, for instance monoliths or even nanofibers [17], [69]. Recently, the use of steric exclusion using hydrophilic cellulose membranes for the purification of these viruses was reported. Although interesting results have been achieved, a deeper study is necessary to enable the scale-up of these devices. Overall, when compared to some of these technologies, the novel affinity resin showed a competitive performance.

3.4. Quality control and vector characterization

Currently, the number of clinical trials using LVs for regulatory approval is increasing. As a result, clinical production at large-scale, according to current good manufacturing practice (cGMP), is becoming more stringent thus requiring an extensive vector characterization for batch release.

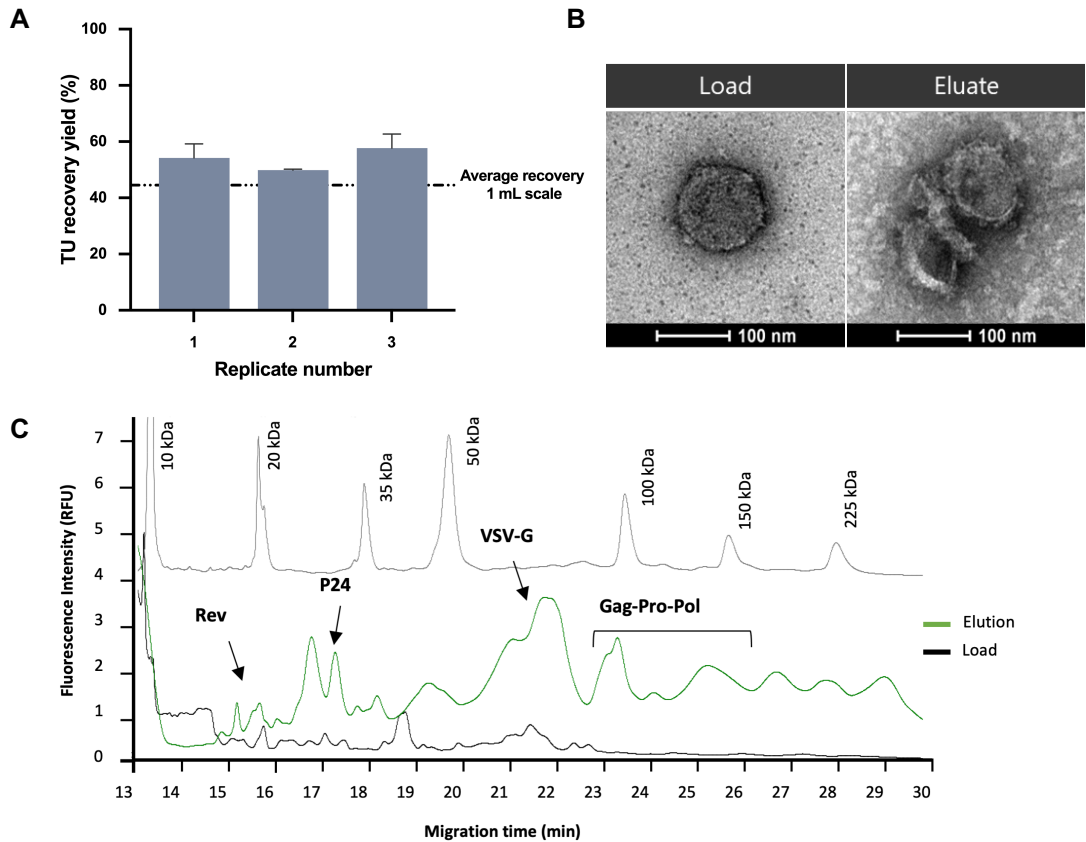


Figure 4.6. Intermediate scale up and characterization of purified VSVG-LV. (A) VSVG-LV purification at 10 mL scale. In the graphic is represented of the TU yield (%) for each replicate (n=3). Dash line shows the average recovery yield obtained from 1 mL scale ($45 \pm 10\%$). Error bars are the standard deviation obtained from the titration method. (B) TEM analysis of VSVG-LV initial sample (left image) and after purification with the Lenti VSVG Affinity Matrix (right image). Scale bar 100 nm. (C) Electropherograms representation of the Molecular Weight marker (gray line), the clarified VSVG-LV sample (black line) and VSVG-LV purified with the Lenti VSVG Affinity Matrix (green line) using CE-SDS with a LIF detector. Major VSVG-LV proteins are indicated.

Consequently, analytical methods have a decisive role during process development since it has been shown that the impurity level can impact the transducibility of γ -retroviral vectors and LVs [61], [70].

Affinity chromatography allows process simplification, high recovery yields and high purity in the final product. To characterize the purity level of purified VSVG-LVs using the novel affinity resin, different analytical techniques were used. A total protein removal higher than 92 % and a total DNA removal higher than 80 % were observed after the chromatographic step, being most of these impurities being washed out in the flowthrough samples. Moreover, the high impurity clearance observed is in agreement with results obtained for different technologies in the LVs purification field [23], [61], [71]. For some cases, the total DNA removal attained in this work was even superior [35], [72]. Another important quality attribute is the capacity of removing host cell proteins (HCP) derived from the producer cell line (HEK293T). An overall clearance of 99 % was achieved, obtaining a total of $1.5 \pm 0.2 \mu\text{g}$ of HCP for a viral dose of 10^9 TU. The novel affinity resin was able to match (e.g., $0.484\text{--}1.631 \mu\text{g}$ of HCP / 10^9 TU) or even decrease (e.g., $5.9 \pm 0.2 \mu\text{g}$ of HCP/ 10^9 TU) HCP content in a single step, when compared to similar viral doses obtained after a full purification process [23], [71].

In addition to impurity clearance, viral quality in terms of the ratio between total particle and transducing units (TP/TU) is also relevant. In **Table 4.2**, a summary of TP/TU ratio progress during the purification process is presented. The results indicate a quality improvement by the reduction of the TP/TU ratio after the elution step. Although no reference value was established in current guidelines, most clinical grade LVs reported higher values around 10^3 [22], [73].

