# Downstream process development of novel valuable bio-therapeutics

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Dissertação para obtenção de grau de mestre em Biotecnologia Farmacêutica. Este trabalho foi desenvolvido no Instituto de Biologia Experimental e Tecnológica (IBET) sob orientação científica da Dra. Cristina Peixoto Lisboa (Dowstream Process Laboratory) e do Dr. João Nuno Moreira (Faculdade de Farmácia).

**On the cover – Representative illustration of mucin detection and quantification (top); and implementation of hepatitis C virus purification strategy (bottom).**

"Far and away the best prize that life offers is the chance to work hard at work worth doing".

**Theodore Roosevelt**

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## Abbreviations

AAL – *Aleuria aurantia* lectin

AB – Alcian blue

AEX – Anion exchange

Asn – Asparagine

BCA – Bicinchoninic acid

BEVS – Baculovirus expression vector system

BLI – Biolayer Interferometry

BSA – Bovine serum albumine

BSM – Bovin submaxillary gland mucin

BV – Baculovirus

CF – Cystic fibrosis

COPD – Chronic obstructive pulmonary disease

CV – Column volumes

D – Dimerization

DNA – Deoxyribonucleic acid

dsDNA – Double strand DNA

DSP – Downstream processing

EGF – Epidermal growth factor

ELISA – Enzyme-linked immunoabsorbent assay

EMA – European Medicines Agency

FDA – Food and Drug Administration

FT – Flow-through

Fuc – Fucose

GalNAc – N-acetyl-D-galactosamine

GLC – Glucose

GlcNAc – N-acetylglucosamine

GLN – Glutamine

GLU – Glutamate

GNA – Snowdrop lectin

HBV – Hepatitis B virus

HCV – Hepatitis C virus

HCPs – Host cell proteins

HCV-LPs – Hepatitis C virus-like particles

HID – High iron diamine

hpi – Hours post-infection

HPLC – High performance liquid chromatography

HPV – Human papillomavirus

HRP – Horseradish peroxidase

IC – Insect cell

IEX – Ion exchange

LAC – Lactate

LOD – Limit of detection

LOQ – Limit of quantification

MAL – *Maackia amurensis* lectin

MCC – Mucociliary clearance

PNA – Peanut agglutinin

PTM – Post-translational modification

PTS – Proline, threonine and serine

PVDF – Polyvinylidene fluoride

q-PCR – Real-time quantitative polymerase chain reaction

RFC – Radial flow chromatography

RNA – Ribonucleic acid

RT-PCR – Real-time PCR

SAX – High precision streptavidin

SEA – Sperm protein, enterokinase and agrin

SEC – Size exclusion cromatography

Ser – Serine

SDS – Sodium dodecyl sulfate

SDS-PAGE – Sodium dodecyl sulfate polyacrylamid gel electrophoresis

SNA – *Sambucus nigra* agglutinin

SUVs – Subunit vaccines

Thr – Threonine

TMP – Transmembrane pressure

UV – Ultraviolet

VLPs – Virus-like particles

VBBs – Virus-based biopharmaceuticals

VNTR – Variable number of tandem repeats

WGA – Wheat germ agglutinin

## Abstract

The development of novel recombinant Virus-like particles (VLPS) has been putting the spotlight in vaccine's biomanufacturing. However, establishing purification and quantification methods for complex therapeutic products with a short time to market represents an engineering challenge.

The first aim of this work was to use BLI technology to improve mucin detection and quantification in a fast, simple and label free way. The experiments were performed using streptavidin (SAX) biosensors that binds biotinylated lectins, which can recognize mucins carbohydrate structures. Two different mucins were detected and quantified: Bovine Submaxillary Gland Mucin (BSM) and human MUC5B mucin. Different assay conditions were optimized and the most suitable lectin, *Aleuria aurantia* lectin (AAL), was selected. Additionally, competition assay allowed the identification of the minimal concentration to inhibit AAL-mucin association. These results can be applied for future mucin purification improvement, for example, in affinity chromatography.

The second part of this work reports the development and implementation of a flow-through purification strategy for Hepatitis C virus-like particles, using anion exchange chromatography. Exploratory evaluations were performed using scale-down tools, in one mL scout column. The results obtained allowed the translation to radial flow chromatography, an alternative to standard axial packed columns. The global recovery yield of the developed strategy was 66%. Impurity clearance efficiencies with a log reduction value of 2 and 1.4 for baculovirus and DNA, were achieved.

Overall, valuable tools were reported and can be used to improved and optimized new and already established purification processes.



**Keywords:** BLI technology, mucins, enveloped virus-like particles, downstream processing

## Resumo

O desenvolvimento de novas partículas recombinantes semelhante a vírus (VLPs) tem colocado grande foco na produção de vacinas. Contudo, implementar métodos de purificação e quantificação para produtos terapêuticos complexos, de forma a entrarem rapidamente no mercado, representa um desafio significativo.

O primeiro objetivo deste trabalho foi utilizar a tecnologia de *Bio-layer interferometry* (BLI) de modo a melhorar a deteção e quantificação de mucinas, de uma forma rápida, simples e sem recorrer ao uso de marcadores. O método foi implementado com recuso a biossensores de streptavidina (SAX), com afinidade para lectinas biotiniladas, que são capazes de reconhecer estruturas de hidratos de carbono existentes nas mucinas. Foram avaliadas, deteção e quantificação, duas mucinas distintas: a mucina da glândula submaxiliar bovina (BSM) e a mucina humana MUC5B. Depois de otimizadas diferentes condições foi selecionada a lectina mais adequada, *Aleuria aurantia* lectina (AAL). Adicionalmente, ensaios de competição permitiram a identificação da concentração mínima para inibir a associação AAL-mucina. Estes resultados podem ser aplicados futuramente, nomeadamente para otimizar a purificação de mucinas, por exemplo, em cromatografia de afinidade.

A segunda parte deste trabalho descreve o desenvolvimento e implementação de uma estratégia de purificação por cromatografia em modo negativo para VLPs de Hepatite C, recorrendo a cromatografia de troca aniónica. Foram realizadas avaliações com o uso de materiais para escalas laboratoriais, em colunas de um mL. Os resultados obtidos permitiram a transição para cromatografia de fluxo radial, que representa uma alternativa às colunas tradicionais de fluxo axial. A percentagem da recuperação global do método desenvolvido foi de 66%. Quanto à remoção de impurezas foram obtidos *log reduction values* de 2 para baculovírus e 1.4 para DNA.

No geral, foram referidas neste trabalho ferramentas de grande valor que podem ser utilizadas para melhorar e otimizar métodos de purificação novos e já existentes.



**Palavras-chave:** *Bio-Layer technology*, mucinas, partículas semelhantes a vírus envelopadas, processos de purificação

# 1. Introduction

## 1.1 Complex Biopharmaceuticals

Biopharmaceuticals, term first used in the 1980s, refers to large complex biomolecules produced in living organisms using recombinant technology (Walsh, 2007)(Olech, 2016). Also known as biologicals, these "medicinal products" are remodelling therapeutic approaches in many diseases since the first biopharmaceutical approval, recombinant human insulin (Humulin) in 1982 (Johnson, 1982)(Walsh, 2014). In fact, due to the emergence of these new and more specific biomolecules there are numerous candidates, both approved and under development, appearing every year in the biopharmaceutical market (Josefsberg and Buckland, 2012) (Lim and Suh, 2015).

Biologics include molecules with different levels of complexity. Most of these molecules are proteins but can also comprise vaccines, toxins, antitoxins, allergenic products, or other tissue and cellular products (Kanter and Feldman, 2012). In comparison to chemically synthesized small-molecule drugs, biopharmaceuticals manufacturing process is much more complex and could result in unexpected changes to the final product (e.g. absence or different glycosylation patterns) and making their full characterisation extremely challenging (Ebbers *et al.*, 2016). Moreover, modern downstream processes face different challenges with the emergence of these new classes of bioparticles, requiring more robust and scalable techniques to address the desired purity, potency and quality of the final product (Vicente *et al.*, 2011). Thus, the establishment of more demanding guidelines by the regulatory agencies, are imposing the need for process optimization.

Due to all these characteristics and challenges, biologicals present some unique downstream and analytical challenges that are giving rise to a new and more robust purification, quantification, and characterization techniques.

## 1.2 Mucins

The mucosal barrier is a viscoelastic gel that coats nonkeratinized epithelial tissue, which can be found in mammalian organs and is responsible for the epithelium protection from external environment (Ridley *et al.*, 2014). Epithelium is a thin layer of connected cells that covers external and internal surfaces of the body, requiring several defence mechanisms to protect and maintain its integrity (Kufe, 2009).

Considered as first line of defence, mucosal barrier is susceptible to environmental, physiological and immunological stimuli (Hasnain *et al.*, 2013). Comparing different epithelial tissues, numerous differences in composition, properties and functions between their respective secretions (mucus) can be found, showing the complexity of this barrier. As an example, In respiratory tract the mucus layer is capable to trap foreign pathogens or particles. In addition, it is a movable barrier which facilitates the removal of trapped material by ciliary transport or cough (Thornton, Rousseau and McGuckin, 2008).

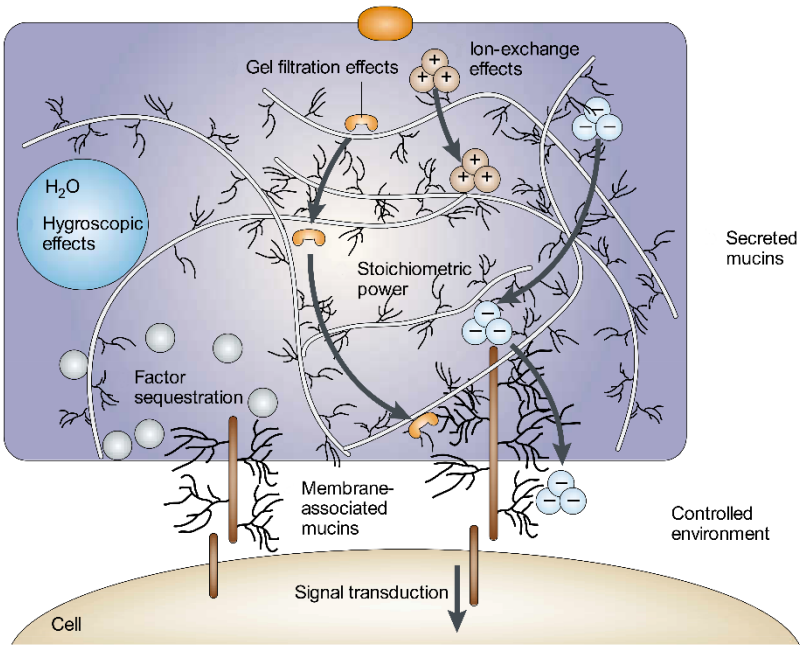


**Figure 1:** Mucins assume the molecular environment control of aerodigestive epithelial-cell surfaces. Membrane-associated mucins and Secreted mucins are two examples of mucins localized in this surface. In this case, mucins play important roles, such as molecular sensors, the secretion of a coating, a gel filtration-like system to small molecules and the binding of important factors.

Despite mobility, hydration and lubrication properties, mucus is a dynamic and complex secretion containing also antibacterial, antiviral and antifungal factors such as defensins, protegrins, cathlecidins, histatins, antibodies, collectins, lysozyme and nitric oxide (Thornton, Rousseau and McGuckin, 2008)(Thornton, 2004). However, the major components of these barriers are the biopolymers known as mucins (Linden *et al.*, 2008).

Mucins are a heterogeneous family of complex high-molecular-weight glycoproteins (0.25 – 50 MDa), produced by all the epithelial cells (Lakshmanan *et al.*, 2015). At least, more than 20 human genes (MUC genes) are known to be involved in their production (Frenkel and Ribbeck, 2015). During the last few decades, mucins have been associated with several important functions like microenvironment regulation, homeostasis maintenance and cell protection on epithelial cell surface. Since, these proteins are subject to countless environment variations (pH, hydration, ion concentration), mucins can easily be exposed to glycosidases, proteases, lipases, acids and toxins. For that reason and due to their particular structure, mucins play an important role on cell survival in these harsh conditions (Hollingsworth and Swanson, 2004).

Besides their structural heterogeneity, high-molecular-weight and the presence of variable number of tandem repeats (VNTR) domains are features that define these glycoproteins. VNTR domains are rich in Proline, Threonine and Serine (PTS or mucin domains) and present high variability in their specific sequence and number of repeats (Brown and Hollingsworth, 2013). Genetic polymorphisms, usually associated to VNTR, are responsible for size variation observed in mucin domains, due to a huge variety of O-glycans that are attached to PTS domains via N-acetyl-D-galactosamine (GalNAc) linkage sugar. The extensive O-glycosylation found in mature mucins results in high carbohydrate mass (up to 80%) and it is promoted by serine and threonine hydroxyl groups (Davies, Wickström and Thornton, 2012). One of the most important functions performed by VNTR domains is the protein scaffold where oligosaccharide structures are produced. These versatile structures can increase the concentration of molecules such as: proteins, carbohydrates, or even glycopeptides, in that specific microenvironment. As a result, mucin-type oligosaccharides can participate in receptor-ligand interactions creating a dense hygroscopic (ability to absorb and retain water (Jang *et al.*, 2015)) and, probably, charged environment. All together contribute to mucus protective barrier function and facilitate selective transport into epithelial tissue (Hollingsworth and Swanson, 2004)(Brown and Hollingsworth, 2013). During the past few decades, several studies have supported mucins clinical significance (Lakshmanan *et al.*, 2015)(Bergstrom *et al.*, 2016). This glycoprotein family has been linked to a variety of

inflammatory diseases and cancer, making it a good candidates to generate potential cancer vaccines and therapies (Kufe, 2009)(Gaidzik, Westerlind and Kunz, 2013)(Kimura *et al.*, 2013).

### 1.2.1 Mucins Family

Regarding their relative location at the epithelium surface, mucins can be classified as: cell-tethered mucins (cell surface, transmembrane mucins) or secreted mucins (Macha *et al.*, 2015). Present at the apical surface of epithelial cells, cell-surface mucins are membrane anchored glycoproteins. They are the most prominent constituent of carbohydrate-rich glycocalyx, which limits the access of cells and huge molecules (Linden *et al.*, 2008)(Hattrup and Gendler, 2008). Typically, they are formed by two distinct subunits sustained by a non-covalent sodium dodecyl sulfate (SDS) bond. The smaller subunit (C-terminal) is constituted by: a short extracellular, a transmembrane and a cytoplasmic tail region. In particular, this last structure plays an important role since it is responsible for coupled signal transduction between the cell and external environment. The most common example of this interaction is how MUC1 is phosphorylated by tyrosine kinase receptors at the cell surface. MUC1 is internalized and consequently binds to promoters that can control patterns responsible for gene expression.

