A Lab-on-a-chip Device for the Purification of Monoclonal Antibodies

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Biotechnology

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Abstract

The demand for monoclonal antibodies (mAbs) has been steadily increasing in last years, due to their high efficacy in the treatment of several life-threatening diseases, and efforts are being made to develop more efficient processes that are able to produce large quantities of pure mAbs at reasonable costs. Downstream processing has been one of main points addressed in order to improve mAbs production, with main efforts directed to improve yield, productivity and purity. Lab-on-a-chip platforms have revolutionized process development, due to their miniaturization, integration and automation potential.

The host cell proteins secreted by two different CHO cell lines have been characterized in terms of size and charge and allowed to identify promising chromatographic conditions. Two different anion exchanger resins were then chosen, to allow the binding of the impurities while the antibody flows through the column without being retained. Q-Sepharose, a strong anion exchanger, was found to have a higher binding capacity and was selected to perform further optimization studies at microfluidic scale.

Breakthrough curves and binding/elution assays were performed in a microfluidic column using first model proteins (BSA and the purified mAb), then an artificial mixture of both proteins and afterwards the CHO proteins

– CHOPs (fractionated by protein A chromatography). Adsorption assays showed that BSA and CHOPs exhibit high retention under the conditions studied. Unspecific binding of mAb to the column can be reduced by increasing the ionic strength of the adsorption up to 20 mM NaCl, without affecting the binding of the impurities.

Keywords

Monoclonal antibodies; CHO cells; CHO host cell proteins; Ion exchange chromatography; Lab-on-a-chip

Resumo

A procura por anticorpos monoclonais (mAbs) tem aumentado constantemente nos últimos anos, devido à sua eficácia no tratamento de diversas doenças fatais, e têm sido feitos esforços para o desenvolvimento de processos mais eficientes que consigam produzir grandes quantidades de mAb puro a preços razoáveis. Os processos de purificação têm sido um dos principais enfoques para melhorar a produção de mAbs, com esforços

a serem direcionados para o melhoramento do rendimento, produtividade e pureza. As plataformas *Lab-on-a-chip* revolucionaram o desenvolvimento de processos, devido ao seu potencial de miniturização, integração e automação.

As *host cell proteins* secretadas por duas linhas celulares diferentes CHO foram caracterizadas no que diz respeito ao seu tamanho e carga, o que permitiu identificar condições cromatográficas promissoras. Duas resinas de troca aniónica foram escolhidas para permitir a adsorção das impurezas, enquanto que o anticorpo não é retido na coluna. A Q-Sepharose, uma resina de troca aniónica forte, foi determinada como tendo uma maior capacidade de adsorção e foi seleciondada para realizar ensaios de otimização à escala microfluídica.

Curvas *breakthrough* e ensaios de adsorção/eluição foram realizados em colunas microfluídicas usando inicialmente proteínas modelo (BSA e o mAb purificado), depois uma mistura artificial de ambas as proteínas e por fim proteínas CHO – CHOPs (fracionadas por cromatografia usando proteína A). Os ensaios de adsorção mostraram que a BSA e as CHOPs têm uma retenção longa nas condições estudadas. A ligação não específica do mAb à coluna pode ser reduzida pelo aumento da força iónica da adosrção até 20 mM NaCl, sem afetar a capacidade de ligação das impurezas.

Keywords

Anticorpos monoclonais; células CHO; CHO *host cell proteins*; Cromatografia de troca iónica; *Lab-on-a-chip*

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List of Abbreviations

2D-PAGE	2D polyacrylamide gel electrophoresis
AEX	Anion Exchanger
APS	Ammonium persulfate
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
CDR	Complementary-determining region
CEX	Cation exchanger
CHO	Chinese Hamster Ovary cells
CHOPs	CHO Host Cell Proteins
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DSP	Downstream processing
DTT	Dithiothreitol
EDTA	Ethylenediamine Tetraacetic Acid
Fab	Antigen binding fragment
FACS	Flourescence-activated cell sorting
FBS	Fetal bovine serum
FBS UL	Fetal bovine serum ultra-low IgG
FDA	Food and Drug Administration
gDNA	Genomic DNA
GS	Glutamine synthase
HTPD	High-throughput process development
IEF	Isoelectric focusing
Ig	Immunoglobulin
IL	Interleukin
FT	Flow-through
mAb	Monoclonal antibody
MMC	Mixed-mode chromatography
MTX	Methotrexate
PBS	Phosphate buffered saline

PDMS	Polydimethylsiloxane
pI	Isoelectric point
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tris-Base	Tris(hydroxylmethyl)aminomethane