Afterward, viral integrity was investigated by transmission electron microscopy (TEM). TEM analysis confirmed the presence of LVs particles on

the initial and elution fractions (**Figure 4.6B**): both presented a vector size slightly larger than 100 nm. Comparing both images, it was observed that shape and morphology were maintained after the purification step using Lenti VSVG Affinity Matrix. In fact, in the eluate image it was also possible to discriminate the envelope proteins displayed on the virus surface. Undeniably, these results corroborate the good compatibility between LVs particles and the gentle nature of the established elution step. Lastly, capillary electrophoresis (CE) is a promising technique that has demonstrated its potential in the viral analytical field. **Figure 4.6C** illustrates the comparison between the obtained electropherograms of the clarified and affinity purified VSVG-LV. CE-SDS LIF analysis was used to assess the protein profile of the purified VSVG-LVs and it was possible to identify four LV proteins: Rev (19kDa), p24 (24kDa), Gag-Pol-Pro (80-160kDa) and the envelope protein VSV-G (58kDa) [44], [74].

4. Conclusions

Nowadays, there is a fast-growing demand for gene therapy and cell-based products due to their tremendous potential for personalized medicine. Regarding LV-based therapies, downstream processing remains challenging due to the lack of simple and scalable purification trains.

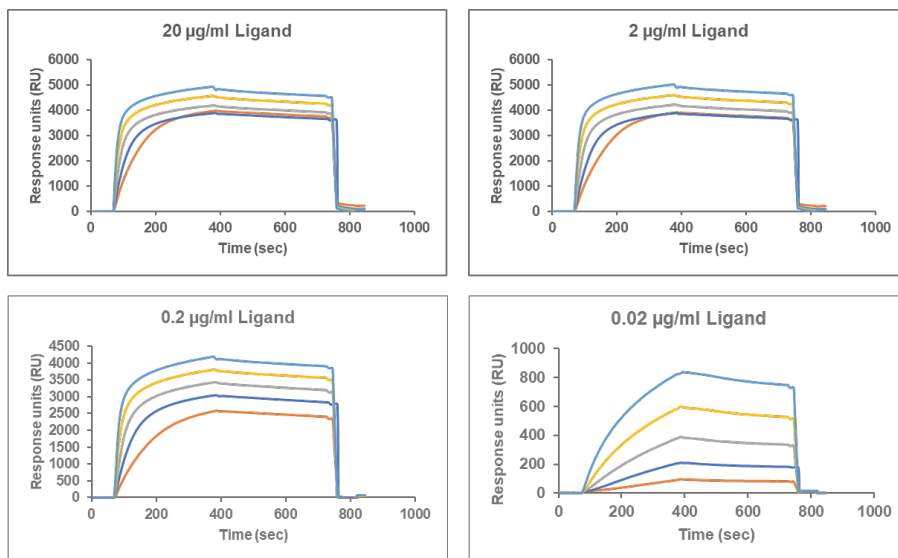
This work describes the discovery and implementation of a novel affinity matrix, based on CaptureSelect™ technology, which enables for the first time the capture of LVs pseudotyped with a VSV-G envelope. The novel adsorber achieved competitive yields and impurity clearance, when compared with the available technologies. The results achieved in this work demonstrate the applicability of this new adsorber which offers high selectivity, high purity, scalability and gentle elution conditions. These features make the Lenti

VSVG Affinity Matrix a reliable alternative to the traditional AEX chromatography. Moreover, the efficient purification and process simplification achieved can decrease time-to-market transition and open new possibilities to address the challenge of large-scale LVs purification.

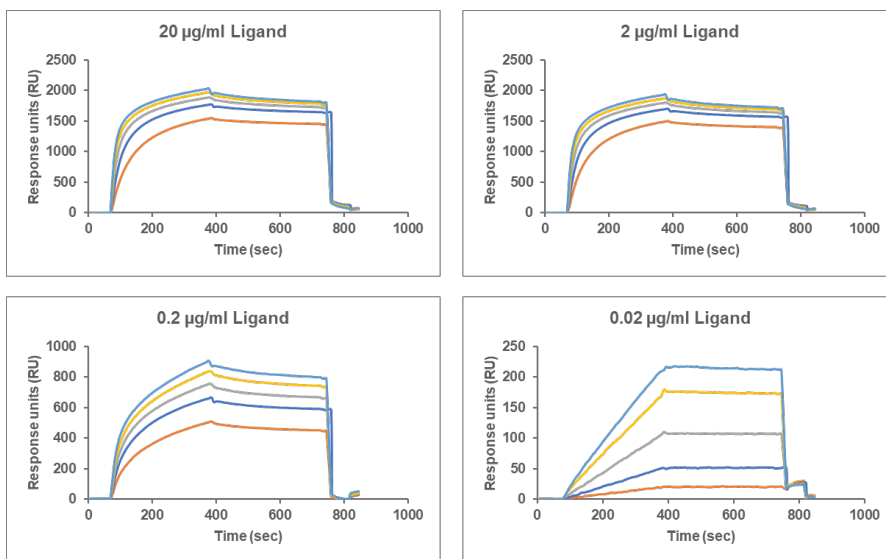
5. Acknowledgements

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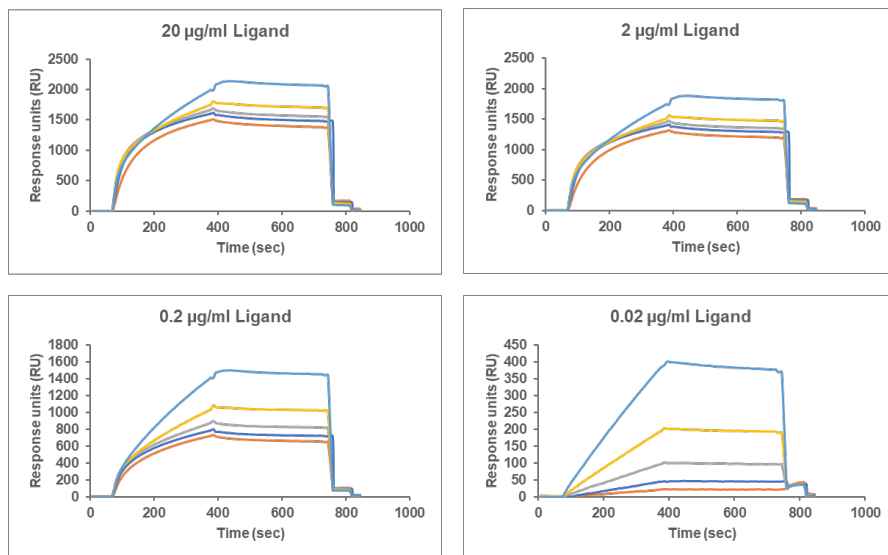
6. Supplementary information