In the other hand, the larger subunit (N-terminal) of transmembrane mucins is constituted by: an extracellular mucin domain that is heavily glycosylated, sperm protein, enterokinase, and agrin (SEA) domain or epidermal growth factor (EGF)-like domain which contains a proteolytic cleavage site (Hattrup and Gendler, 2008)(K, 2011). Taking into account their monomeric structure and membrane protein characteristics, transmembrane mucins do not form gels. Moreover, the extracellular VNTR domains located in glycocalyx allows a wide range of interactions between mucins glycosylated domains and external environment. MUC1, MUC4, MUC16 are examples of membrane cell-tethered mucins (table 1).

Produced mainly by goblet cells or granular mucus cells, secreted mucins are the most prominent constituent of secreted mucus layer and can be divided in two classes: gel-forming and non-gel-forming mucins (Hasnain *et al.*, 2013)(Thornton, Rousseau and McGuckin, 2008). Polymeric mucins or gel-forming mucins present a vast size and mass variation (2-50 MDa). Given their extreme size, pre-formed mucins are stored in secretory vesicles ("mucin granules") until regulated secretion is activated, what results in their release on the cell surface (Davis and Dickey, 2008). Usually, polymeric mucin can be found at the surface of respiratory, gastrointestinal, urinary and reproductive tracts and also in breast and eyes. Generally, these proteins share the follow structure: N- and C- terminal domains, a central PTS domain. This

domain is extremely glycosylated, and it is possible to find cysteine-rich regions that vary in number between mucins. Gel-forming properties of these polymeric mucins results from multiple cross-linking through disulphide bridges, O-glycosylation and N-glycosylation (much less frequent), dimerization, C-mannosylation coupled with other modifications (not so relevant).

Particularly, disulphide bounds arise from N- and C- terminal domains that are enriched with cysteine residues and due to other covalent and non-covalent bonds. Furthermore, mucin gels are also formed by mucin core oligomerization that are originated by D domains. The name of these domains was originated by the similarity between Willebrand factor dimerization (D) domains and dimerization mucin domains.

MUC7, an example of non-gel-forming mucins, belongs to a secreted mucins class with a very different molecular weight range (200-250 kDa). One of the main reasons that explain this weight difference is the lack of cysteine-rich motifs that participate, as referred above, in disulphide bonds assembly. Consequently, non-gel-forming mucins are synthetized as monomers. Although monomeric mucins do not participate directly in mucus gel formation, MUC7 can interact with bacteria since it contains histatin-like-domains which present antifungal activity.

MUC5B human mucin was the gel-forming-mucin analysed during this work. More specifically MUC5A and MUC5B are the major constituents of human respiratory tract, but MUC2 can also be detected. Particularly, MUC5A and MUC5B are responsible for protecting cell surface from infection and to bind microorganisms and particles that are then removed by mucociliary clearance (MCC) (Roy *et al.*, 2014)(Perez-Vilar, Randell and Boucher, 2004). MUC5B has a generic structure for polymeric mucins, as already describe above (Frenkel and Ribbeck, 2015)(Ridley *et al.*, 2016). Therefore, to better understand mucins behaviour, several studies on mucin amount, size and type have been performed, in healthy individuals and patients with respiratory diseases, for a better understanding of mucins behaviour. As an example, in a healthy subject, the mucus secretion plugging the airways is approximately 0.6mg/mL, while in *status asthmaticus* it is about 40 mg/mL (Kirkham *et al.*, 2002). Other results have indicated that one of MUC5B variants predominate in mucus associated with chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) (Thornton, 2004)(Kirkham *et al.*, 2002)(Livraghi-Butrico *et al.*, 2016). This evidence may suggest a connection between MUC5B production with infection/inflammation.

**Table 1**: The family of mucin genes, describing chromosomal location, PTS domain tandem repeat size and tissue distribution (Corfield, 2015).

| Mucin | Chromosome | Tandem repeat size (amino acids) | Main tissue expression |
|---|---|---|---|
| **Secreted mucins:** | | | |
| Gel-forming | | | |
| MUC2 | 11p15.5 | 23 | Jejunum, ileum, colon, endometrium |
| MUC5AC | 11p15.5 | 8 | Respiratory tract, stomach, conjunctiva, endocervix, endometrium |
| MUC5B | 11p15.5 | 29 | Respiratory tract, submandibular glands, endocervix, |
| MUC16 | 11p15.5 | 169 | Stomach, ileum, gall bladder, endocervix, endometrium |
| MUC19 | 12q12 | 19 | Evidence for MUC19 protein not reported |
| Non-gel-forming | | | |
| MUC7 | 4q13-q21 | 23 | Sublingual and submandibular glands |
| MUC8 | 12q24.3 | 13/41 | Respiratory tract, uterus, endocervix, endometrium |
| MUC9 | 1p13 | 15 | Fallopian tubes |
| **Membrane-associated:** | | | |
| MUC1 | 1q21 | 20 | Breast, pancreas, duodenum, ileum, colon, trachea, bronchii, cornea, conjunctiva, fallopian tubes, uterus, endometrium, endocervix, ectocervix, vagina |
| MUC3A/B | 7q22 | 17 | Small intestine, colon, gall bladder |
| MUC4 | 3q29 | 16 | Breast, respiratory tract, small intestine, colon, conjunctiva, cornea, endocervix, ectocervix, vagina, endometrium |
| MUC12 | 7q22 | 28 | Colon, pancreas, prostate, uterus |
| MUC13 | 3q21.2 | 27 | Colon, trachea, prostate, kidney, small intestine |
| MUC15 | 11p14.3 | none | Colon, respiratory tract, small intestine, prostate |
| MUC16 | 19p13.2 | 156 | Ovary, cornea, conjunctiva, respiratory tract, endometrium |
| MUC17 | 7q22 | 59 | Stomach, duodenum, colon |
| MUC20 | 3q29 | 18 | Placenta, colon, respiratory tract, prostate, liver |
| MUC21 | 6p21 | 15 | Respiratory tract, thymus, colon |

This relation can be the key for new and more specific therapies for respiratory diseases. So, it is important to find new tools that allow a better detection and quantification of mucins.

For that purpose, a commercial mucin was also evaluated in this study used, mostly, for assay optimization. Bovin Submaxillary gland Mucin (BSM) is a commercial available mucin that have been used for different experimental goals, for example as biomaterial coating (Sandberg, Blom and Caldwell, 2009). There are diverse studies dedicated to purify and characterize BSM structure and all reported the presence of sialic acid, hexosamine, fucose and galactose (Tettamanti and Pigman, 1968)(Wu, Csako and Herp, 1994) also present in MUC5B.

### 1.2.2 Mucin Glycosylation and analysis challenges

Glycosylation is one of the most important post-translational modifications (PTM) that proteins can undergo. In glycoprotein N- or O- glycosylation can be found depending where the glycans are attached. In N-linked oligosaccharides, the attachment occurs commonly via N-acetylglucosamine (GlcNAc) to an Asparagine (Asn) residue in a consensus sequence Asn-X-Ser/Thr (Jensen, Kolarich and Packer, 2010). In O-glycosylation, there is a higher diversity given that glycans attachment can be made with Ser or Thr via O-linkage to N-acetylgalactosamine (GalNAc), usually found on mucins, or via O-linkage to N-acetylglucosamine, mannose, fucose and other sugars (Brown and Hollingsworth, 2013)(Tarp and Clausen, 2008).

O-glycans constitute about 50-90% of mucins mass and are related to their biological properties such as: protease resistance, water and ions binding or even pathogen trap. Moreover, mucin hygroscopic properties contribute to a high degree of hydration and modulation of mucus visco-elastic characteristics. O-linked oligosaccharides can suffer sialylation and sulfation, resulting in increased volume which can also be relate with mucins network formation. Sulfation can confer, at the same time, chain protection from bacteria glycosidases since it adds a significant negative charge which lead to ionic repulsion or attraction to charged molecules. Sulfated species abundance vary with cell and tissue type, adding even more variation to mucin isoforms.

Although mucins are known to be rich in O-glycans, they can also present N-glycans in their structure (Brown and Hollingsworth, 2013)(Corfield, 2015). N-glycans are found mainly in membrane-associated mucins, such as MUC1, MUC4 and MUC16, and have different patterns. Mucins N-glycans occur typically in cysteine-rich regions and are possibly involved in protein folding. This heterogeneity in structures and site occupancy leads to a challenging

glycoanalysis. Additionally, the nonexistence of a universal enzyme to detach O-glycans from proteins in contrast to N-glycans, makes it even more difficult.

Complementary techniques are used for glycan structure analysis, which include lectin blotting, liquid chromatography and mass spectrometry techniques. Glycoanalysis allows a better understanding about mucins nature and structure. Commonly, mucin detection and quantitation is made using two different approaches: with chemical stains, taking advantage of their glycosylated structure; or using antibodies, specific for known peptide sequences. For chemical detection high iron diamine (HID), alcian blue (AB) and Periodic Acid/Schiff's (PAS) reagent are generally used.

**Table 2:** Lectins, competitive sugars and conditions used in octet assays and lectin blotting analysis.

| Lectin | Name | Organism | Affinity | Salts |
|---|---|---|---|---|
| MAL | *Maackia amurensis* lectin | *Maackia amurensis* | Neu5Ac/Gcα2-3Galβ1 4GlcNAcβ1-R | No |
| PNA | Peanut Agglutinin | *Arachis Hypogaea* | Galβ1-3GalNAcα1 Ser/Thr (T-Antigen) | 1 mM $CaCl_2$ + 1mM $MnCl_2$ |
| SNA | Elderberry lectin | *Sambucus nigra* | Neu5Acα2-6Gal(NAc)-R | No |
| AAL | *Aleuria aurantia* lectin | *Aleuria aurantia mushrooms* | Fucα1-2Galβ1-4(Fucα1-3/4)Galβ1- 4GlcNAc; R2-GlcNAcβ1-4(Fucα1-6)GlcNAc-R1 | No |
| WGA | Wheat Germ agglutinin | *Triticum vulgaris* | GlcNAcβ1-4GlcNAcβ1-4GlcNAc, Neu5Ac (sialic acid) | No |
| GNA | Snowdrop lectin | *Galanthus nivalis* | α 1-3 and α 1-6 linked high mannose structures | No |

HID reagent in mucins presence, stains the sulfate residues black (Mantle and Allen, 1978) (Ridley *et al.*, 2016). Similarly, alcian blue can detect mucins presence by colouring sulfate residues at pH 1, but can also react with sulfate and sialic acid groups at pH 2.5 (Harrop, Thornton and McGuckin, 2012). PAS is also a colorimetric assay for polysaccharides measurement. This method is divided in two parts: firstly, periodic acid is used to oxidize sugar residues then a reaction with Schiff's reagent allows to obtain a pink-purple colour (Mantle and Allen, 1978). These assays are useful for mucins' detection in intermediate purification steps of known samples. However, they lack specificity since they stain all glycoproteins, being

this major disadvantage of chemical methods. On the other hand, mucins detection using antibodies is a very attractive approach since it uses a specific known sequence against mucins. However, antibodies are not useful since they were raised against sequences in mucins' PTS domains that also have affinity to mucins precursors. Moreover, they are not effective for mucin detection in secretions and cell culture media. Although deglycosylation could increase the feasibility of this method, there is not a universal enzyme to detach O-glycans to proteins yet (Tarp and Clausen, 2008).

Overall, in view of extensive glycosylation, heterogeneity's and not fully understood structure coupled with gel-forming characteristics, it represents a huge challenge to work with.

### 1.2.3 Bio-layer Interferometry

Nowadays, in contrast to traditional techniques (Western Blot, ELISA and HPLC), industry requires tools that are time saving, robust and able to analyse in-process complex crude and purified samples. During bioprocess development it is necessary to have available tools that allow a fast protein analysis in order to make careful process decisions. The Octet ® System (Fortébio, Inc. Menlo Park, CA) is a label-free technology for measuring molecules interactions through kinetic and quantitation assays, that provide a simple and fast analysis with real-time results. Label-free technology interest and applications have grown during the last decades (Rich and Myszka, 2007). This technique is capable of detecting molecule binding to tips of disposable fiber-optic biosensors, using BioLayer Interferometry (BLI) (Martin *et al.*, 2011).

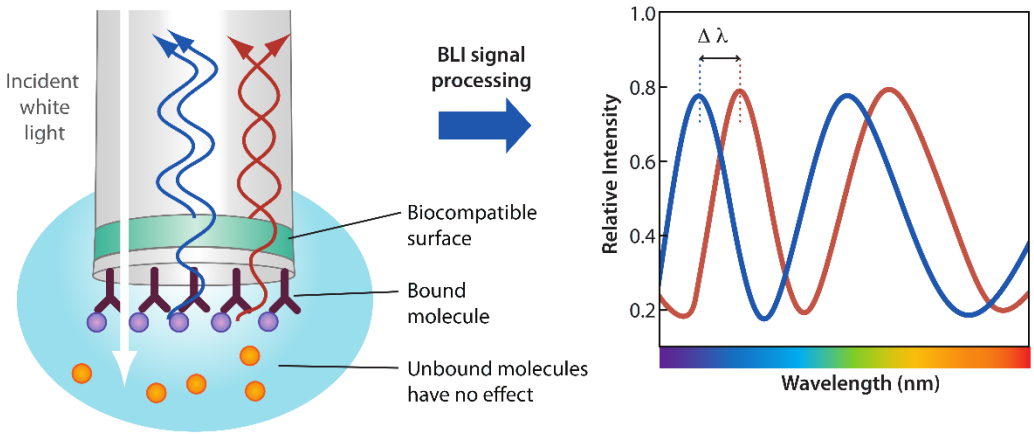


**Figure 2:** BLI technology analyses the interference pattern of white light reflected from two surfaces. Changes in the number of molecules bound to the biosensor causes a shift in the interference pattern that is measured in real time (Tobias, 2013).