I. Introduction

I.1. Monoclonal Antibodies (mAbs), an Overview

The pioneer work developed by Kohler and Milstein in 1975 (which led to a Nobel prize win in 1984) using mouse hybridoma cells for the production of monoclonal antibodies had a huge impact on medicine, paving the way to the development of a great amount of new and innovative sources for diagnostic and therapeutics based in monoclonal antibodies (mAbs). To date, there are more than 50 mAb-based drugs approved by the various regulatory agencies, proving this molecules high and growing importance in the biopharmaceutical status.[1]

The first mAb approved by U.S. Food and Drug Administration (FDA) was Orthoclone OKT3® (muronab-CD3), in 1986, which was produced by hybridoma cells technology. However, in the early times, there were several issues with the immunogenicity of these molecules due to their murine origin. To overcome this limiting factor, chimeric antibodies with the fusion of human constant regions and mouse variable domain ones where engineered. This chimeric mAbs (with "-ximab" prefix), Figure 1, are made of about 65% human content to minimize the risk of giving origin to undesired reactions against these molecules and one of the first to be released to the market was Infliximab®, however there were still some issues with the immunogenicity effect of the foreign parts.[1]

The next step on the improvement of mAb to become a viable therapeutic technology was the development of humanized mAbs ("-zumab"), **Figure 1**, by grafting the hypervariable regions that bind to the antigen (complementary-determining regions – CDRs) into a human antibody backbone, reducing the immunogenicity to 9%. Moreover, in the last few years, the efforts are focused on the development and production of fully human mAbs ("-umab"), such as Humira® (adalimumab) which was the first one to be developed, for the treatment of rheumatoid arthritis. This type of monoclonal antibody can be produced, namely, by technologies based on human hybridomas, however there is only a few of this hybridoma-derived mAb nowadays in the market due to the fact that is difficult to generate stable hybridomas secreting antibodies with functional activity and high affinity, and by immunized mice and by antibody libraries.[2, 3, 4]

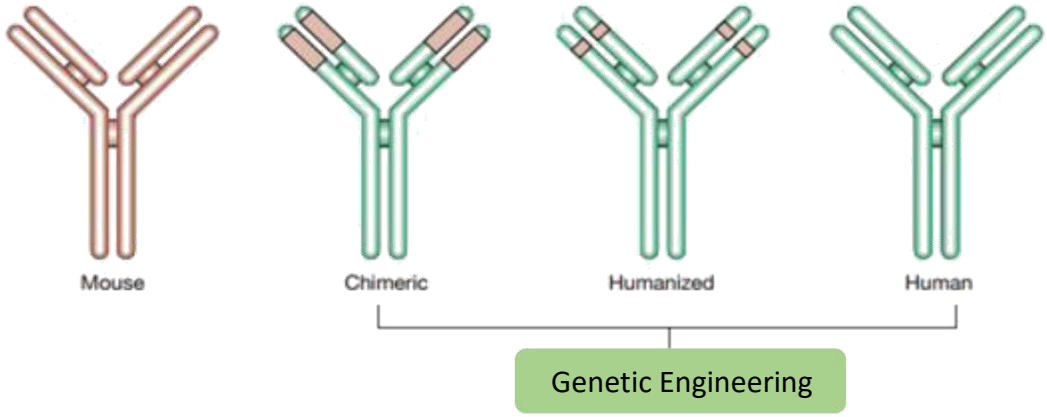

Figure 1 – Types of therapeutic mAbs. Mouse aminoacid sequences are shown in brown and human sequences in green. The variable domains of antibodies contain three such hypervariable CDRs, which form the antibody-binding site. Therefore, antigen specificity can be conferred by grafting appropriate mouse CDRs onto human variable regions, using genetic engineering methods.

I.1.1. Structure and Different Types

Antibodies, usually called immunoglobulins (Ig) are Y shaped and relatively heavy (with about 150kDa) molecules that belong to the glycoproteins family. They are produced by B-lymphocytes and can be secreted by these cells in their soluble form or can be attached to their surface and act as B-cell receptors (BCR). This BCRs allow the differentiation of B-cells into plasma B cells (antibody-secreting ones) or memory B cells (allow the body to react faster when an encounter with same antigen occurs), by binding to a specific antigen.[5]

This molecules are composed of four polypeptide chains: two identical heavy chains (HC) and two identical light ones (LC), covalently linked together by disulphide bonds in order to keep its shape (**Figure 2**). At the N-terminal end of the polypeptide chains there are regions that are variable in terms of its amino acidic composition from antibody to antibody, and they are called variable regions (V). The antibody has a bifunctional ability to bind to an antigen, through the Fab region (antigen binding fragment) and to immune cell surface receptors and some proteins through the Fc region (crystallisable fragment) in order to initiate and regulate host defense mechanisms. The specificity of an antibody is determine by the variable regions composition, especially in the CDR regions where the direct contact with the antigen occurs.[5]