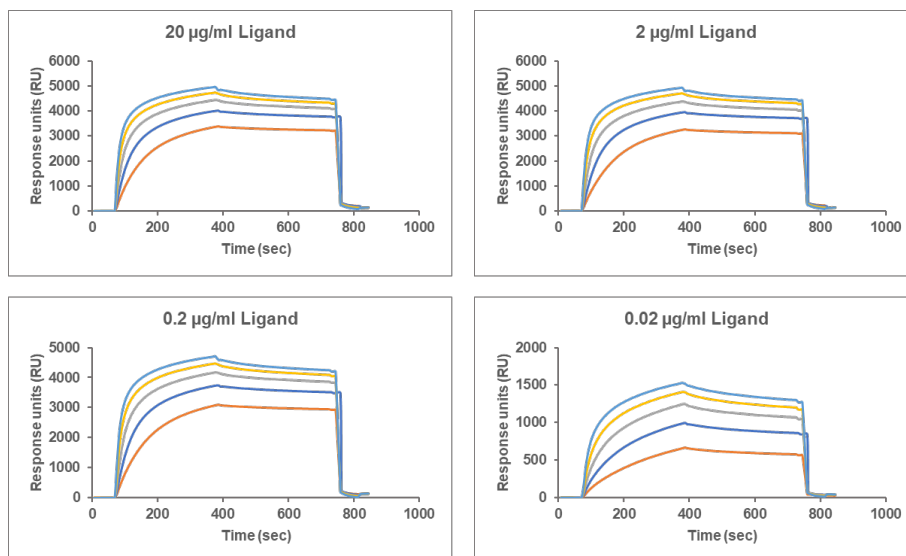
Supplementary Figure S1. Representation of the SPR Sensorgrams containing the relative response (RU) versus time (s) for ligand A. Different binding responses were obtained by varying the dilution of a VSV-G pseudotyped LVs feedstock injected (2- fold dilutions from 10 to 160 times). Colour code: blue represents the 10 times dilution, green represents 20 times dilution, yellow represents 40 times dilution, red represents 80 times dilution and gray represents 160 times dilution.



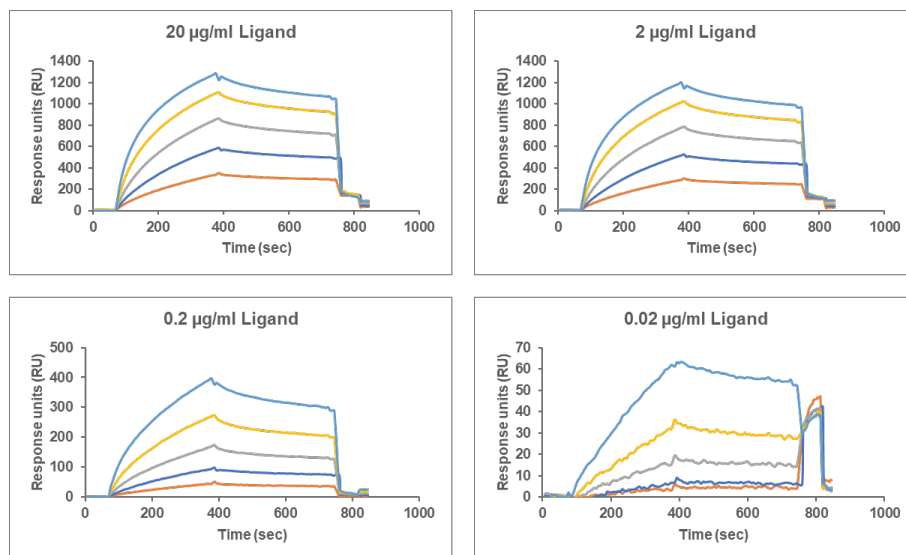
Supplementary Figure S2. Representation of the SPR Sensorgrams containing the relative response (RU) versus time (s) for ligand B. Different binding responses were obtained by varying the dilution of a VSV-G pseudotyped LVs feedstock injected (2- fold dilutions from 10 to 160 times). Colour code: blue represents the 10 times dilution, green represents 20 times dilution, yellow represents 40 times dilution, red represents 80 times dilution and gray represents 160 times dilution.



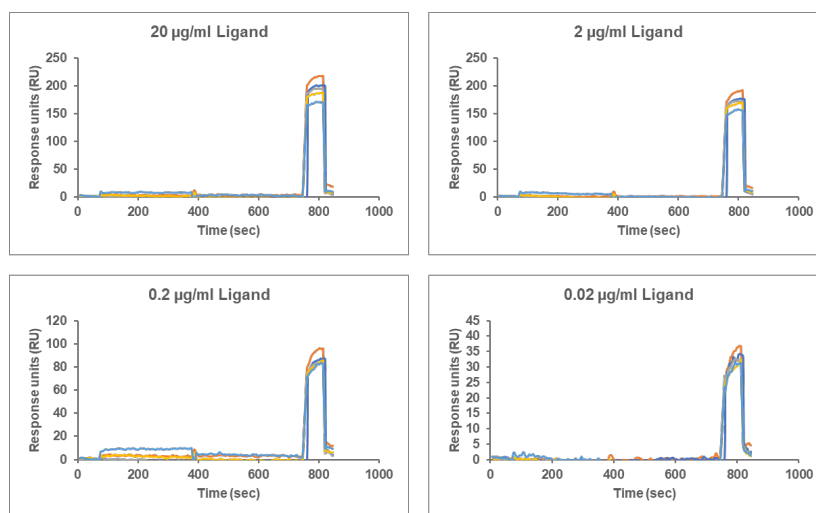
Supplementary Figure S3. Representation of the SPR Sensorgrams containing the relative response (RU) versus time (s) for ligand C. Different binding responses were obtained by varying the dilution of a VSV-G pseudotyped LVs feedstock injected (2- fold dilutions from 10 to 160 times). Colour code: blue represents the 10 times dilution, green represents 20 times dilution, yellow represents 40 times dilution, red represents 80 times dilution and grey represents 160 times dilution.