The biosensors used are composed by two-dimensional binding surface, where the outer layer is made by a biocompatible matrix that minimizes non-specific binding. Briefly, label-free technology is an optical analytical tool that uses interferometry principle. A layer of molecules is immobilized on the tip surface of an optic fiber which reflects a white light from two surfaces, and consequently creates an interference pattern at the detector. When an alteration in the number of molecules bound to the tip occurs, it causes a pattern shift. The interferometry wave patterns are monitored in real-time.

In an attempt to overcome all the challenges that mucins family offers in terms of quantification and detection, a different approach was used in this work. Taking into account the sample complexity and the steps required, mucins quantitation was assessed using the octet kinetic mode. High Precision Streptavidin (SAX) biosensors were used to perform quantitation studies using their widely known interaction with biotin. For binding to the SAX biosensor biotinylated lectins were used. Lectins are sugar-binding proteins with agglutination capacity and can be isolated from several origins (Singh, Kaur and Singh, 2014). These proteins bind reversibly specific glycan structures, have hemagglutinating activity and are of non-immune origin (Singh, Kaur and Singh, 2015). Lectins have high carbohydrate specificity and can recognize even slight variations in carbohydrate structures. Consequently, they have been used for characterization, purification and drug targeting of different glycoprotein classes (Singh, Bhari and Kaur, 2015).

The assays comprise five steps: Baseline, Loading, Second baseline, Association and Dissociation. On the association step, mucins are exposed to the sensor surface already blocked with biotinylated lectins. The lectins evaluated are enumerated in table 2.

## 1.3 Hepatitis C virus

### 1.3.1 Vaccines: state-of-the-art

The last few decades witnessed an increased interest in novel biopharmaceuticals development and the market insertion of new-virus based biopharmaceuticals (VBBs). VBBs can be described as any virus-derived component or virus-based particle with therapeutic or prophylactic application, including diverse bioproducts such as: VLPs, vaccine vectors, viral vectors for gene therapy and oncolytic virotheraphy (Rodrigues *et al.*, 2014). More particularly, vaccines are entering in a period of unprecedented development increase after three decades where only few changes were introduced (Nestola *et al.*, 2015)(Almond, 2007). Several vaccines against microbial diseases have improved the health of millions of people. In fact, these

biopharmaceuticals make possible the elimination or reduction of major scourges number, including smallpox, poliomyelitis, measles, pertussis, tetanus, yellow fever, and diphtheria (Moxon and Siegrist, 2011).

Nowadays, the combination of new available technologies in different areas (genetics, structural biology and biochemistry) is enabling the expansion of vaccines against respiratory infections and diarrhoeal diseases (Moxon and Siegrist, 2011)(Rodrigues *et al.*, 2015). Consequently, being the most cost-effective tool for viral disease control, pharmaceutical companies and scientists in public sector have recently shown a great deal of interest and resources in new vaccines development (Lua *et al.*, 2014) (Roldao *et al.*, 2010).

**Table 3:** Succinct description of vaccine product classification. Adapted from (Josefsberg and Buckland, 2012)

| Product classification | Licensed vaccines |
|---|---|
| Live attenuated virus | Smallpox, polio, measles, mumps, rubella, chicken pox, rotavirus, shingles, influenza, and yellow fever |
| Inactivated purified virus | Inactivated polio, japanese encephalitis, hepatitis A, influenza (seasonal and pandemic), and rabies |
| Live attenuated bacterium | Tuberculosis and typhoid |
| Whole inactivated bacterium | Whole cell pertussis |
| Purified protein | Acellular pertussis |
| Purified protein toxoid | Tetanus, anthrax, and diphtheria |
| Purified VLPs | HBV and HPV |
| Purified polysaccharide | Pneumococcal for adults and tyohoid |
| Polysaccharide conjugated to carrier protein | Pneumococcal for infants, haemophilus type B, and bacterial meningitis |

For several reasons they continue to be an appealing health care tool and a sustainable business: (i) due to emerging markets, vaccine demand has grown substantially; (ii) diverse medical needs cannot be totally fulfilled and vaccines may play a decisive role; and (iii) advances in immunology and microbiology may now allow vaccination for previously intractable targets (Nestola *et al.*, 2015)(Smith, Lipsitch and Almond, 2011). Moreover, this is one of the less affected areas by profit decrease, owing to patent expiration. Thus, vaccines high complexity constitutes a barrier to new competitors emergence, also contributing to business sustainability (Smith, Lipsitch and Almond, 2011). On the other hand, the constant increasing demands on product purity and product safety by regulatory authorities, such as the US Food and Drug Administration (FDA) and European Medicines Agency (EMA), clearly affects vaccines costs (Wolf and Reichl, 2011).

In the beginning, vaccines consisted mainly of live attenuated viruses (e.g. Smallpox,) or inactivated bacteria (e.g. Pertussis) (Josefsberg and Buckland, 2012) (Vicente *et al.*, 2011).

These days, a great number of viral vaccines currently marketed are usually divided in two classes depending on the pathogen delivery state. The first class is based on the live-attenuated form of the virus. This type of vaccines makes use of the weakened virus in the host limited replication, causing an immune response similar to a natural infection (Plummer and Manchester, 2011). Also, a single dose is normally sufficient for an effective immunization. The second class is based on chemical inactivation of virus or infected tissues (termed "killed" vaccines). Since the genetic material or global structure of the virus is destroyed replication does not occur. This characteristic generally renders this class of vaccines less effective thus requiring a multi dose administration along time to maintain protection. In spite of the great success of these vaccines against viral infectious diseases, some have, however, shown adverse effects. The potential for reversion of an attenuated virus or an incompletely inactivated vaccine, are one of vaccination biggest disadvantages (Baylor, 2016). These situations can lead to the appearance of side effects on vaccinated individuals, supporting the urge need to produce safer vaccines development, which is a global desire nowadays (Kushnir, Streatfield and Yusibov, 2012). Therefore, recombinant DNA technologies and genetic engineering have been used in order to generate isolated viral components (Table 1), equally capable of removing pathogens from the system. These isolated viral components, called subunit vaccines (SUVs), achieve immunization by delivering subset immunogenic viral proteins, usually located on the virus capsid (Kushnir, Streatfield and Yusibov, 2012). This type of vaccines is safer as a result of its lack of replication potential. Nonetheless, they present lower immunogenicity, generally, comparing to pathogen-based inactivated or live attenuated vaccines and thereby requiring higher doses.

### 1.3.2 Emergence of VLPs

Virus-like particles (VLPs)-based vaccines are a distinct class of SUVs that represent a new molecular tool for control and containment of infectious diseases and are opening new advances in vaccine development field. Since the successful development and introduction of the hepatitis B virus (HBV) surface antigen and the human papilloma virus (HPV) capsid protein L, vaccines against hepatitis B and HPV-induced cervical cancer respectively, VLPs have emerged has good candidates for vaccine development (Vicente *et al.*, 2011) (Zeltins, 2013) (Effio and Hubbuch, 2015).

VLPs are viral proteins that can self-assembly into highly ordered structures of empty DNA. (Kalnciema *et al.*, 2015). These viral proteins are capable of trigger a protective humoral and cellular immune response, since they display intact and active antigens that resemble the native virions. The absence of genetic material makes them replicative- and infectious-incompetent (Lua *et al.*, 2014)(Noad and Roy, 2003)(Fernandes *et al.*, 2013). Altogether it is an appealing model of SUVs: having a viral capsid conformation similar to native viruses, and being non-infectious due to their lack of viral genome thus avoiding the drawbacks seen with live attenuated viral vaccines. One of the most important VLPs utilities it is the possibility to used them for vaccination against to the corresponding virus that they are derived (Fernandes *et al.*, 2013).

Presently, there are a few VLP-based-vaccines against human diseases that have been already commercialized (Zhao *et al.*, 2013)(Rappuoli, Black and Lambert, 2011). An overview about vaccine candidates in different clinical phases, and even licensed products are present in table 4.

VLPs can be classified by their original virus taxonomy (e.g. adenovirus, etc), based on their architecture and, finally, to the synthesis method (e.g animal, plant, yeast, plant, etc) (Naskalska and Pyrc, 2015). According to their architecture, they can be distinguished between native or chimeric VLPs. Chimeric VLPs can be formed through modification of VLPs gene sequence(s), so that the fusion of VLPs proteins and foreign vaccine proteins are assemble into VLPs during *de novo* synthesis (Lv *et al.*, 2014). Because of their diversity, they also can be structurally classified as non-enveloped (capsid-based) or enveloped, and in both situations they may have a single or multiple layers of the same or different proteins. Non-enveloped VLPs can be constitute by a single- or multiple-capsid protein VLPs and are known to be composed of one or more pathogen components. This approach has been used for example to produce VLPs from Hepatitis E. In contrast, enveloped VLPs consists of a lipid membrane derived from the respective expression host that involves the proteins matrix. This membrane arises during assembly and budding from the cells and integrate, at the same time, glycoproteins (target antigens) in this lipid layer. Consequentially, enveloped VLPs are more flexible and can easily integrate more antigens from the same or different pathogens but less uniformed than non-enveloped (Kushnir, Streatfield and Yusibov, 2012). The most common examples of this group are the VLPs designed to express vaccine targets to influenza virus, retroviruses and hepatitis C virus (HCV) (Kushnir, Streatfield and Yusibov, 2012).

**Table 4:** Example list of VLP vaccines containing some of their features and corresponding development stages.

| VLP type | Production system | Indication | Product name | Status |
|---|---|---|---|---|
| Non-enveloped[a] | Mammalian | HBV | GenHevacB® | Licensed |
| Non-enveloped[a] | Plant | HBV | | Phase 1 |
| Non-enveloped[a] | Yeast | HBV | Engerix-B®, Recombivax HB® | Licensed |
| Non-enveloped | Insect/baculovirus | HPV | Cervarix® | Licensed |
| Non-enveloped | Yeast | HPV | Gardasil® | Licensed |
| Non-enveloped (chimeric) | Yeast | Malaria | RTS,S | Phase 1 |
| Non-enveloped (chimeric) | Bacteria | Malaria | MalariVax | Phase 3 |
| Non-enveloped (chimeric) | Bacteria | Influenza A | ACAM-FLU-A | Phase 1 |
| Enveloped | Plant | Influenza A | | Phase 1/2 |
| Enveloped | Insect/baculovirus | Influenza A | | Phase 2 |
| Enveloped (virosome) | Cell-free | Influenza A | Inflexal® V | Licensed |
| Enveloped (virosome, chimeric) | Cell-free | Malaria | PEV3 | Phase 1/2 |

[a] Contain host cell lipids.

A distinct but also important characteristic about VLPs it is the synthesis method. There are a few systems that can expressed viral structures proteins such as: bacteria (mostly *E.coli*), mammalian cell lines, plant cells, yeast and baculovirus expression vector/insect cell system (BEVS/IC). Even though there are more than one system available, the specific target function on the VLP and existence of PTM, are important factors to take into account when choosing an expression system (Zeltins, 2013)(Naskalska and Pyrc, 2015). For example, one of the major disadvantages of bacteria and yeast systems it is the absence or different PTM patterns. One the other side, mammalian cells lines are the most convenient expression system due to their ability to carry out complex PTM usually required for proper protein folding. However high production costs, scale-up difficulties and safety concerns, are the main limitations. Nowadays, BEVS system it was recognized for offering significant advantages on recombinant protein production and its large genome size enables insertion of large or multiple genes for expression of complex proteins structures (e.g. VLPs) (Y.-C., K. and T.-Y., 2008). This system is less expensive, facilitates process scale-up and it is also capable of PTM or recombinant protein modification like in mammalian cells, even though they are not equal (Zeltins, 2013) (Fernandes *et al.*, 2013).

Although this expression system is appropriate for large-scale production, one of the most significant limitations is the concomitant production of infective baculovirus (BV) particles. This

poses a challenge for the purification stage in manufacturing as BV particles have an average size comparable to VLPs. Inactivation of BV present in VLPs-based immunogens can be achieved by chemical procedures or through the use of several processing steps (Rueda *et al.*, 2000); these can impact VLPs quality and affect its intended immunogenic effect (Cruz *et al.*, 1998). Similar to viruses, VLPs present varying morphologies and surface characteristics; therefore, purification usually requires a combination of different unit operations.

### 1.3.3 Hepatitis C virus

HCV is a single strained RNA virus and considered as the main cause of chronic liver diseases. Although there is no indication about HCV chronic infection prevalence, 2010 analysis estimate about 130-170 million people infected with HCV, having a risk of developing hepatosteatosis (lipids accumulation in the liver), liver fibrosis, cirrhosis and hepatocellular caricinoma (Bartenschlager *et al.*, 2011). Moreover, HCV causes approximately 500 000 deaths each year (Lanini *et al.*, 2016). Therefore, the available antiviral is expensive, associated with side effects and only a small number of patients can be cured. Thus, a prophylactic vaccine it is a major medical priority (Bellier and Klatzmann, 2013).

HCV encodes the structural core protein and two enveloped glycoproteins E1 and E2 (Badia-Martinez *et al.*, 2012) (figure 3). It was reported that HCV-like particles (HCV-LPs), expressed in recombinant BEVS, it is capable of assembling into virus-like structures, have a lipid bilayer envelope and a similar biophysical, ultrastructural and antigenic properties to HCV (Bellier and Klatzmann, 2013). HCV VLPs have in their native sequence of E1 and E2 proteins, epitopes with neutralizing potential. Although, it was described that insect cell-derived E2 antigens were not able to produce a protective antibody response in primates, insect cell-derived HCV VLPs have produced encouraging results for HCV vaccine design (Bellier and Klatzmann, 2013). Thus, it is necessary to improved HCV VLP immunogenicity (Torresi, Johnson and Wedemeyer, 2011).

Currently, the treatment applied to patients with HCV consists in the administration of interferon alpha and ribavirin. The percentage of cured patients with this treatment is 80% to 90% for patients infected with genotypes 2 and 3 and 40% to 50% for genotype 1, this being very long therapy, toxic and expensive (Yu and Chiang, 2010). Overall, an effort is being made in order to develop new therapies. A future vaccine candidate of HCV VLP based could make it possible to deliver important neutralising antibody and core specific T cell epitopes in a single vaccine construct that will most closely resemble mature virions antigenically.