There are five different immunoglobulin classes, distinguished between themselves by the type of heavy chain that they contain. IgA exist in both monomeric and dimeric forms and its secretory form (the dimeric one) objective is not to destroy antigens, but to prevent the entry of foreign molecules into the circulatory system. IgD is a monomeric isotype which is expressed in the plasma membrane of immature B-lymphocytes and produced in a secreted form as well (found in smaller amounts in blood serum), however its function is still unknown. IgE is a monomeric antibody only found in humans and responsible for the body's response against parasitic invasion and allergic reactions. IgG is the most predominant class of immunoglobulins present in the human serum (about 75%) and takes part in the secondary response of the body to a specific antigen. IgM occurs

as a pentamer or monomer (expressed on the plasma membrane of B-cells) and its action takes place, predominantly, during the primary immune response to most antigens. Several information about each one of the five classes of immunoglobulins will be presented in **Table 1**.

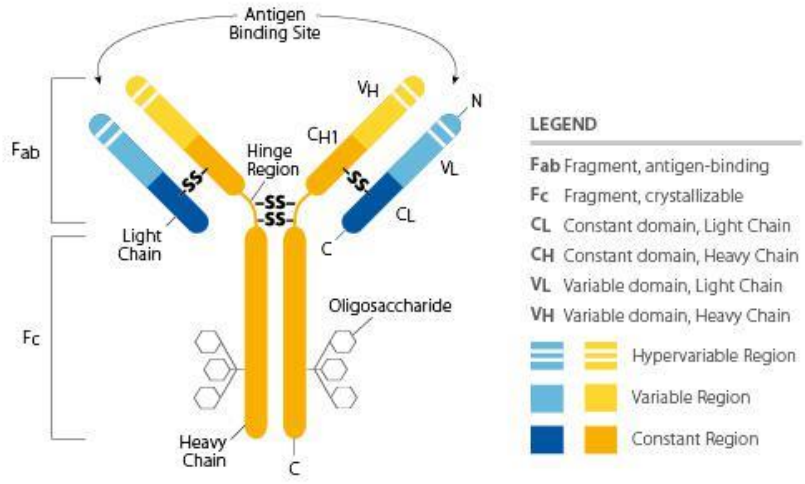

Figure 2 – Immunoglobulin (Ig) molecule. All immunoglobulin monomers are composed of two identical light (L) chains and two identical heavy (H) chains. Light chains are composed of one constant domain (CL) and one variable domain (VL). The heavy chains are covalently linked in the hinge region and the light chains are covalently linked to the heavy chain. The variable domains of both the heavy and light chains compose the antigen-binding of the molecule, termed Fv. Within the variable domains there are three loops designated complementary-determining regions (CDRs) 1,2, and 3, which confer the highest diversity and define the specificity of antibody binding.

Table 1 – Different antibodies classes in terms of function, structure, molecular weight, location, and serum concentration.

Type of Ig	Type of Heavy Chain	Function	Structure	Molecular Weight (~kDa)	Location	Serum Concentration (mg/mL)
IgA	α	Defense against invading pathogens by blocking antigens binding to epithelial surfaces	Monomer **Dimer**	320	Intestinal tract and external secretions	1-4
IgD	δ	Unknown	Monomer	180	Lymphocyte surface	0-0.4
IgE	ε	Parasite invasion response; allergic reactions	Monomer	200	Serum; Eosinophils, mast cells and basophils surface	$1x10^{-5}$-$4x10^{-4}$
IgG	γ	Infection control of body tissues, predominantly during secondary immune response	Monomer	150	Serum and extracellular fluid	10-16
IgM	μ	Primary response to antigens	**Pentamer** Monomer	900	Serum	0.5-2

I.1.2. Monoclonal and Polyclonal Antibodies

The activation of a large number of B-cells is usually involved in the response to the entry of an antigen in the body, resulting in the production of an enlarged number of antibodies with different targets/specificity, each targeting a different and specific binding site (epitope – antibody binding site). This mixture of antibodies is called polyclonal antibodies. Monoclonal antibodies are generated by one single B-cell and only recognize one specific epitope of the antigen in question.[6]