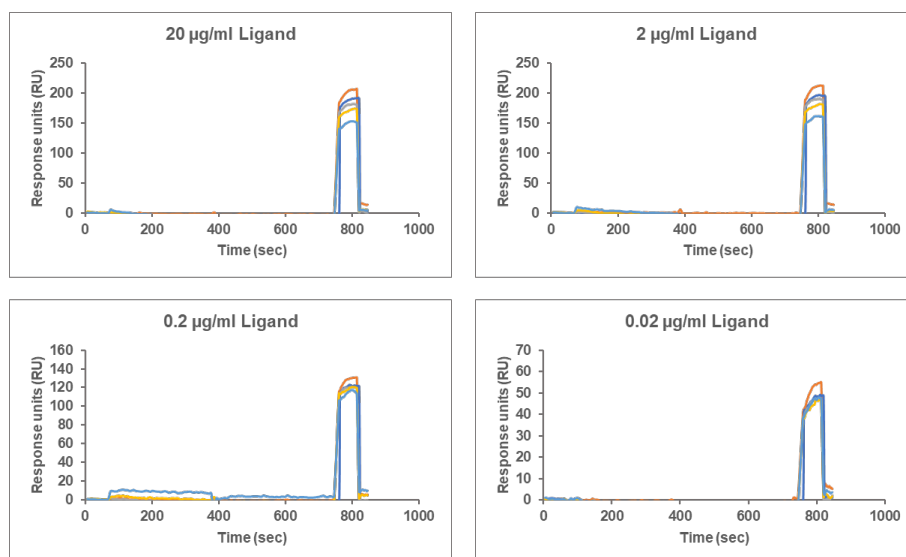
Supplementary Figure S4. Representation of the SPR Sensorgrams containing the relative response (RU) versus time (s) for ligand D. Different binding responses were obtained by varying the dilution of a VSV-G pseudotyped LVs feedstock injected (2- fold dilutions from 10 to 160 times). Colour code: blue represents the 10 times dilution, green represents 20 times dilution, yellow represents 40 times dilution, red represents 80 times dilution and gray represents 160 times dilution.



Supplementary Figure S5. Representation of the SPR Sensorgrams containing the relative response (RU) versus time (s) for ligand E. Different binding responses were obtained by varying the dilution of a VSV-G pseudotyped LVs feedstock injected (2- fold dilutions from 10 to 160 times). Colour code: blue represents the 10 times dilution, green represents 20 times dilution, yellow represents 40 times dilution, red represents 80 times dilution and gray represents 160 times dilution.



Supplementary Figure S6. Representation of the SPR Sensorgrams containing the relative response (RU) versus time (s) for the negative control Bacuclear ligand. Different binding responses were obtained by varying the dilution of a VSV-G pseudotyped LVs feedstock injected (2- fold dilutions from 10 to 160 times). Colour code: blue represents the 10 times dilution, green represents 20 times dilution, yellow represents 40 times dilution, red represents 80 times dilution and gray represents 160 times dilution.



Supplementary Figure S7. Representation of the SPR Sensorgrams containing the relative response (RU) versus time (s) for the negative control AAVx ligand. Different binding responses were obtained by varying the dilution of a VSV-G pseudotyped LVs feedstock injected (2- fold dilutions from 10 to 160 times). Colour code: blue represents the 10 times dilution, green represents 20 times dilution, yellow represents 40 times dilution, red represents 80 times dilution and gray represents 160 times dilution.

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5

Discussion and future perspectives

Author contributions to the chapter:

Ana Sofia Moreira wrote the chapter.

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1. Discussion

Cell and Gene therapy (CGT) is a fast-growing transformative field that offers innovative solutions for unmet needs in the medical field [1], [2]. Advanced Therapy Medicinal Products (ATMPs) can nowadays be an alternative treatment for patients suffering from cancer, inherited or immune diseases, and other therapeutic areas [3]–[5]. CGT can be commonly described as a strategy used to correct abnormalities or induce new traits by transferring genetic material into the patient's cells [6]. Despite the challenging journey that has been encountering several hurdles and adverse effects, after 30 years, CGT is now thriving towards clinical translation with almost 40 products on the market [7]. This success has resulted in a massive investment of more than 20 billion US dollars globally in 2021 [8]–[10].

Viral vectors are one of the most used delivering vehicles in CGT, for both *in vivo* and *ex vivo* modalities [11]. The success of viral vectors as a gene delivery method for cells/tissues has been widely demonstrated [12]. Within the subset of viral vectors that reached clinical use, Lentivirus have come across as an exceptional tool due to their ability to stably integrate the genome of dividing and non-dividing cells and present a payload up to 12kb. Particularly, LV-based therapies have the potential to address cancer and numerous genetic disorders and could benefit between 12-15 million patients worldwide each year [13]. Currently, due to their demonstrated safer integration profile when compared with γ -retroviruses, LVs are the third most used vectors in clinical trials. However, only 7 LV-based products were approved despite the high number of studies submitted annually making imperative the development of enhanced production platforms.

The manufacturing of clinical-grade LVs should be fast, efficient, scalable, cGMP compliant, and cost-effective to support the increasing industry demand. LVs' traditional production platform relies on adherent cell

culture methodologies and transient transfection using multi-tray vessels. The low titers obtained in the harvested feedstock usually lead to extended processing times and low recovery yields [6], [14].

Different LVs purification technologies have been described for both laboratory and industry settings. Most of these processes are based on batch chromatography and membrane technology with overall yields ranging from 20 – 40 % [15]–[17]. LVs are sensitive to most critical process parameters (CPP) contributing to the loss of biological activity during the purification process. Undeniably, significant efforts have been put into the improvement of the manufacturing capacity and in decreasing the associated costs. However, assembling efficient LVs processes at large-scale remains challenging mostly due to the unique features of this viral vector [8], [18], [19].