Moreover, future approaches comprise VLP-based vaccines which have been pointed out to prevent HBV or HPV infections (Bellier and Klatzmann, 2013)(Torresi, Johnson and Wedemeyer, 2011).

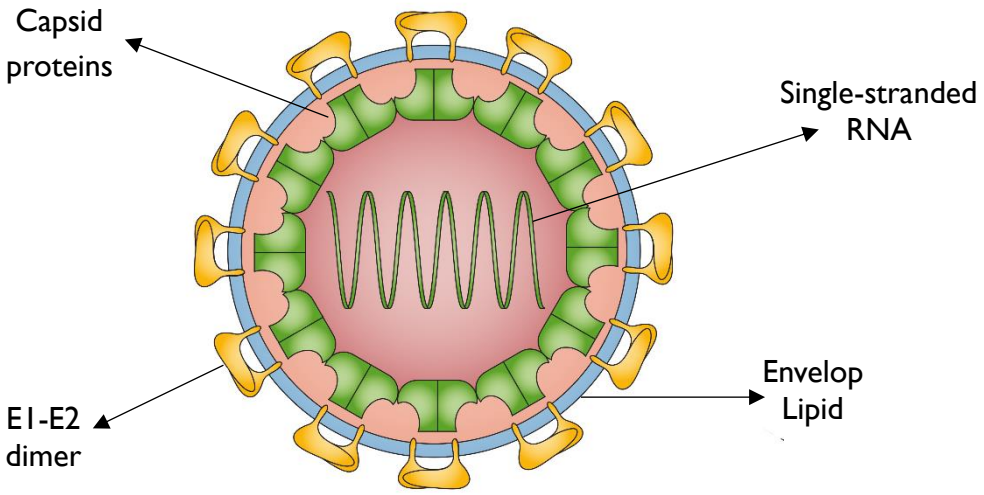


**Figure 3:** Morphology of Hepatitis C virus. The HCV core protein interacts with viral genomic RNA to form the nucleocapsid. Two membrane-associated envelope glycoproteins, E1 and E2 are embedded in a lipid envelope which is derived from the host (Wakita *et al.*, 2010). Adapted from (Morales and Aguado, 2012).

### 1.3.4 Downstream processing of enveloped VLPs

The downstream processing (DSP) of biopharmaceuticals main goal is the recovery and purification of biological products. Generally, it is more cost-intensive than the equivalent upstream processes in biotech industry and can account for up to 80% of total production cost. One of the challenges that are slowing down manufacturing processes and development of new VLP-based products it is the design and scale-up of DSP (Effio and Hubbuch, 2015).

Downstream processing of bioparticles, such as VLPs in genetically modified cells, is aassociated with two major impurities subgroups: process- and product-related contaminants. Process-related impurities should be removed in the beginning of the processes, whereas product-related contaminants in the final steps. Media components, anti-foam agents, host cell impurities (e.g cell debris, host cell proteins (HCPs), DNA, proteases, endotoxins and lipids) and baculovirus, as well as products used during downstream and upstream processing (e.g. nucleases, stabilizers, proteases) are usually described as process-related contaminants. Product-related impurities, more specifically viral particle-related, comprise VLPs aggregated, misfolded, disassembled particles (Effio and Hubbuch, 2015). Residual HCPs or endotoxins, as an example, can induce immunogenic responses in patients. Due to safety concerns, industry

set up specifications for contaminants and regulatory agencies (US FDA and EMA) provide guidelines concerning, as an example, acceptable ranges of DNA (normally bellow 10 ng per dose) (Vicente *et al.*, 2011)(Nestola *et al.*, 2015)(Wolf and Reichl, 2011).

Analytical techniques should be developed for measuring both, product and impurities, in and to assess the potency of the final product. A compromise between purity (high product quality), potency (higher titer) and scalability (large quantities) in a cost-effective manner, is the recipe for "the ideal" downstream process (Rodrigues *et al.*, 2006).

### 1.3.5 Standard process purification

Viruses possess different biological and biochemical properties and therefore purification conditions must be established specifically for each virus (Lightfoot and Moscariello, 2004). Although a well-defined platform for VLP purification does not exist, it is still possible to define a set of stages that compose a typical (DSP) train of operations (Rodrigues *et al.*, 2007) (figure 4).

One of the first stages of purification trains is the bioreactor harvesting. There is influenced by the bulk characteristics like cell density and viability, as well as if the product will be present intra- or extra-cellular. Normally, protease inhibitor and stabilizing agents addition are essential to this stage (Nestola *et al.*, 2015). The method used to reduce the amount of host nucleic acids in extracts is the addition of nucleases, such Benzonase ® (Merck Millipore), even though it is very expensive. A clarification stage aiming to remove cell debris, process impurities (DNA and HCP) and large aggregates takes place (Bandeira *et al.*, 2012).

A second stage, intermediate purification, have as main goal volume reduction and stabilization; this task is especially important in the case of low production titers and thus diluted bulk material (Vicente *et al.*, 2011). In this step, the aim is to remove low molecular weight HCP, fragmented HC DNA and fragmented product-related impurities. Volume reduction eases subsequent operations by reducing the size of the equipment and infrastructure required (Nestola *et al.*, 2015). The last stage in DSP can be regarded as two distinct steps: the first step, purification, has the purpose of removing bulk impurities while the second step, polishing, aims at the removal of residual impurities. Although ultracentrifugation is a powerful method for selective fractionation of viral particles, adsorptive techniques are the most popular choice for purification and polishing (Wolf and Reichl, 2011). Axial resin packed bed columns are the benchmark for liquid chromatography, offering a wide diversity of adsorptive surfaces and chemistries. Nevertheless, due to the relative dimensions of viruses or VLPs when compared to typical pore sizes of commercial adsorbents (30 – 80

nm), adsorption of these species is restricted to the bead surface with material transport governed by pore-diffusion or pore-exclusion phenomena, while impurities such as host cell DNA and protein will access the porous surface area within the beads. It is also important that elution conditions should not compromise product stability; this is particularly relevant in the case of enveloped viral particles. Physiological conditions should, therefore, be maintained and impact of salt addition as elution modulator in high concentrations (>1 M) should be assessed in terms of product quality (Cruz *et al.*, 2006) (Morenweiser, 2005).

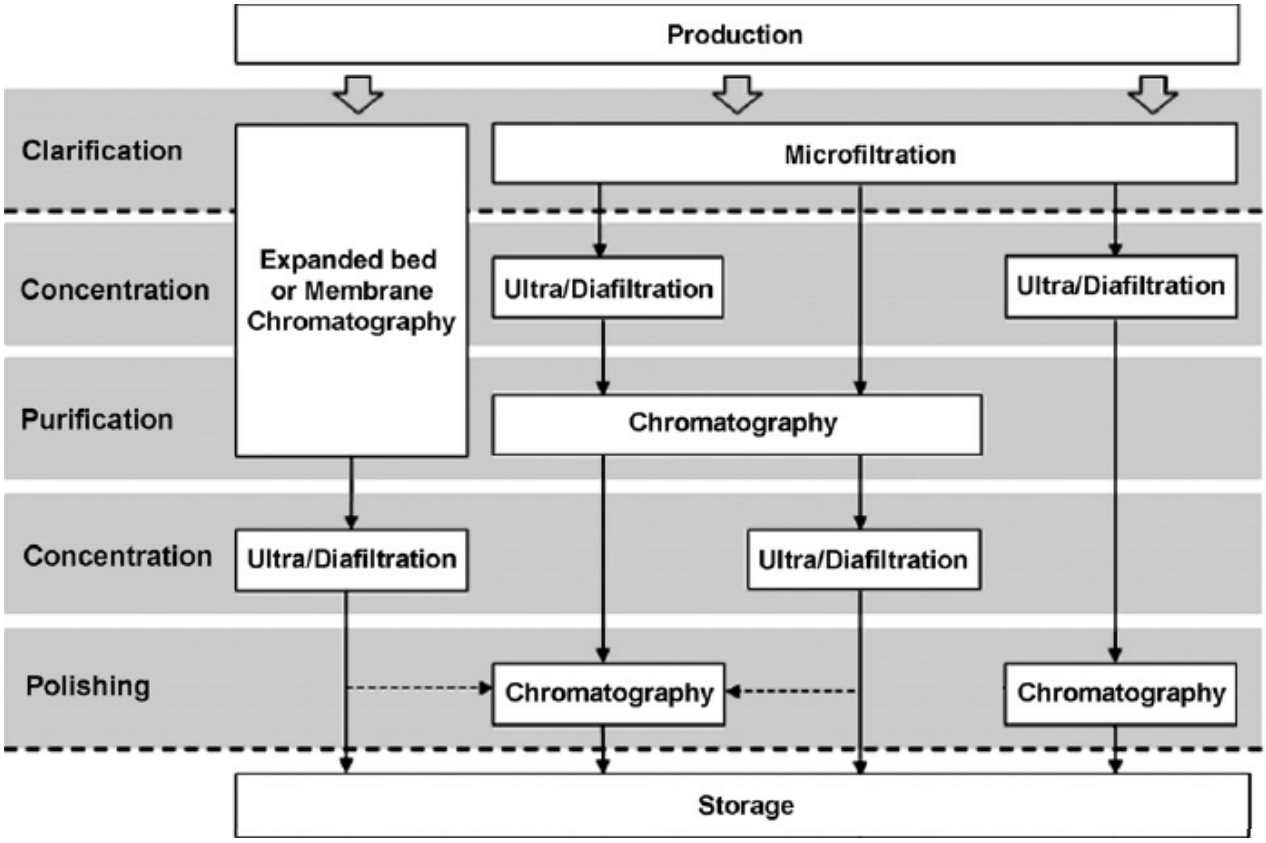


**Figure 4:** Schematic representation of a possible standard downstream process for VLP purification. Adapted from (Rodrigues *et al.*, 2007)

Chromatographic operation with axial flow packed beds of resins can, in larger scales, be limited by factors such as pressure drop across the packed bed and pressure resistance of resins (Besselink *et al.*, 2013). These parameters should be addressed when a chromatographic separation is designed, as they impact process productivity. Radial Flow chromatography (RFC) is a potential alternative to overcome pressure-related issues in conventional axial-bed columns. In the RFC column design, the medium is held between two concentric cylindrical porous frits and the mobile phase is directed from the outer surface to the inner surface, or vice versa across the radius of the column. Potential for large scale application of RFC has already been demonstrated elsewhere (Cabanne *et al.*, 2007)(Besselink *et al.*, 2013), with

proven benefits in scale-up, performance maintenance (Huang *et al.*, 1988), and potential productivity increase. Coupling RFC with appropriate chromatographic resins and hardware has the potential to provide a good option in vaccine manufacturing.

Ion exchange chromatography (IEX) is often used to reduce residual impurities (HCP and DNA) that need to be removed to fit acceptable threshold values (Effio and Hubbuch, 2015). Purification of viruses and VLPs by IEX chromatography is often operated in positive mode, also known as bind-elute mode. In this type of strategy, impurities are mostly collected in the flow-through (FT) fraction, whereas the virus particles and impurities with higher charge are retained in the resin (Wolf and Reichl, 2011)(figure 5). Product is then fractionated and eluted with the use of a buffer gradient with increasing ionic strength. Applications of FT strategies are commonly encountered in MAb purification, where a polishing step is used, collecting the product in the FT fraction as it does not adsorb in the chromatographic support, contrarily to the associated impurities (Marichal-Gallardo and Álvarez, 2012)(Vicente *et al.*, 2009).

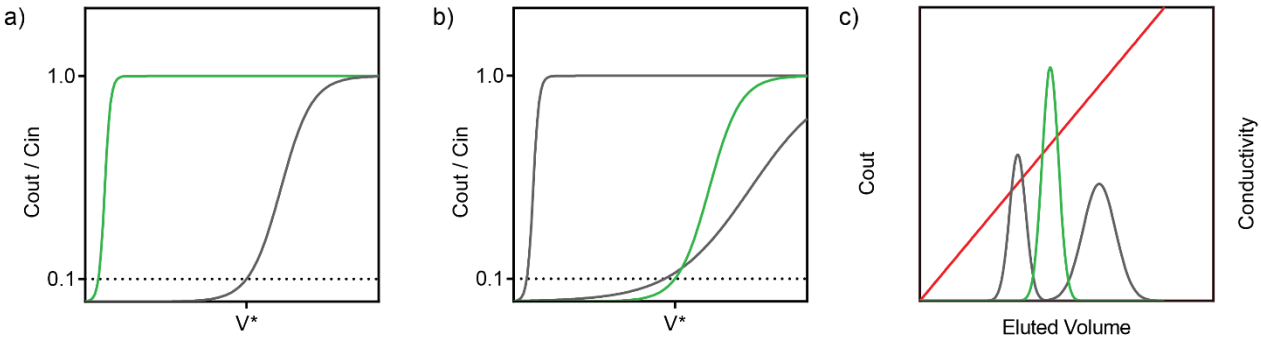


**Figure 5:** Schematic graphic of negative-mode or flow-through chromatography (V* correspond to critical volume). (B) Schematic graphic of positive-mode or bind-and-elute chromatography (V* correspond to critical volume). (C) Gradient operation of the positive-mode example. The green lines represent the product of interest, the gray the impurities and the red curve the ionic strength. Adapted from (Silva *et al.*, 2015)

## Objectives

The biopharmaceutical industry is growing at an unprecedented rate especially regarding complex therapeutic molecules. The development of novel recombinant VPLs and the constant research for new and safer vaccine candidates has been putting the spotlight in vaccines production. However, the large size and complexity are still on of major bottlenecks of downstream processing, since they should follow requisites such as: high capacity and throughput, low cost, efficient impurity removal to a level acceptable by regulatory standards and easy scale-up. Process development occurs in parallel to the development of analytical methods for measuring impurities as well as assess product potency. During this thesis it is described the study of two different complex biopharmaceuticals.

The first part of this work aims to validate BLI technology as a quantitation tool for Mucins. Mucin family has drawn off attention during the last few decades, for example, as a possible candidate for cancer vaccines. But due to their high complexity and extensive glycosylation poses huge downstream and analytical challenges. Thus, it is necessary to design faster and accurate quantification assays.

The second part of this work aims to develop and implement a flow-through purification strategy for Hepatitis C virus-like particles VLPs using anion exchange chromatography. HCV production was carried out using insect cell-based expression with recombinant baculovirus. Due to the product diversity, variability, and physicochemical properties, purification processes of HCV still offer multiple challenges.