The choice for one of the other type of antibodies depend on the purpose, having both their own advantages. MAbs are highly specific for one type of epitope, having great advantages when the study is related to therapeutic uses and, as said before, are mainly produced in mice, by hybridoma technology. Polyclonal antibodies are produced by immunization of a chosen animal, followed by serum collection and purification, and can be obtain much faster, cheaper and with less work load than mAbs.[6]

I.1.3. Market

Since the first mAb was launched to the market in 1986, the growth of approved products as increased rapidly over the years, to about 75 million USD in sales in 2003, which represented approximately half of the total sales related with all biopharmaceutical products (**Figure 3**). It is expected that about 26% of mAb products that enter in Phase 2 human clinical trials will be approved and reach the market in a period of seven years. Since there is about four new approvals *per* year, it is expected that by 2020 there will be around 70 mAb products commercialized and at the growth rate observed, by this year sales will reach a value of 125 billion USD.[7]

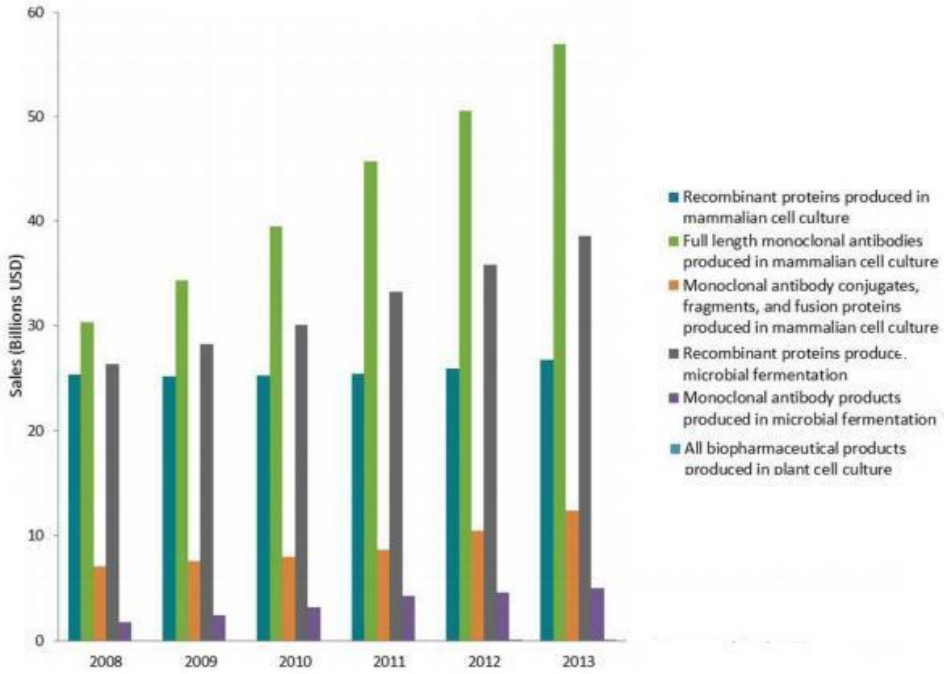

Figure 3 – Sales of Biopharmaceutical products as a function of product type.

The immense demand for the development of new and more efficient mAbs resulted in a large improvement in the methods used for their production, as well as in approaches taken to design and optimize the processes used. There are several explanations for this increased demand on mAb products such as: the advances made in understanding diseases at molecular level, providing mAbs a faster and lower risk platform for the initial studies (if successful can easily be launched first than other products) or the need for the development of new and more effective products against cost-sensitive conditions, like asthma and cancer, result in opportunities for this products to increasingly dominate the market.[7]

I.2. Monoclonal Antibodies Production

Nowadays, the treatment of innumerous diseases is possible via the use of recombinant proteins (particularly mAbs) as therapeutic drugs. These proteins are produced by cultivating genetically engineered cells that host the genes encoding for the product of interest. Therapies related with the use of mAbs usually require a large dosage, for a long period of time, increasing the need for the development of large-scale processes, which at the same time must be cost and time efficient.[8]

Although mammalian cells are difficult to work with and have a low yield and shear sensitivity, they are widely used for mAb production purposes since it is possible to enhance cells specific productivity by medium or environmental parameters optimization. Despite the existence of many different mammalian host cell culture platforms for the production of therapeutic proteins, the most commonly used include NS0 murine myeloma cells, PER.C6® human cells and especially Chinese hamster ovary (CHO) cells.[8]