The work developed herein aimed at optimizing the process workflow and implement alternative strategies to accelerate the LVs bioprocesses. Therefore, we took advantage of previously developed purification schemes to build an enhanced downstream process to alleviate the low titers obtained when using adherent cell cultures. On the other hand, following the industry transition to suspension technologies, a novel clarification step was developed to address the different properties of this new feedstock. Finally, two distinctive chromatographic strategies, AEX and AC, were explored and optimized for the capture of LVs. The main achievements obtained for **Chapters 2 to 4** are summarized in Figure 5.1.

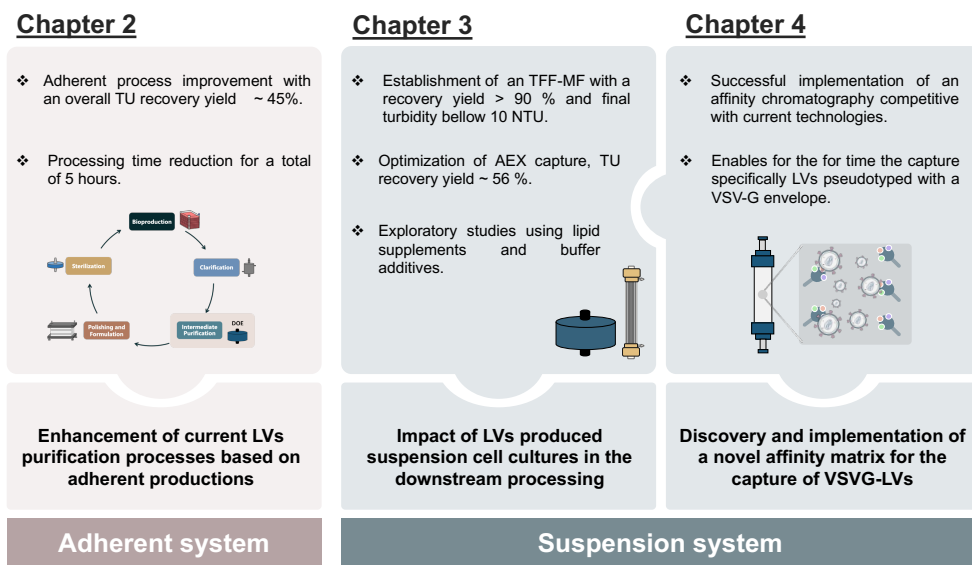


Figure 5.1. Schematic representation of thesis aims and major achievements of each chapter (2-4) towards accelerating downstream processing of LVs. TU – Transducing units; TFF-MF – tangential flow microfiltration

1.1. Process optimization for Lentiviral vectors from adherent productions.

The success of LV-based products reinforced the need for a robust and efficient manufacturing process. Traditionally, these viruses are produced in mammalian cell lines, usually HEK 293 or HEK 293T, in the presence of Fetal Bovine Serum (FBS) and mainly by transient transfection [14], [17]. The industry choice commonly lies on HEK 293T due to the expression of SV40 T antigen that results in higher cell growth and transfection efficiency when compared with the HEK 293 parental cell line [17].

In the past, anchored-dependent cell lines were capable to provide enough viral material to sustain clinical trial development, however, they present clear limitations in terms of process scalability when high dose/high demand is required. Recently, other production technologies were developed for the production of cGMP-grade LVs: i) the use of microcarriers (Fibra-Cel®) in a rock motion bioreactor [20]; ii) a hollow fiber bioreactor (Quantum™) [21] and iii) fixed bed bioreactors (scale-X™ and iCELLis®) [19], [22]. Fixed bed bioreactors are one of the breakthrough technologies in this field over the last few years. They are fully closed systems that offer scalability, a small footprint, and present some advantages for intracellular (facilitating lysis step) and extracellular products (reducing initial turbidity). One example that has already progressed to GMP manufacturing is the iCELLis® technology from Pall Corporation [23]. Therefore, being aware that are still clinical processes that rely on adherent technologies, **Chapter 2** outlines the strategy developed to enhance the efficiency of these manufacturing processes.

The primary goal of downstream processing is to provide high concentration, and high purity material meeting the standard requisites from

the regulatory agencies (FDA and EMEA) at minimal costs [17], [24]. The establishment of efficient LVs processes is heavily dependent on the initial production characteristics and virus intrinsic properties. The purification process usually starts with a clarification step where cells, cell debris, and cellular products are removed [25], [26]. From the manufacturing perspective, to select the best clarification filter is crucial to consider not only filter throughput but also other factors such as economic feasibility and availability of ready-to-use supports with GMP-compliant certification. In **Chapter 2**, filtration technologies were used for this step and several membrane materials were evaluated. Since most of the cells are attached to the multi-tray supports, all filters presented high turbidity reduction and recovery yields superior to 80%. Similarly, to results already reported [27], the PP filter was identified as the best performer and a capacity of 2150 L/m² was observed with no turbidity dependence.

The intermediate purification and concentration of the LVs, which might include tangential flow filtration [TFF] or chromatography (e.g., anion exchange chromatography [AEX], affinity chromatography [AC]) operations, are critical units due to their sensitivity to high conductivities and high shear rates. Not only several materials can be chosen for AEX and TFF but also their sequence can vary. In this work, our strategy was to directly load the clarified material onto the AEX membrane avoiding an initial concentration step that can be time-consuming. DoE was used to maximize viral recovery by optimizing the AEX membrane, viral load, ionic strength, and elution pH. Regarding the LV elution profile, the results obtained showed an elution of 37 % and 56 % of TU in the 0.4M NaCl and 1.5M NaCl steps. Higher ionic strength led to higher recovery yields for Mustang Q as was observed in different studies [28]–[31]. Since most of the proteins were washed out in the flowthrough fraction (99 %) and co-eluted in the 0.4M fraction (1 %), only the virus eluted at 1.5M NaCl were used for further processing to increase the

purity of the final product. Furthermore, the results obtained were confirmed by a process scale-up of 4 L. The benefits of an appropriate viral formulation (e.g., stability or shear protection) have been demonstrated by different authors [19], [32]–[34]. For that reason, the impact of sucrose addition earlier in the process, during the chromatography instead of in the TFF step, was studied. Despite the absence of significant differences in LVs recovery, we decided to maintain this early formulation in the final downstream process due to its potential benefits for the subsequent steps.