# 2. Methods

## 2.1 Mucins Quantification and Characterization

### 2.1.1 Biolayer interferometry

Lectin - Mucin binding characterization was accessed using an Octet RED96 System (fortéBIO, Pall Corp., USA). Lectins evaluated were *Aleuria aurantia* lectin (AAL), Peanut Agglutinin (PNA), Wheat Germ Agglutinin (WGA), *Maackia amurensis* lectin (MAL), *Sambucus nigra* agglutinin (SNA), Snowdrop lectin (GNA). All of them were purchased from Galab Technologies, Germany, and kindly provided by Glycobiology Laboratory, ITQB. Two distinct mucins were used: bovine submaxillary mucin (BSM) (84195-52-8, Sigma Aldrich®) and Human 5B Mucin (isolated from Human saliva, provided by David Thorton, UK). Briefly, biotinylated lectins were diluted with Sample diluent (18-5028, fortéBIO, Pall Corp., USA) and loaded onto High Precision Streptavidin (SAX) Biosensors (18-0037, fortéBIO, Pall Corp., USA), previously hydrated with the same buffer. Mucin samples were then associated to the different lectins and association and dissociation profiles were measured. The assays were defined with five steps: Baseline (120 s), Loading (600 s), Baseline (120s), Association (900s) and Dissociation (300s). Experiments were performed at 25 °C and sample plates were agitated at 1000 rpm. Data Analysis v9.0 software (fortéBIO, Pall Corp., USA) was used for data fitting and mucins' concentration calculation.

### 2.1.2 Limit of Detection and limit of Quantification

To establish Limit of detection (LOD) and limit of quantitation (LOQ) parameters, the FDA Guidelines were followed (ICH, 1996). From the several possible approaches, the determination of both limits was defined based on the standard response deviation and the slope of the calibration curve:

$$LOD = \frac{3.3\sigma}{S} \qquad \text{(Equation 1)}$$

$$LOQ = \frac{10\sigma}{S} \qquad \text{(Equation 2)}$$

where σ is the standard deviation of the response and S the slope of the calibration curve (Armbruster and Pry, 2008). For all the experiments, quantifications were performed using the initial values (0 to 100 s) of the binding responses.

### 2.1.3 Size exclusion Chromatography (SEC)

A BSM solution (4,25 mg/mL) was prepared with 20 mM PIPES, 300 mM NaCl buffer (working buffer), at pH 5, and filtered with a 0,45µm Minisart® High Flow Hydrophobic PES syringe filter. SEC was conducted using a Superdex 200 Increase 10/300 GL column (GE Healthcare, USA) coupled to an ÄKTA™ avant 150 liquid chromatography system (GE Healthcare, USA) equipped with UV and conductivity/pH monitors. System operation and data gathering and analysis was performed using the UNICORN™ 6.3 software (GE Healthcare, USA). The column was loaded with 1 mL of BSM at a constant flow rate of 0,6 $mL.min^{-1}$. Working buffer was used as eluent and the eluted fractions were collected for further analysis. Elution of BSM was monitored at 230, 260 and 280 nm.

### 2.1.4 SDS-PAGE and lectin blotting

BSM samples were analysed by SDS-PAGE, in 10% acrylamide gels (T=30.8%, C=2.6%). BSM mucin was prepared and solubilized in reducing sample buffer (0,08% (w/v) M Tris-HCl pH 6,8; 77-86-1Carl Roth®; 2% SDS, 151-21-3 Sigma Aldrich®; 5% ß-mercaptoethanol, 460691CARLO ERBA Reagents; 10% glycerol 56-81-5 Sigma Aldrich®; 0,005% Bromephenol blue 115-39-9 Sigma Aldrich®) and incubated at 99 °C, for protein denaturation. After solidification, the gel was transferred to an electrophoresis tray and filled with running buffer 1x (25 mM Tris, 201-064-4 Carl Roth®, 192 mM Glycine, 200-272-2 Sigma Aldrich®, 0,1% SDS). Samples were loaded (15 µL) and the electrophoresis was performed using running buffer at 180 V during 60 minutes. Gel was transferred to PVDF membranes. Lectin blotting was performed with the lectins described in Biolayer Interferometry section. Blots were blocked with 3% BSA biotin free (292-322-5 Carl Roth®) in TBST for 1 hour, and incubated with each lectin for 1 hour and washed (D-PBS, 0,1% Tween 20, 8221840500 Merk Milipore, USA) four times, during 5 minutes. Incubation was performed during 1 hour with 0,1µg/mL streptavidin-peroxidase (S5512, Sigma) followed by washing with the corresponding buffer. Detection was performed with the Immobilon Western chemiluminescence HRP substrate (WBKLS0500 Millipore, USA). As control for non-specific binding AAL lectin was incubated in the presence of its competitive sugar, fucose (Fuc).

## 2.2 Hepatitis C virus studies

### 2.2.1 Preparation of hepatitis C VLP feedstock

Production of Hepatitis C (HCV) VLPs was carried out in a disposable stirred-tank bioreactor. Sf9 cells were cultured in 2L working volume disposable Mobius® 3L Bioreactors (EMD Millipore, Billerica, MA). Cultivations were carried out at 27 ◦C and controlled with a heating mantle, $dpO_2$ of 30 % and an aeration rate of 0.01 vvm. After 24 h inoculation ($1x10^6$ cells/mL) cells were co-infected at a MOI of 2 for BV-Gag and BV-HCV. Bioreactors bulk harvesting was performed at 96 hpi.

### 2.2.2 Clarification and Concentration

Upon harvesting, bulk clarification was performed using disposable Optiscale depth filtration devices with Polygard® CN membrane material (EMD Millipore, Bedford, MA) pre-equilibrated with 50 mM HEPES, pH 7.4, 150mM NaCl. The filtration flux was set to 988 $Lm^{-2}h^{-1}$ using a Tandem 1082 Pump (Sartorius Stedim Biotech, Germany) and Masterflex®14/16 tubing (Masterflex Group, Germany). Pressure was monitored by an in-line pressure transducer (080-699PSX-5, SciLog®, USA) according to the manufacturer's instructions. Clarified bulk concentration was carried out using UF cassettes (Pellicon® XL, EMD Millipore, Bedford, MA) with a nominal pore size of 300 kDa, composed of composite regenerated cellulose. The concentration was performed until a volume reduction factor of 5 was achieved.

### 2.2.3 Anion exchange chromatography

Anion exchange (AEX) chromatographic media, Fractogel® TMAE (Merck Millipore, Darmstadt, Germany) was used HCV VLP purification. The resin was used either in slurry format for batch adsorption studies or packed in different chromatographic column formats.

For packed bed formats column equilibration was performed with 5 column volumes (CV) of equilibration buffer (50 mM HEPES, 150 mM NaCl, pH = 7.4) and regenerated using 0.5 M of sodium hydroxide after each experiment. An Äkta explorer 10s (GE Healthcare, Sweden) equipped with UV, conductivity, and pH detectors, and a fraction collector FRAC-950 (GE Healthcare, Sweden) was used for all column chromatography experiments. Sample injection was performed using a 50 mL superloop (GE Healthcare, Sweden).

Scouting of the range of NaCl concentration needed to recover adsorbed HCV VLP in the AEX media was carried out with a 1mL pre-packed axial column (Merck Millipore, Darmstadt, Germany). After column equilibration, 10 Cv of the HCV VLP feedstock were injected. After

sample application, the column was washed with 5 Cv of equilibration buffer and a linear gradient was performed by modulating NaCl concentration in the range of 150 to 2000 mM. Radial flow chromatographic experiments were carried out using a pre-packed 5 mL column (Procxys, Netherlands).

### 2.2.4 Static adsorption experiments

Small scale batch adsorption studies were conducted using a solid/fluid ratio of 4. To an aliquot of 250 μL settled adsorbent, 1.0 mL of fluid with a fixed concentration of HCV VLPs was added. Concentration of NaCl in each tube was adjusted to 150, 300, 450 and 575 mM. The samples were incubated at 25◦C for 80 min under agitation. After incubation, supernatant was recovered and analysed for HCV VLP, BV, DNA and HCP composition.

## 2.3 Analytical methods

### 2.3.1 Host Cell Protein quantification

Sf9 host cell protein (HCP) concentration was measured using a two-site immunoenzymetric assay (F020, Cygnus Technologies, Inc., USA) without changes to the manufacturer's protocol.

### 2.3.2 Hepatitis C VLPs quantification

VLPs were quantified following Gag p30 protein content in process samples using a commercially available QuickTiter MuLV core antigen ELISA kit (MuLV p30) according to the manufacturer's instructions (Cell Biolabs, USA).

### 2.3.3 Total Protein Quantification

Total protein was quantified using the BCA Protein Assay Kit (23225, Thermo Fisher Scientific, USA) according to the manufacture's protocol. Bovine serum albumin (BSA) was used for the calibration curve (23209, Thermo Fisher Scientific, USA). In order to avoid matrix interference, the samples were diluted between 2-256 fold. The assay took place in a clear 96-well microplate (260895, Nunc, USA) and the absorbance at 562 nm was measured on Infinite® 200 PRO NanoQuant (Tecan, Switzerland) microplate multimode reader.

### 2.3.4 Total dsDNA Quantification

Total DNA was quantified using the fluorescent-based Quant-iT™ Picogreen® dsDNA assay kit (P7589, Invitrogen™, (UK) according to the manufacturer's instructions. In order to avoid matrix interference, the samples were diluted between 2–1024 fold with the provided reaction buffer. The assay took place in a Black 96-well microplate, flat transparent (3603, Corning, USA) and the fluorescence was measured on Infinite® 200 PRO NanoQuant (Tecan, Switzerland) microplate multimode reader at 480 nm for excitation wavelength and 520 nm for emission. In all experiments, the gain was optimized.

### 2.3.5 Real-time PCR

Baculovirus viral DNA was extracted and purified using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Germany) using the manufacturer's instructions. The number of genome containing particles were monitored by real time quantitative PCR (q-PCR) using LightCycler® FastStart DNA Master SYBR Green I (following the protocol described elsewhere (Vicente *et al.*, 2009)). The Rea-time PCR reactions were executed and analyzed on Light Cycler® 1.5 (Roche) equipment and software as described in table 5.

**Table 5:** Real-Time PCR amplification program for recombinant baculovirus quantification.

| Program | Temperature target (°C) | Hold time (sec) | Slope (°C/sec) | Cycles |
|---|---|---|---|---|
| Pretreatment | 95 | 600 | 20 | 1 |
| Amplification | 95 | 30 | 20 | 45 |
| | 63 | 5 | 20 | |
| | 72 | 6 | 20 | |
| Melting | 95 | 0 | 20 | 1 |
| | 70 | 15 | 20 | |
| | 95 | 0 | 0,1 | |
| Cooling | 40 | 30 | 20 | 1 |

# 3. Results and Discussion I

## Mucin studies

### 3.1.1 Design of mucin detection and quantification assay

The diversity of mucin family and their glycosylated properties make them resistant to proteolysis and able to hold water, giving rise to its gel-like properties found in mucosal barriers (Brown and Hollingsworth, 2013) (Johansson and Hansson, 2016) . Due to these particular characteristics, mucins quantitation represents a complex challenge. Bilayer interferometry (BLI) analysis was used to set up mucins detection and quantitation assay. The method uses high precision functionalized streptavidin (SAX) biosensors, that have high affinity to biotin (Klumb, Chu and Stayton, 1998), and biotinylated lectins that can recognize mucins carbohydrate structures (Jeffers *et al.*, 2010). Mucins quantitation was assessed using the octet kinetic mode which is illustrated in figure 6. Briefly, biosensors are pre-equilibrated and blocked at the initial baseline step, followed by lectins loading. Then, a second baseline step is performed to wash unbound lectins before the mucin association and, lastly, the dissociation step.

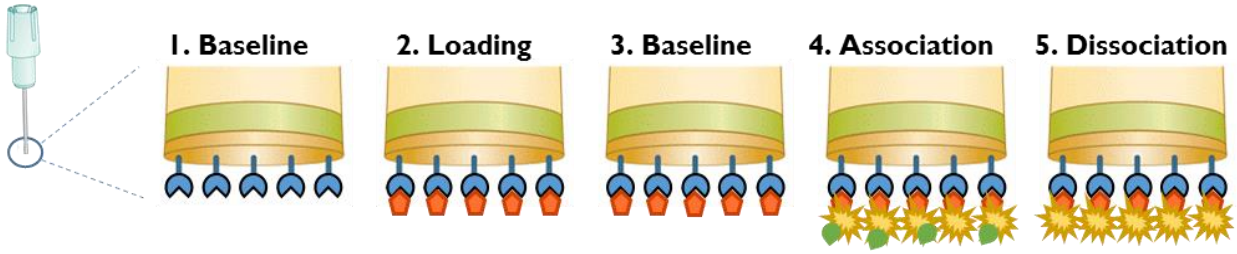


**Figure 6: Schematic representation of mucin octet detection and quantification method, using Streptavidin biosensors.** After an initial baseline step (1) in sample diluent, biosensors are dipped into biotinylated lectins (2). A second baseline step (3) is performed followed by mucins association (4) and dissociation of other process components (5).

The most suitable lectin for mucin association, lectin loading concentration and mucin concentration (maximum and minimum) in the association step were optimized. Mucins' dissociation values obtained were negligible in comparison to association values for the evaluated mucins and lectins.

Two distinct mucins were evaluated during this study: Bovine Submaxillary Gland Mucin (BSM) and human MUC5B. Since these mucins present a high content of O-glycans, a plethora of lectins were chosen, which are reported to bind those structures, in order to assess mucin detection and subsequent quantification (Jeffers *et al.*, 2010).