Concerning the TFF step, hollow fibers and cassettes with different cut-offs (100 kDa, 300kDa, and 750 kDa) were explored as tools for viral concentration. The results obtained showed recovery yields of around 75 % regardless of the device used which are in agreement with the values reported in the literature [19], [35], [36]. Depending on the envelope protein of choice, the establishment of an efficient concentration step can be more challenging. Different authors have reported higher resistance to physical damage for VSV-G pseudotyped LVs when compared with GaLV-TR envelope [37]–[39]. A distinct aspect to consider is the therapeutic modality (e.g., *ex vivo* application or *in vivo* administration). The viral dose required for each medical disorder will dictate the final concentration factor needed in the concentration step. This observation emphasizes that LV purification is not a one-fits-all process and may require slight adjustments based on the industry demand. Viral sterilization has been identified as a challenging stage in the LVs bioprocessing, with potential viral losses of up to 50 % [14], [19], [40]. Ensuring the aseptic conditions of the final sample is crucial to meet the regulatory guidelines. As an alternative to avoid the final sterilization, some authors have decided to sterilized LVs before TFF and perform the sample concentration in an aseptic configuration [40]. Remarkably, we were able to establish a sterilizing filtration with a recovery yield higher than 85 %. Overall, the final optimized process accomplished in this thesis is composed of four

operation units allowing a total processing time of 5 h with a global recovery yield of 45 %. The implementation of such strategies could have a positive impact on the number of clinical batches per year and consequently, increase manufacturing efficiency.

1.2. Impact of adherent to suspension transition on downstream process.

Adherent cell lines have been the gold standard for LVs production. However, the use of adherent cultures comes with several challenges starting from the scale-up to regulatory compliance concerns. The majority of anchored-dependent cell lines required serum supplementation. Although the presence of albumin and lipid sources contributes to viral stabilization [41], adding serum to the production media can increase the risk of contamination with adventitious agents and batch-to-batch variability, contributing to driving material cost up and being dependent on market availability [42], [43]. Additionally, all these culture-medium derived impurities must be eliminated during the purification process. Therefore, there is an imperative drive to serum-free platforms. The biopharmaceutical industry is moving towards suspension systems and several advantages can be found by comparing them with their adherent counterparts. The transition to suspension cell lines offers in-line monitoring (e.g., pH, pO₂, pCO₂); is less time-consuming; eases scale-up; and allows new modes of operation (e.g., perfusion) [44]. As already stated, LVs are being produced mainly using HEK 293 and HEK 293T cell lines which can be easily adapted to suspension conditions [45]. However, this might not be a smooth transition since an impact on viral productivity, 10⁶ TU/mL or even below, was observed in the first studies reported [46]. In recent years, several HEK 293 variants have

been adapted to grow in suspension conditions envisioning LVs manufacturing. Efforts on refining the existing cell media and supplements to improve and create new chemically defined media have also been made [41], [47], [48]. To this date, viral titers reported for LVs production in serum-free media are already comparable with the traditional production methods ranging from 10^7 TU/mL to 10^8 TU/mL [49], [50]. Given all these advances, we also implemented a serum-free production using an in-house adapted HEK 293T cell line.

After establishing the production platform, it was necessary to reassess how we approached the clarification step. Filtration technologies are frequently used in the clarification units being known for their efficiency, scalability, disposable options available and to enable simplified cleaning and validation procedures [45], [51]. Several filtration materials and/or configurations are frequently applied on LVs clarification, although for smaller scales low-speed centrifugation has also been used [52]. There are two filtration modalities: tangential flow filtration (TFF) and normal flow filtration (NFF) which includes depth, surface, or membrane filters. To avoid pore-clogging, a normal flow filter cascade can be designed where the membrane pore size will progressively decrease until $0.45\ \mu\text{m}$ to avoid loss of viral activity or envelope damage [53], [54]. Following the industry trends of continuous processing and process intensification, we decided to implement a tangential flow microfiltration step. In this way, the process integration between upstream and downstream might be facilitated as TFF devices allow cell retention and perfusion modes. Hence, the first challenge addressed in **Chapter 3** was the implementation of an alternative clarification step. As previously mentioned, this initial step is heavily dependent on the initial production characteristics, thus it was necessary to adapt this unit to cope with the increased feedstock turbidity. A few studies using the Tangential Flow Depth Filtration™ (TFDF) for LVs production and harvest

have been published in recent years [55], [56]. In this work, we aimed to evaluate different commercially available TFF devices that could be applied to LVs manufacturing. TU recovery yield, membrane throughput, and turbidity reduction were the parameters used to compare the performance of each device. In our strategy, we wanted to maintain not only the viral biological activity, but also the cells integrity to facilitate process integration and allow flexibility between different operation modes. For that reason, all experiments were performed at 3000 s^{-1} shear rate that demonstrated to be compatible with both parameters. Regarding device performance, BiOptimal™ MF-SL achieved 90 % TU viral recovery and final turbidity on the clarified stock below 10 NTU. Preliminary experiments showed an average flux of 162 L/h/m^2 and no pressure increment was observed with the increase in the filtrate throughput. Thus, to assess the full capacity of this microfiltration device, the V_{MAX} was determined which allows us to perform a scale-up prediction. The results revealed a maximum throughput of 300 L/m^2 and the establishment of a clarification step that might be capable of processing 1000 L of LVs feedstock in less than one hour. The integrity of the viral particle was also confirmed by TEM, supporting the use of this device for sensitive particles such as LVs. To be continued, still missing the discussion with AEX membranes and suspension LVs.

In the second part of this chapter, the main goal was to understand the impact suspension cell cultures transition in the capture of LVs by AEX. The adoption of a new expression system can affect the chromatographic step since the level of virus impurities (nucleic acids and proteins) might change. This can impact virus-membrane interactions and potentially reduce the capacity of these devices. Additionally, as mentioned before, the binding mechanism of AEX depends heavily on charge. Therefore, any viral envelope modifications that outcome from the depletion of animal serum can affect the process efficiency [14], [47], [52]. Thus, LVs were produced by

transient transfection in both culture systems (adherent and suspension) and their purification was compared. The results obtained showed a decrease by half in the overall TU recovery yield for LVs produced in suspension, as well as a modification of the elution profile. To overcome the reduced recovery yield, two strategies were proposed. The use of serum-free production processes can change the cell media composition, influencing cell metabolism and the production of viral-specific components [41], [57], [58]. Particularly, changes in lipid and cholesterol levels on the cell membrane could affect lipid bilayer and lead to loss of viral infectivity [58]–[60]. Therefore, two lipid supplements were explored in this study. Results showed that lipid supplementation has impact on viral elution profile. One of the studied supplements presented a 2-fold increase in the fraction 0.4 M of NaCl resulting in an increase of the overall yields. This finding might suggest that lipid supplementation leads to more resistant LVs and able to cope with the purification process. Lastly, the addition of sucrose to the buffer system was evaluated during the LV chromatographic step. LVs sensitivity to most CPP emphasize the importance of ensuring an appropriate viral formulation to accomplish an efficient purification process [19], [33], [61]. In this study two conditions were considered 0 % and 17 % of sucrose. The highest sucrose concentration showed a 2-fold increase, which might suggest a beneficial effect, however further studies must be carried out to confirm this preliminary result. Finally, a last purification run was performed using a single elution step of 1.5 M of NaCl. A TU recovery yield of 56 % was obtained, with neglectable loss in the FT fraction. Adopting a new expression system can result in the increase of cell-based impurities, (e.g., host cell proteins), thus adding new challenges to the suspension LVs DSP. Hence, the impurity clearance of this modified purification scheme was addressed. A total protein removal higher than 95 % and a DNA removal of approximately 90 % was achieved. Moreover, the quantification of HCP showed the presence of $5.5 \pm 0.82 \mu\text{g}$ of HCP for a viral dose of 10^9 TU. The high impurity reduction

achieved is consistent with findings from other purification techniques [19], [62], [63]. As discussed in Chapter 3, it was reasonable to anticipate a potential increase in HCP levels of the final sample. However, remarkably, even with only two purification steps, the attained HCP content was comparable to the four-step process reported in Chapter 2. Moving forward, it is imperative to undertake a more comprehensive and rigorous characterization, as well as a comparative analysis of the LVs generated in both production systems. This investigation can significantly contribute to understand the mechanisms responsible for the modifications observed in the elution profile during the chromatography step.

The results in this chapter demonstrate the potential for large-scale LVs purification, and some insights were gained in the field of viral stabilization to be applied on this modified production system.

1.3. Establishment of an affinity chromatography for VSV-G pseudotyped LVs.

Chromatography is a widely used and versatile technique that enables the separation of complex mixtures, making it particularly valuable for purifying complex macromolecules and viral vectors on a large scale [51], [64]. As mentioned before, AEX is a commonly employed chromatographic method for purifying large particles. However, high salt concentrations might be needed for viral elution requiring immediate dilution to maintain viral activity [52], [65]. The binding mechanism of AEX relies on electrostatic interactions, consequently, there is no specificity towards the particle of interest as the adsorption of impurities also occurs (e.g., nucleic acids and proteins) and are after co-eluted with the viral particles. The hurdles enumerated underline the necessity of developing new alternatives.

Affinity chromatography (AC) can be a powerful tool for viral vector purification [66], [67]. This chromatography modality explores highly selective and reversible interactions between the immobilized ligand and the product of interest, offering high purity, high fold concentration, and scalability in a single step [66], [68]. Additionally, it has the potential to reduce the number of unit operations and promoting process standardization, simplification and offer economic benefits [67]. Different strategies are described in the literature for the purification of LVs using affinity chromatography such as: Immobilized metal affinity chromatography (IMAC) using a histidine tag (his-tag) [69]; the expression of biotin onto the LVs surface for streptavidin interaction [18]; LVs labeled with cTag8 where a biotin mimic was genetically encoded and pseudotype-independent [18]; or even applying heparin chromatography [70]. Nevertheless, significant concerns have been raised about the use of harsh desorption agents resulting in viral inactivation (e.g., imidazole or EDTA) or the possible adverse effects in subsequent clinical applications (e.g., proteases, his-tag ligands, metal ions leakage) [65], [71]. Despite the efforts to establish affinity chromatography for LVs capture, the major challenge remains in the ability to elute at mild conditions without compromising the biological activity [66]. **Chapter 4** presented for the first time the establishment of an AC designed to bind specifically VSV-G pseudotyped LVs based in CaptureSelect™ technology. Several ligands have been designed using camelid-derived single-domain antibody fragments (V_{HH}) to be applied on biopharmaceutical products, including different AAV serotypes [68], [72]. Recently, a similar technology was reported for baculovirus removal in insect cells manufacturing process [73]. V_{HH} ligands being small, highly specific, allowing mild elution conditions and free from animal components, can be an option to addressing some of the concerns associated with affinity approaches.

Primarily, a set of ligands were generated by phase display and a comprehensive study based on their specificity and elution efficiency at gentle conditions was performed. To maximize viral recovery, different buffer systems were assessed such as monovalent (sodium chloride) and divalent (calcium chloride) salts and also arginine. As was already observed for other AC operations [74], [75], the use of arginine showed higher and consistent elution efficiencies across the ligands evaluated. When establishing a novel elution step, viral integrity and viral activity must be taken into consideration [52]. For that reason, the effect of pH on viral release was also studied and the final buffer formulation selected. Thus, the performance of the five candidates was screened in terms of the functional recovery yield. In the final evaluation, the top three ligands behaviour at different ligands densities was characterized and the final candidate was selected and referred to as Capture Select Lenti VSVG Affinity Matrix. To characterize the full potential of the new assemble resin several parameters were analysed (residence time and dynamic binding capacity). After the prototype optimization, it was achieved a dynamic binding capacity of 1×10^{11} TU/mL. The small size experiments (1 mL) retrieved an average of 45 % TU recovery yield at 2 minutes of residence time. VSVG-LVs affinity matrix robustness was assessed at 10 mL scale showing a slight improvement with an average recovery yield of 54 % of TU, a 5-fold increase in TU concentration and a VCF of 12. Regarding the impurity profile, the HCP content obtained in a single step was similar to the reported values after a full purification process [62], [76]. Considering the existing technologies, the new generated affinity resin demonstrated to be a competitive support for LVs bioprocessing, providing high recovery yields, high purity, and allowing mild elution conditions compatible with LVs labile nature. As next steps, it would be interesting to apply this matrix for LVs produced in stable cell lines. Additionally, since this work was developed using a serum-free production system, the performance of the LVs produced in serum containing cultures

should be addressed. Another crucial aspect that must be assessed is the lifetime of the resin. It is fundamental that a resin could be repeatedly re-used in downstream purification processes of biologics without experiencing a decline in its capacity. Multiple factors can contribute to the deterioration of the resin lifetime with repeated usage. Impurities and residual products have the potential to persistently adhere to the resin matrix, impeding access to the surface ligands.