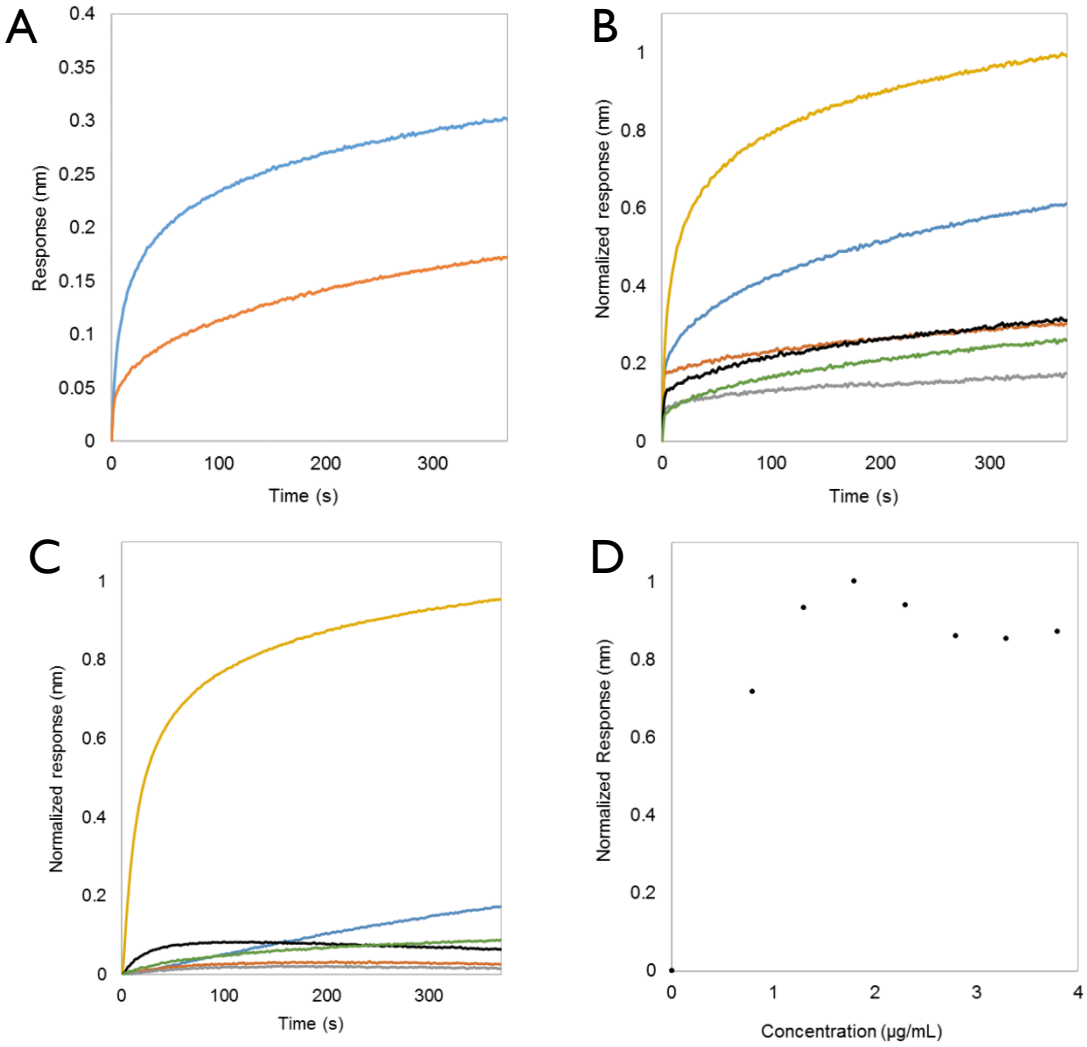


**Figure 7: Lectin selection and loading optimization.** Evaluation of the mucin association response for AAL (blue line) and MAL (orange line) lectins (A). Representative association response for BSM (B) and MUC5C (C) at 1.5 µg/mL for all lectins – MAL (blue line), PNA (orange line), SNA (grey line), ALL (yellow), WGA (black line), GNA (green line). Optimal loading concentration (1.5 µg/mL) was assessed for AAL lectin ranging 0.8 to 3.8 µg/mL (D).

To evaluate the most suitable lectin for this assay, the association of BSM (Figure 7B) and MUC5B (Figure 7C) to MAL, AAL, PNA, SNA, WGA, GNA (table 2) was performed. MAL and AAL lectins were selected due to their higher loading values on the biosensor (figure 7A). AAL lectin showed higher association response levels and it was selected to further experiments. This mucins-AAL was expected, since this last one is described for having sugar-binding specificity to L-fucose, structures (Nagata *et al.*, 1991), present in both mucins in study (Wu, Csako and Herp, 1994) (Thomsson *et al.*, 2002).

Aiming to verify mucin-lectin binding specificity, lectin blots of AAL with BSM and MUC5B were performed (Figure 8). Fucose (Fuc), the competitive inhibitor for mucin-AAL binding, was used as control (Olausson *et al.*, 2011). As observed, both mucins presented specific

binding to AAL lectin. MUC5B exhibited a higher signal, in comparison with BSM, on the lectin blot even with lower concentrations (0.5 µg/mL versus 2.5 µg/mL). This result may be explained by different amounts of fucose-containing structures present on both mucins. Additionally, the higher purity level of MUC5B sample could also be a valid explanation. This was also observed in octet binding and will be discussed later.

In order to assess the ideal concentration of immobilized AAL, several concentrations (ranging from 0.8 µg/mL to 3.8 µg/mL) were loaded onto the biosensor (figure 7D). To determine the absence of non-specific bindings to the biosensor a buffer sample without AAL was also evaluated. Because it was observed non-specific bindings to the biosensor, the obtained results were analysed and normalized after. For higher concentrations (above 2 µg/mL) the response was not concentration-dependent, presenting an erroneous behaviour, due to biosensor saturation. In the loading step, higher concentrations of ligand can rapidly saturate the biosensor, whereas the lowest concentrations do not reach saturation. The conditions of an ideal assay should comprise a significant loading signal with a slow initial binding without biosensor saturation. Altogether, the loading concentration selected should be the lowest value, where an acceptable association signal is achieved (Tobias, 2013). Therefore, to avoid biosensor overcrowding and to minimize mucin binding interferences, the optimal lectin concentration selected was 1.5 µg/mL, since it is just before the saturation plateau.

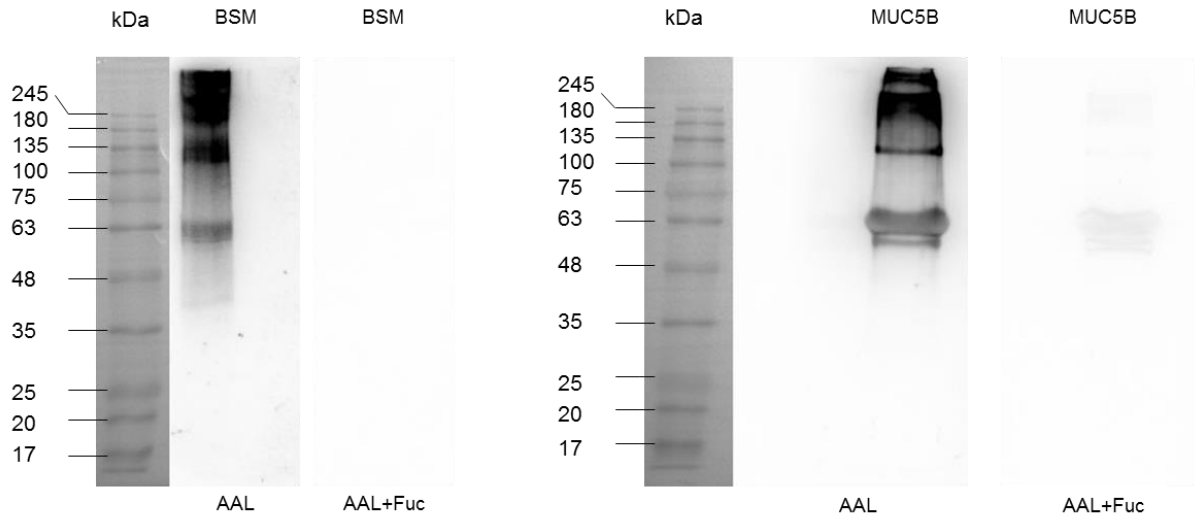


**Figure 8: Comparison of BSM and MUC5B affinity to AAL**. BSM lectin blotting (A) and MUC5B lectin blotting (B) with biotinylated AAL. Control with competitive sugar, L-Fuc, are shown in the left panel (Cummings, RD; Etzler, 2009). The lanes contained 2.5µg and 0.5 µg of BSM and MUC5,C respectively.

### 3.1.2 Mucin detection and quantification calibration

As already mentioned, MUC5B presented higher binding responses than BSM. Several studies reported the presence of impurities, or non mucin biomolecules (e.g. albumin, immunoglobulins and salts), on most commercial mucins (Nikogeorgos, Madsen and Lee, 2014)(Lundin *et al.*, 2009). The major impurity of BSM is bovine serum albumin (BSA) that representing up to 9% of the total mass of "as-received" BSM. For that reason, there are numerous purification methods described on the literature that can remove most of these impurities (Madsen *et al.*, 2014)(Madsen *et al.*, 2015). In an attempt to understand if the sample purity influences the association response values, BSM sample was purified using size exclusion chromatography. After the purification step, the association response was higher than the one obtained for the commercial available sample (figure 9). This result confirms that sample purity influence association responses.

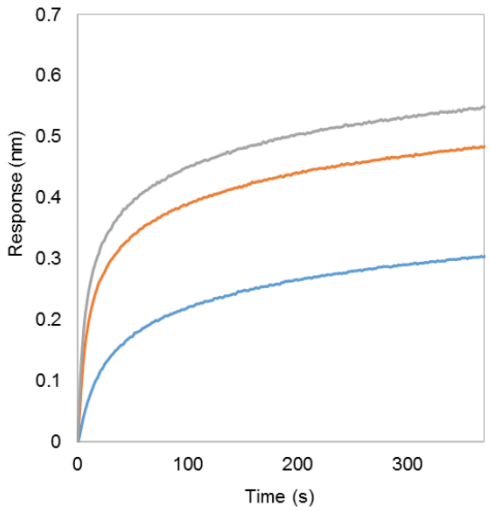


**Figure 9: Response comparison of BSM crude sample and after purification.** Association of BSM sample, as commercial available and purified using size exclusion chromatography (SEC). Association responses for the two SEC peaks were evaluated. Orange and grey lines correspond to peak 1 and 2, respectively.

To evaluate BLI detection and quantification method, BSM and MUC5B samples at different concentrations were analysed according to their different association responses (figure 10A-B). BSM calibration curve was performed using higher concentration values ranging from 25 until 800 µg/mL (figure 10A). However, the response did not follow a linear behaviour above 50 µg/mL, probably due to biosensor saturation and mucin-mucin complex binding. Therefore, the calibration curves for BSM studies were performed only in the linear range (until 50 µg/mL). The dissociation step was also evaluated and for these concentrations the values observed were negligible. The same approach was applied to MUC5B, but using lower

concentrations on the standard curve, ranging from 2.5 until 12 µg/mL. For this mucin a nonlinear behaviour was observed above 5 µg/mL (figure 10B).

Nevertheless, it was important to understand if the found linearity relation was valid. Aiming to verify its linearity, BSM samples with known concentrations were ran against BSM calibration curve (figure 10C). The values calculated using BLI assay were well correlated with the known values, presenting only slightly deviations from the real concentration probably due to the errors associated with the quantification methods.

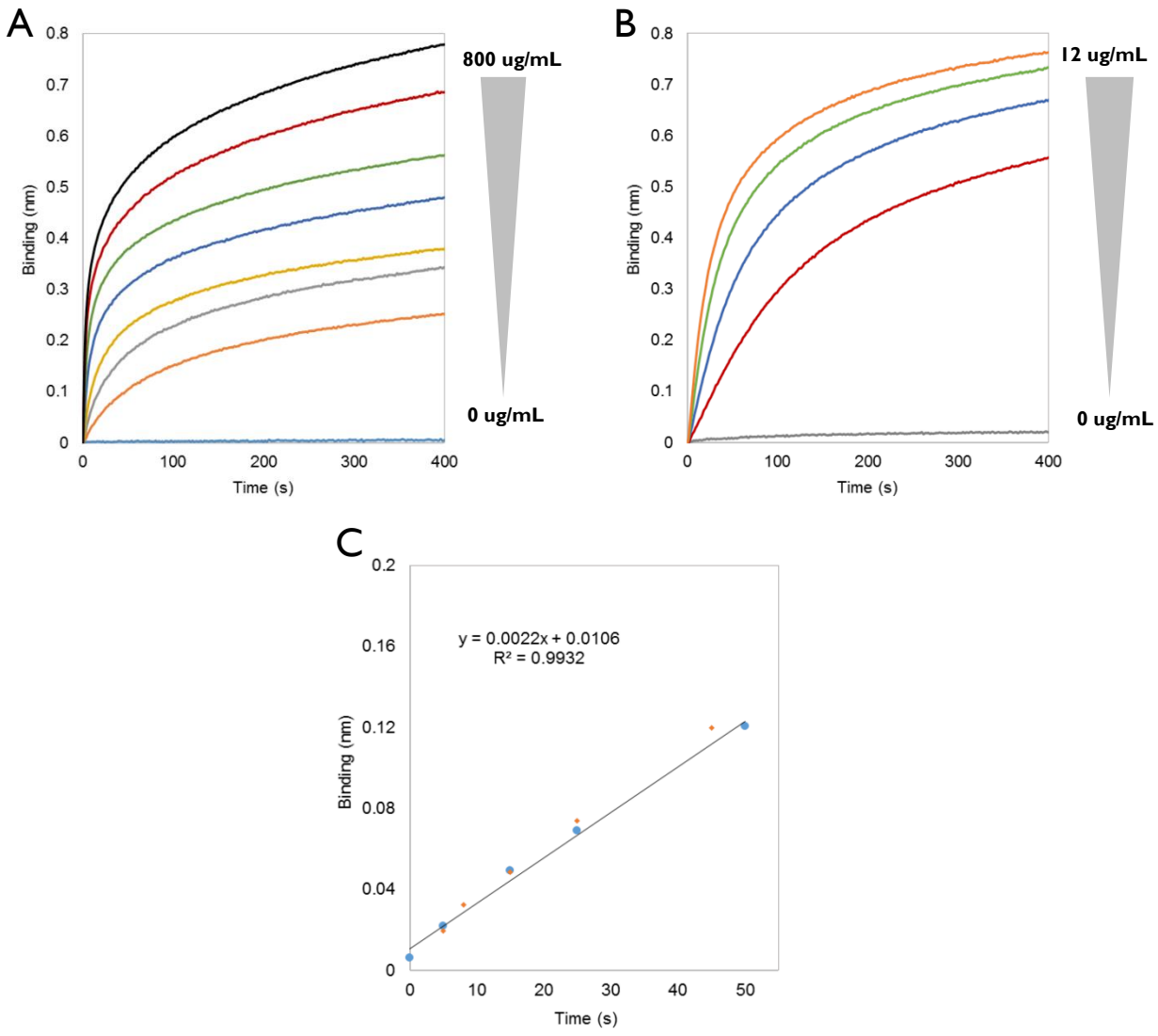


**Figure 10: Calibrations curves for BSM and MUC5B and assay validation.** Illustrative binding curves for BSM (A) and MUC5B (B). BSM calibration curve was performed at a higher concentration curve ranging 25 to 800 µg/mL – 800 µg/mL (black line), 600 µg/mL (red line), 400 µg/mL (green line), 20 µg/mL (dark blue line), 100 µg/mL (yellow line), 50 µg/mL (grey line), 25 µg/mL (orange line) and 0 µg/mL (light blue line). MUC5B calibration curve concentration ranged from 2.5 to 12 µg/mL – 12 µg/mL (orange line), 8 µg/mL (green line), 5 µg/mL (blue line), 2.5 µg/mL (red line) and 0 µg/mL (grey line). BSM samples with a known concentration were ran against BSM calibration curve (C). Blue dots represent the 50 µg/mL calibration curve and the orange ones corresponds to the samples ran against the curve.

Presently, there are no mucins' absolute quantification methods available most due to their saccharides heterogeneity (Schömig *et al.*, 2016). For this reason, crossing several analytical methods with different principles are the recommended. Moreover, the detection methods available are time-consuming, an important parameter during bioprocess development (Vicente *et al.*, 2011) . The method developed here can detect successfully different mucins in a wide range of concentrations. Despite the BLI assay limitations it is a fast and simple method for mucins quantification. Limit of Detection (LOD), and Limit of Quantitation (LOQ) are terms used to describe the smallest concentration of a measure that can be reliably measured by an analytical procedure. LOD is the lowest analyte concentration which detection is feasible, whereas LOQ is the lowest analyte concentration sufficient to produce analytical signals that meet predetermined targets. Typically, LOQ will be found at a higher concentration than LOD. Limit of Detection (LOD) and Limit of Quantitation (LOQ) were calculated and compared with the available methods (Shrivastava, Gupta and Article, 2011). It was obtained a LOD of 3.8 µg/mL for BSM and 0.2 µg/mL for MUC5B. The LOQ for BSM as 11.7 µg/mL and 3.8 µg/mL for MUC5B. Most ELISA assays can only detect mucins when their concentration reached a nanograms scale (Zhang *et al.*, 2014). Therefore, this method exhibited a higher sensitivity and an improvement on mucins detection.

### 3.1.3 Competition inhibition assays

Mucin-lectin binding can be exploited to develop mucins' purification methods (Cheng-Siang Lee, Arivalagan Muthusamy, Puteri Shafinaz Abdul-Rahman, 2013). Besides choosing the lectin with higher binding affinity, the elution conditions also need to be settled. In this case, the best conditions for mucin elution from AAL lectin were evaluated to establish a protocol for affinity chromatography. Fuc is a sugar that competes with mucins for AAL binding sites being ideal for the competitive assays, as elution buffer. The first approach was to block AAL binding sites by incubated with L-Fuc at 0.1 M during the baseline step. However, this incubation did not impair mucin binding to AAL. Only when mucin samples, BSM and MUC5B, and AAL lectin were incubated with L-Fuc at a concentration higher than 100 µM, mucin-lectin association was totally inhibited (figure 11A-B). For BSM mucin, incubation with different L-Fuc concentrations, ranging from 0.1 M until 1 µM was performed (figure 11A). The minimum Fuc concentration required for partial association inhibition was 100 µM. At this concentration, there was association, but it was approximately five times lower than the one observed for BSM without Fuc incubation. Fuc concentrations above 100 µM completely inhibited mucin – AAL binding. At 10 and 1 µM, the association was similar to the control

sample. In the case of MUC5B Fuc concentrations ranging from 100 µM to 1 µM were evaluated (figure 11B). Contrarily to BSM, for MUC5B incubation with 100 µM Fuc was enough for total association inhibition. It was observed that the binding response of BSM was approximately ten times lower than MUC5B. However, the affinity of BSM to AAL appeared to be higher as it is necessary higher concentrations of competitor sugar to impair their association. Overall, it was possible to identify the minimal L-Fuc concentration to inhibited AAL-mucin association, for both mucins. The obtained results can be further used in mucins purification, and the minimal concentration of L-Fuc used for set up elution conditions, for example, in an affinity chromatography.

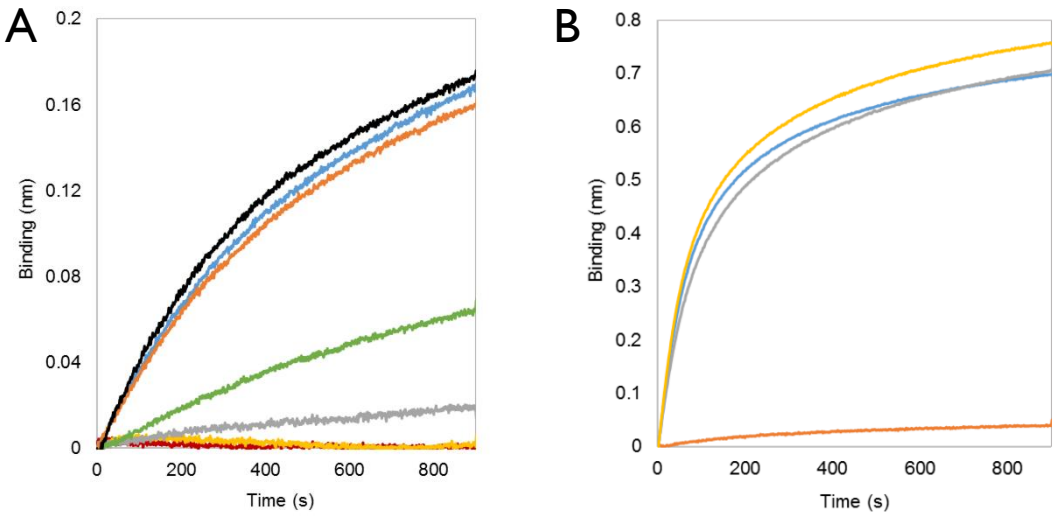


**Figure 11**: **Competition inhibition assays.** Competition assay developed for BSM (A) and MUC5B (B).L-Fuc at 0.1M was used for biosensor blocking and at 0.1M until 1 µM for sample incubation – 0.1M (red line), 10mM (yellow line), 1mM (grey line), 100 µM (green line), 10 µM (orange line), 1 µM (blue line) and 0 µM (black line) (A), MUC5B competition assays (B) were performed in a L-Fuc range from 100 µM to 1 µM – 100 µM (orange line), 10 µM (grey line), 1 µM (blue line) and 0 µM (yellow line).

Analytical techniques are used in nearly all stages of research, process development and manufacturing of biotherapeutics. Traditional methods (ELISA, HPLC) have been successfully used for protein quantification and continue to be the workhorses despite their many limitations. The high cost and lengthy times associated with these methods have forced the biopharma companies to improved efficiency and economics in all stages of development and purification processes. Octet systems, with their label-free biosensor-based binding assays, are increasingly being used. The mucins measurement principles of the method described in this chapter are very similar to the already established immunoassays (ELISA) (Cheng-Siang Lee, Arivalagan Muthusamy, Puteri Shafinaz Abdul-Rahman, 2013). Octet platform provide

important advantages such as real-time monitoring of binding interactions and shorter assay development times. Usually, a common ELISA assay takes, approximately, 5.5 hours to the operator while Octet assays only requires 40 minutes. Moreover, Octet system allows almost a full sample recovery contrary to ELISA assay. Other clear advantage is the possibility to evaluate crude material since adsorption interference is not affected in colored samples and light scattering with turbid samples. This is especially useful in downstream process development, as it is possible to performed sample quantification at different stages at different purification process. Thus, this technique can help to identify the optimal DSP conditions with the desired yield, binding specificity and potency (Tobias, 2013).

Overall, this system can offer a broad utility in protein quantification and characterization with decreased sample preparation requirements, enhance throughput and low cost of operation.

# 4. Results and Discussion II

## Hepatitis C Virus studies

## 4.1 Hepatitis C virus Studies

Over the last decades, several studies reported the use of IEX chromatography in a positive separation mode to purify virus and VLP. Additionally, AEX chromatography is often used in the polishing of mAb products (Gagnon, 2012), with particular relevance in the removal of a variety of viruses, including retroviruses (Norling *et al.*, 2005; Strauss *et al.*, 2009). The focus of the present work was to evaluate and develop a chromatography purification step for a Hepatitis C vaccine candidate, based on pseudotyped retrovirus-like particles, displaying E1 and E2 epitopes of HCV. For that reason, a flow-through (FT) strategy using AEX media was developed, in which binding conditions rather target the adsorption of impurities in detriment to VLP. This approach was selected since previous studies indicate that residence time and ionic strength can be used to modulate product binding (Rodrigues *et al.*, 2006; Weigel *et al.*, 2014).

### 4.1.1 Initial studies

An initial assessment of the non-binding conditions for HCV VLP was performed using a 1 mL scout column and depicted in Figure 12. Elution of HCV VLP occurred in the range of 200 to 600 mM of NaCl to which fraction F1 to F5 are respective.

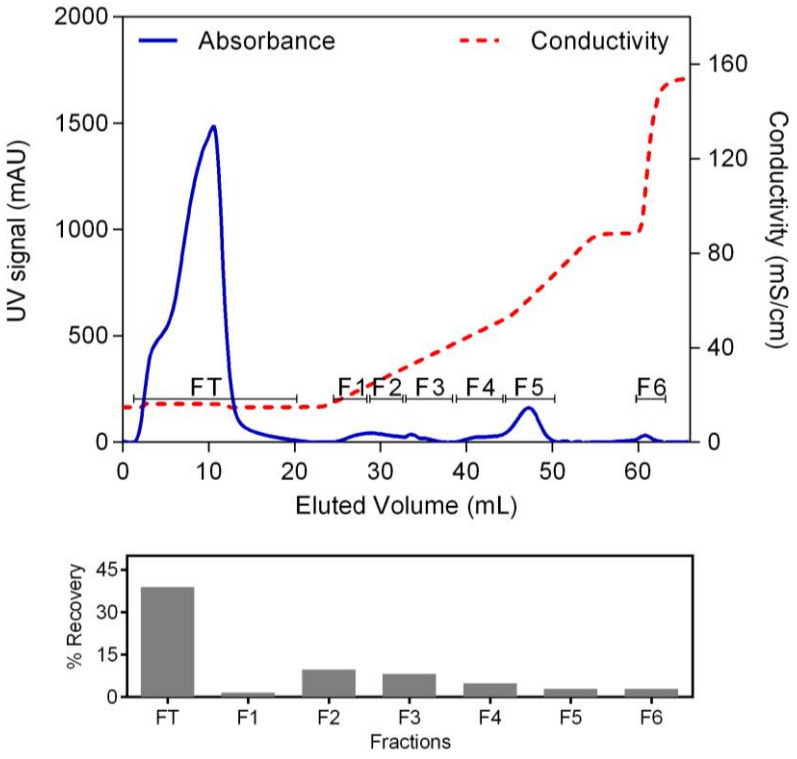


**Figure 12: Non-binding conditions assessment.** Top: Descriptive chromatogram of an exploratory run using a pre-packed 1 mL column with Fractogel® TMAE. The injection volume was 10 CV. Bottom: Recovery of HCV VLP in each fraction collected.

Experimental results suggest that a concentration of NaCl in the range of 200-450 mM was sufficient to elute HCV VLPs from the AEX media (Figure 12). Nevertheless, VLP recovery and impurity clearance should be balanced.

A more detailed study of the effect of NaCl concentration in the loaded sample and its impact on impurity removal was performed with batch adsorption studies. As reported in Figure 13, VLP recovery is mildly affected by NaCl concentration, increasing with NaCl concentration range of 47 % to 74 %. An opposite behaviour is observed for the impurities log reduction value (LRV), with a significant decrease observed on a log scale. The pronounced decrease observed in Figure 13 for HCP, DNA and BV removal, limits the range of NaCl concentration that can be used, with values above 300 mM seriously compromising product purity.

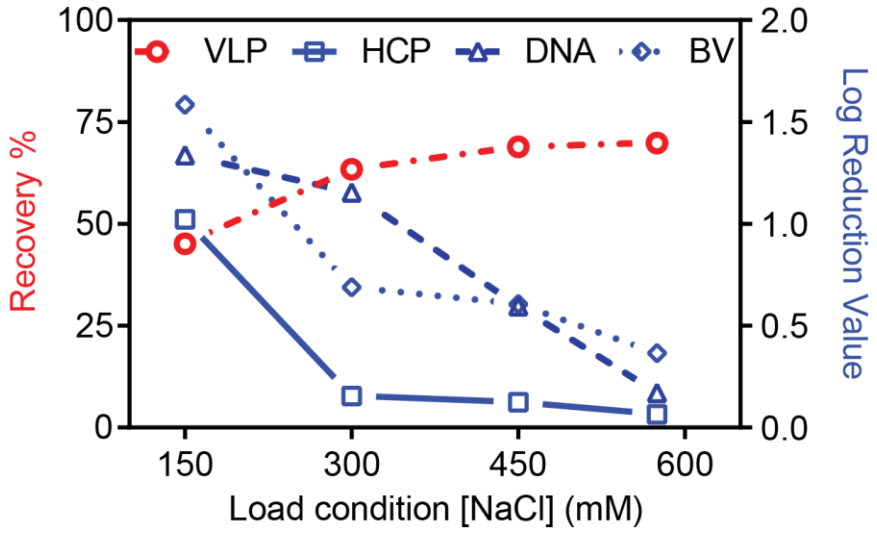


**Figure 13: Evaluation of NaCl concentration in the loading sample and impurities clearance.** Batch adsorption study using Fractogel® TMAE at different load conditions; VLP recovery refers to the left axis: ∘; impurities removal in LRV refer to the right axis: □, Δ and ◊.