The establishment of the first affinity support for the capture of VSV-G pseudotyped LVs constitutes an important milestone for LVs manufacturing field. Not only competitive performance was achieved, as well as improvements on the viral quality were observed. Nevertheless, some concerns can be raised regarding ligand release and consequently their impact on viral infection. More specifically, the camelid heavy-chain-only antibodies (V_{HH}) are robust and compact structures that provide high specificity and high-affinity binding. Due to their high stability, they are compatible with extreme conditions often used during chromatographic operation. The CaptureSelect technology has been used for more than 20 affinity resins suitable for bioprocessing. In general, low ppm levels of leaching (5-20 ng ligand/mg product) have been reported in the elution fraction of the resin. This is comparable with, for instance, ProteinA products, which are the gold standard in monoclonal antibody purification for clinical applications. Since the ligand, chemistry, and base bead are the same for the CaptureSelect Lenti VSVG affinity matrix, it is hypothesized that comparable leaching data for this product is expected. Particularly, elution of VSVG-LVs was performed under mild conditions (50 mM HEPES, 150 mM NaCl, 800 mM Arginine, pH 7.5) and taking into consideration the high stability nature of this ligand and the strong biotin- V_{HH} conjugates binding to streptavidin agarose beads, ligand leaching is negligible. Additionally, to the

extent of our knowledge, the infection impairment by V_HH ligand-derived leaching has never been reported.

With the raising of new clinically relevant envelopes that are even more unstable, the investment of new affinity resins can be a real alternative for the purification of these labile particles.

1.4. Conclusion and future perspectives

Over the past decade, lentiviral vectors have become a fundamental tool for gene and cell therapy, explaining the increasing number of clinical trials which use these viruses. The approval of Kymriah™ [77] and Yescarta™ [78] cell therapeutic products have reinforced the urgent need for simple, fast, and robust manufacturing processes of viral vectors. In recent years, this field has witnessed a tremendous effort to develop and optimize large scale production and purification processes capable of meeting the demanding number of viral doses necessary for clinical use.

In this thesis, the strengths and limitations of each downstream operation unit were highlighted. For chromatography, the use of convective driven devices has been shown to be effective in LVs purification schemes [19], [62] and new stationary phases are emerging with very promising results [79]. Additionally, affinity chromatography using tailor-made ligands might prove valuable due to its high selectivity allowing to decrease the number of purification steps and increasing recovery yields [46], [80].

LVs purification processes have evolved significantly; however, recovery yields remain far from their maximal potential, and the amount of virus required cannot be efficiently obtained using these with current technologies. The transition to new production systems may overcome these hurdles but can introduce different challenges into the downstream processing of LV

[81], [82]. The increase of the production titer will of course benefit the purification train, but the shift for a suspension system will increase the cell mass during the clarification step. Therefore, the DSP has to engage with those challenges to achieve the same high-quality viral stocks [83]. The impurity profile will also change and an increase in process-related impurities will be observed (e.g. genomic DNA). Process development heavily relies on analytical tools, which are crucial for monitoring both upstream and downstream processes. Most of this expression systems are known to co-produced extracellular vehicles (EVs) which present similar sizes and properties making it a challenge to efficiently separate EVs from LVs [84], [85]. Thus, there is a need to build new and improved to cope with these complex systems [84].

The biopharma industry is now moving to new operation modes trying to reach fully continuous and integrated bioprocesses [81]. These improvements are very appealing because they allow high purity and productivity, reducing the overall costs [46], [80]. Much has been done to improve these continuous processes, more specifically in continuous chromatography for other viral particles such as adenovirus [86], influenza virus [87], adeno-associated virus [88] and extracellular vesicles [89]. However, the production of LVs using continuous processes may be hampered due to cytotoxicity, particularly for VSV-G pseudotyped vectors. Until a robust LVs manufacturing process is established, the transition to continuous production and purification will be difficult to apply to a larger scale but research at lab scale is already committed to these new processing operation modes [23], [47], [56], [90].

Notwithstanding the advantages of VSV-G regarding its stability throughout the purification train, in recent years, production and purification schemes for other envelope proteins are being published. Those proteins are derived, for instance, from BaEV (baboon endogenous virus) [49], RD114

(an endogenous feline virus) [79], JSRV (jaagsiekte sheep retrovirus) [91] or GaLV (gibbon ape leukemia virus) [32]. Although still a relatively incipient trend, it might have several reasons: on the one hand, the use of constitutive packaging cell lines implies the use of other pseudotypes than VSV-G, due to its cytotoxicity to producer cells [17]; on the other hand, there are safety concerns regarding insertional mutagenesis. The use of GaLV [92], [93] or BaEV [94], given its high tropism against specific cells, makes possible the reduction of the multiplicity of infection (MOI, i.e. the number of vector copies per cell), which reduces the tumorigenic potential alongside with cytotoxicity to target cells [95]. Additionally, for in vivo delivery, VSV-G LV is inactivated by human serum [96], [97] and its broad tropism may imply the transduction of unwanted cells [98].

Some future directions were identified, and the progress made in the manufacturing of LVs was described. Some success stories have already been reported and novel applications and technological developments are thriving. The trend is likely to continue, as several groups and companies are committed to investing more funding and time to push lentiviral vectors into broader clinical use.

2. References

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Accelerating Lentivirus Manufacturing: An engineering-driven approach

Ana Sofia da Costa Moreira