### 4.1.2 Radial Flow Chromatography

In order to assess the use of RFC in the FT purification of the system studied, and to validate under dynamic conditions the results mentioned before, several injections of increasing volume were performed. The exploratory experiments were carried in the 5 mL radial flow column packed with Fractogel® TMAE media. The sample loaded was supplemented to 300 mM of NaCl, since this condition presented to be the best compromise between HCV VLP recovery and impurity clearance. Recovery of HCV VLP and impurity clearances are described in Figure 14, as well as different injection volumes for this setup.

From the result analysis it was observed, that contrarily to HCV-VLPs recovery, removal of BV and DNA is reduced with increasing injected volumes. Moreover, variation in VLP recovery and LRV of the impurities is less pronounced for higher injection volumes. VLP recovery slightly increases with the increased volume of injected bulk. Therefore, it was interesting to notice that the impurity levels and VLP recovery approach the ones from the static equilibrium experiments depicted in Figure 13 for 300 mM of NaCl. This indicate that an equilibrium was reached with this RT. Operating at lower residence times mildly affect adsorption of DNA and HCP, but will have a more pronounced effect on BV and VLP adsorption.

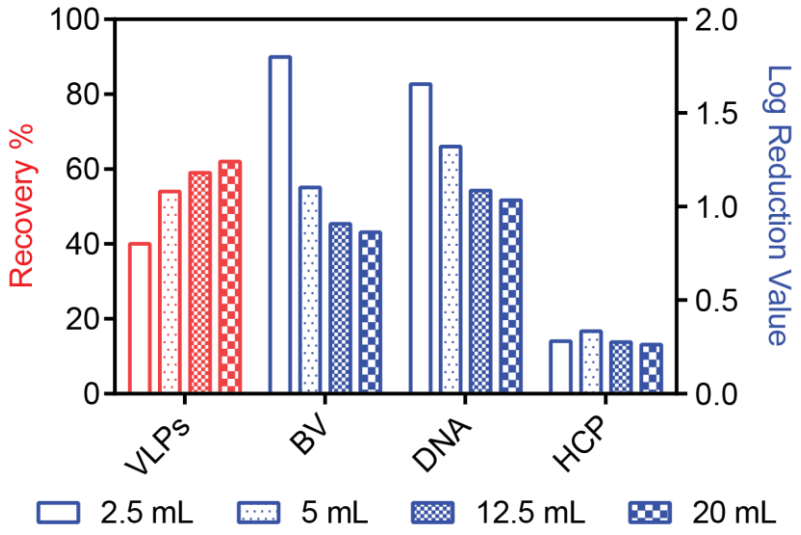


**Figure 14: Effect of loaded sample volume (2.5, 5, 12.5 and 20 mL) in RFC column**. Left axis refers to VLP recovery and right axis relates to the log reduction values of BV, DNA, and HCP.

### 4.1.3 Residence time optimization and impurities clearance evaluation

Determination of the optimal residence time (RT) for the RFC setup was performed by replicating the 20 mL sample injection at successively smaller RT. The relation between RT and both HCV VLP FT recovery and associated impurity clearance is represented in Figure 15. Maximum recovery is achieved at a residence time of 2.5 min, decreasing abruptly for higher or smaller RTs. Optimal removal of BV can also be found at the before mentioned RT. Nevertheless, its variation with respect to RT follows a different trend from HCV VLP recovery. Although the decrease of the BV LRV was moderate for smaller RT, BV LRV decreases abruptly, for higher values of RT. Contrarily to this tendency, both DNA and HCP LRV decrease monotonically with the increase of RT.

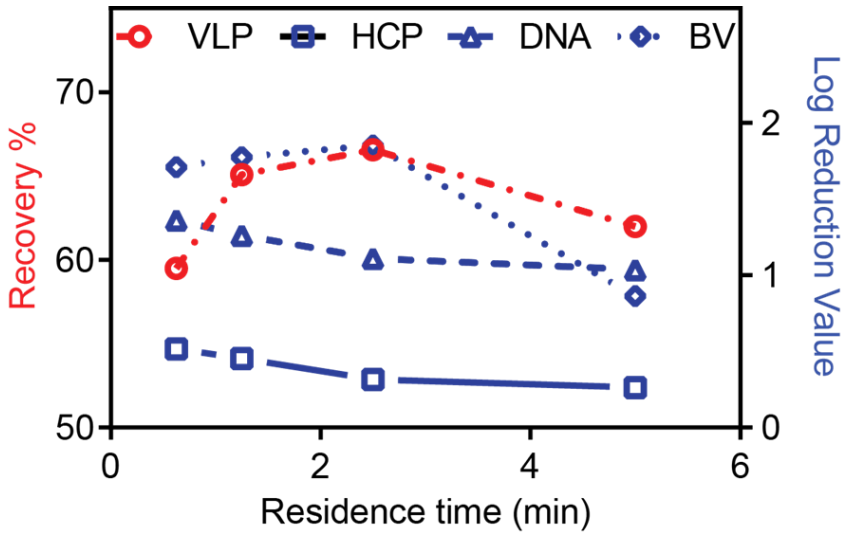


**Figure 15: Residence time evaluation.** Impact of residence time in HCV VLP recovery and Impurity clearance in the used RFC setup. VLP recovery refers to the left axis: ○; impurity removal in LRV refer to the right axis: □, Δ and ◊.

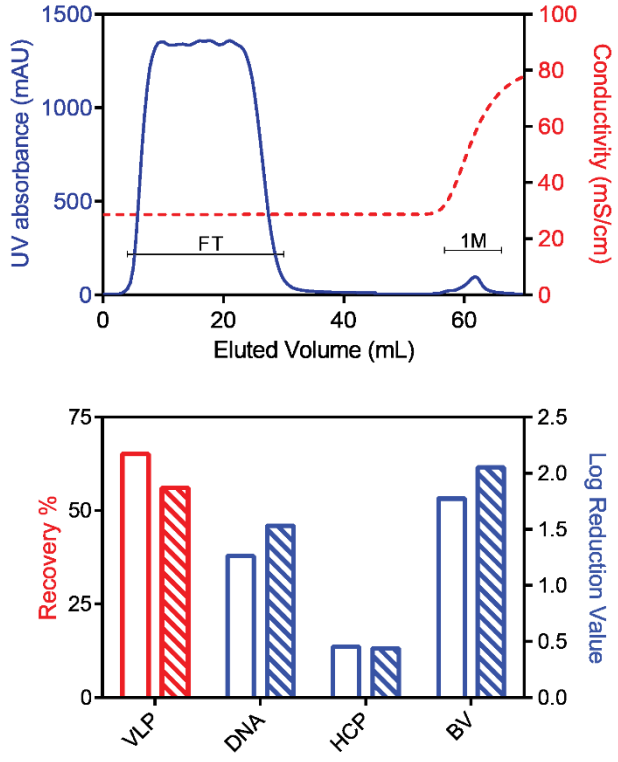


**Figure 16: - Effect of loaded sample ionic strength at a residence time of 1.25 min in the 5 mL radial flow column.** Top: chromatogram of the experimental run performed with a 300 mM NaCl concentration; Bottom: VLP recovery (left axis) and impurity clearance for the FT fraction (right axis) of each experiment, where the empty bars refers to the 300 mM run and the striked bars to the 270 mM experiment; Posterior elution at 1 M in each experiment yielded 13 and 12 % of VLP recovery (not shown).

According to the obtained results, in order to maximize selectivity, one should operate at lower RT. However, an abrupt decrease in VLP recovery is observable. A probable explanation is the increase in shear leading to VLP disruption. Although BV particles have a dimension similar to the VLPs and as such could be affected by shear in the same manner, its capsid structure is more robust, withstanding higher shear rates. Optimal VLP recovery was obtained with RT of 2.25 min. Nevertheless, at this point, a compromise between recovery and purity must be established.

Another RFC run was performed with a RT of 1.25 min and the impact of a 10% reduction on the NaCl concentration in the loaded sample was also evaluated. The chromatogram for the initial experiment and the variation of HCV-VLP recovery and impurity removal for the new conditions are represented in Figure 16. By reducing RT to 1.25 min it was still possible to achieve a VLP recovery above 66% and reach a considerable LRV for the impurities scoped. Fine tuning this strategy is still possible by varying the NaCl concentration in the loaded sample. A reduction of the HCV VLP recovery was observed, from 65% to 56%, and LRV of DNA and BV increased from 1.2 and 1.8 to 1.5 and 2.0, respectively. As seen before, HCP LRV did not evidence any observable variation. As it can be detected, due to the VLP recovery reduction from 66% to 56%, the removal of both DNA and BV can be further increased. HCP removal seems to be insensitive to this modulation in the loading conditions. These findings are in line with other purification strategies for adenovirus (Peixoto *et al.*, 2006) and enveloped viruses (Segura, Garnier and Kamen, 2006).

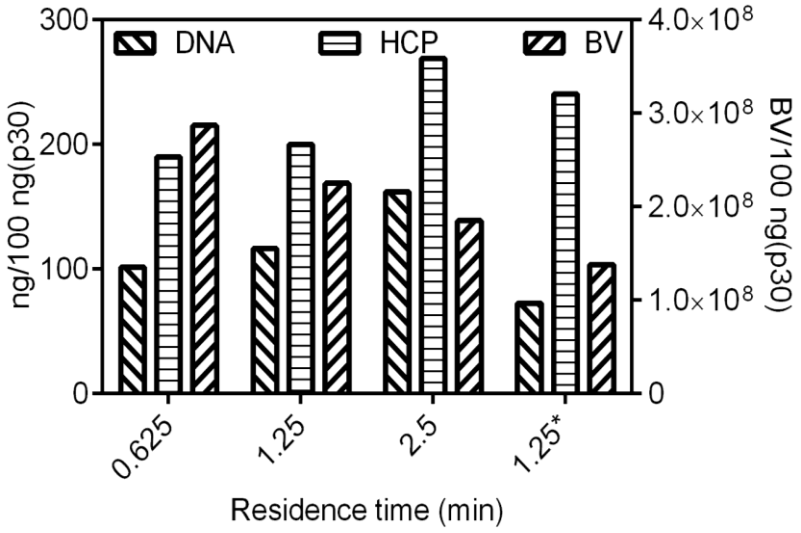


**Figure 17: Residence time optimization and impurities clearance evaluation.** Ratio of DNA, HCP and BV obtained for a basis of 100 ng of p30 protein. The experiment with a residence time of 1.25 marked with * denotes the optimization performed reducing NaCl concentration in the sample loaded.

At the current time, there is not a defined immunological dose for this vaccine, so it is possible to define impurity thresholds in this study. As referred before, VLP quantification is performed using an ELISA assay targeting a capsid protein, Gag p30. In order to understand the influence of the different parameters in the final purity of the product, a 100 ng basis of Gag p30 was used around which DNA, HCP, and BV levels were calculated. A deeper insight of the process performance is represented in Figure 17. The levels of DNA and HCP in the purified fraction increase with RT, contrary to what is observed for BV. This is related to the increase of VLP recovery with the increment on residence time from 0.625 to 2.5 minutes. The reduction of ionic strength of the sample loaded with a residence time of 1.25 minutes yielded the highest purity in terms of DNA and BV.

Therefore, the developed approach should be seen as consider a first step in developing a purification process for the current hepatitis C vaccine candidate, where further investment in other operation units of the process can bring an increase in product purity (Morenweiser, 2005; Segura, Kamen and Garnier, 2006; Wolf and Reichl, 2011). One of these improvements can be the implementation of an endonuclease treatment step, especially if DNA clearance is not adequate. As suggested by the literature, endonuclease digestion reduces the length of nucleic acids, improving DNA removal in diafiltration steps (Bandeira *et al.*, 2012; Cruz *et al.*, 2006). However, the use of endonuclease can increase the cost of the DSP and make the purification process not cost effective. Additionally, since the developed strategy is based on a FT approach, this purification step can be easily integrated with other unit operations such as continuous chromatographic purification (Godawat *et al.*, 2012; Nestola *et al.*, 2014, 2015; Rodrigues *et al.*, 2008; Silva, Rodrigues and Mota, 2012; Silva *et al.*, 2015) as a polishing step, or by combination with ultrafiltration/diafiltration.

# 5. Final Remarks

The production of complex biopharmaceuticals requires high concentrations and purities, making the downstream processing a critical component of the overall process. The upstream process has evolved over the past few decades, contrarily to the downstream processing that did not follow the trend in terms of throughput and scalability (Wolf and Reichl, 2011). The emergence of these complex molecules require more robust and scalable techniques to address the purity, potency and quality of the final product

Interest in mucins has been increasing due to their implications as targets for cancer treatment, in the field of vaccines and antibodies (Bergstrom *et al.*, 2016). However, mucins saccharides heterogeneity poses challenges in the downstream process development and analytical methods (Schömig *et al.*, 2016). The detection methods available are not suitable for process development because the results are not immediate. Moreover, there are no absolute quantification methods, being necessary to cross different tools with different principles (Harrop, Thornton and McGuckin, 2012). The method described in this thesis uses BLI technology to detect and quantify mucins in a fast, simple and label-free manner. This technique was able to identify the most suitable lectin, AAL lectin, to bind BSM and MUC5B mucins. Additionally, the LOD and LOQ obtained showed that the method described exhibit a higher sensitivity compared with the traditional ELISA assay. Moreover, competition assay allowed the identification of the minimal concentration to inhibit AAL-mucin association. These results can be applied for mucin purification strategies in downstream process development. For example, in affinity chromatography, AAL lectin can be used for mucin binding and L-Fuc for set up elution conditions.

The second part of this work described a rational design and implementation of a FT purification strategy for HCV VLP using AEX chromatography. Initially, the impact of process operating parameters and materials was evaluated using scaled down tools – scout on one mL columns and shake flasks experiments. The acquired results were used to translate to a RFC setup, thus providing an alternative to standard axial packed columns. Selected RFC operational parameters, residence time and ionic strength loading conditions were studied and their impact on the purification performance was assessed. Since the application of enveloped VLPs in pharmaceutical field is recent, the DSP for this particles needs to be improved and optimized (Nestola *et al.*, 2014). The described approach is a contribution to the development of a purification process for the current hepatitis C vaccine candidate. In the future several improvements can be made, for example, to increase product purity. The implementation of an endonuclease treatment step, even though increasing the cost of the DSP, can reduce the DNA content.

Finally, valuable tools for detection and quantification as well as for DSP improvement of emerging complex biopharmaceuticals and can be applied to improve new and already established purification processes.

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