I.2.1. CHO Cell Line

The most widely used cell line for the production of mAbs has been, for the past decades, the CHO cell line. These cells are used nowadays for therapeutic protein (including antibodies) manufacturing were originated from an immortalized cell line that came from a primary culture of Chinese hamster ovarian cells. The preference shown is due to the fact that: this cell line allows the overtaking of one of main disadvantages of using mammalian cell lines for the production of proteins, namely low specific productivity, since they have very efficient gene amplification systems; CHO cells can perform post-translational modifications, producing proteins with glycoforms that are bioactive in humans; they are able to adapt and grow in suspension and in chemically defined serum-free medium, allowing reproducibility and eliminating the use of very challenging purification processes; finally, since CHO cells have already been used for several decades, the community is much more familiarized with this platform, such as development/production institutions, regulatory agencies and suppliers, allowing products to be accepted and commercialized faster.[9]

The first biotherapeutic protein produced in CHO cells was Activase® (a tissue plasminogen activator) in 1986 and, nowadays, over 70% of approved and commercialized recombinant proteins are produced in CHO cells (the major slice concerns to mAbs, **Figure 4**).[10]

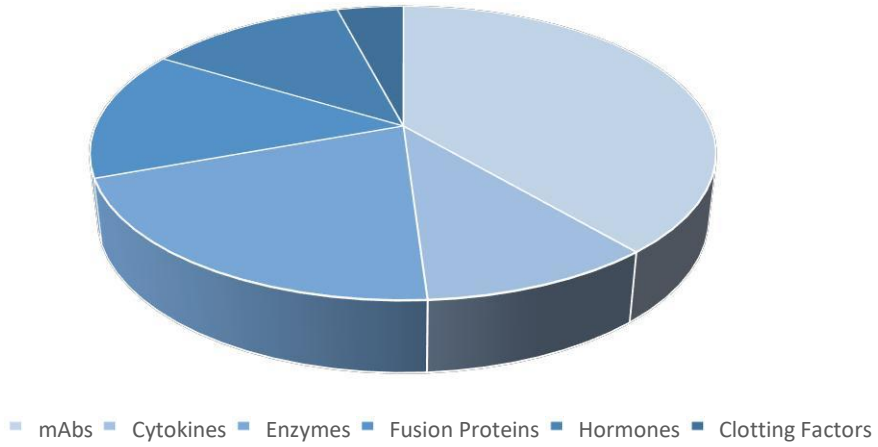

Figure 4 – CHO cell line derived products

I.2.2. Cell Line Development

The production of mAbs in mammalian cells is possible by transient or stable transfections, however the latter is used the most in large-scale production. The cell line development begins with the transfection of the cells chosen with plasmid vectors, already optimized for mAb production, carrying genes encoding for the antibody's light and heavy chains, for selection markers, which can be metabolic or antibiotic, and to enable the plasmid to replicate in bacteria. To achieve higher levels of antibody expression there is the possibility to also include in the plasmid a strong promoter (usually viral – CMV) and an elongation factor alpha (EF1α) promoter.[9]

There are two gene amplification systems that are used the most for the selection of CHO cells: the dihydrofolate reductase (DHFR) system that uses methotrexate (MTX) resistance, and the glutamine synthase (GS) system, which uses methionine sulphoximine (MSX). [9]

- The DHFR/MTX system is usually used together with DHFR-deficient CHO cells like DG44 or DXB11. The DHFR enzyme takes part in the reduction of dihydrofolate to tetrahydrofolate, which is necessary for the nucleic acid metabolism. The selection is performed in a medium without hypoxanthine and thymidine so that only the cells that incorporated the DHFR gene may survive. Next, the amplification, is done by the addition of increasingly concentrations of a folic acid analogue, MTX that inhibits the activity of DHFR, need for the cells to amplify the DHFR gene copy and, consequently, to survive. The genes encoding for the mAb chains, which are located in the same vector, will also be amplified;
- The GS/MSX system acts similarly to the one present before. The GS catalyzes the formation of glutamine from glutamate and ammonia, allowing the cells that were well transfected to survive in a medium without glutamine. In mammalian cells (with endogenous levels of GS), there is the need for the use of a GS inhibitor that acts as MTX in the DHFR/MTX system, the MSX, forcing the cells to amplify

the GS and the product of interest genes. Furthermore, this system requires a lower number of recombinant gene copies per cell, making the selection of high-producing cells process much faster.

After transfection, there is the need for choosing single clones to perform the scale-up and characterization of the final product. This step of selecting high mAb-producing clones is more time and labor-consuming because of the random integration and amplification that occurred. To overcome this difficulties and to improve processes' consistency, people started to rely on automation. One of this new methods developed was flow cytometry and fluorescence-activated cell sorting (FACS) for the high-throughput selection of high mAb producing cells (the level of secreted proteins is proportional to the amount of protein on the cell surface).[10]

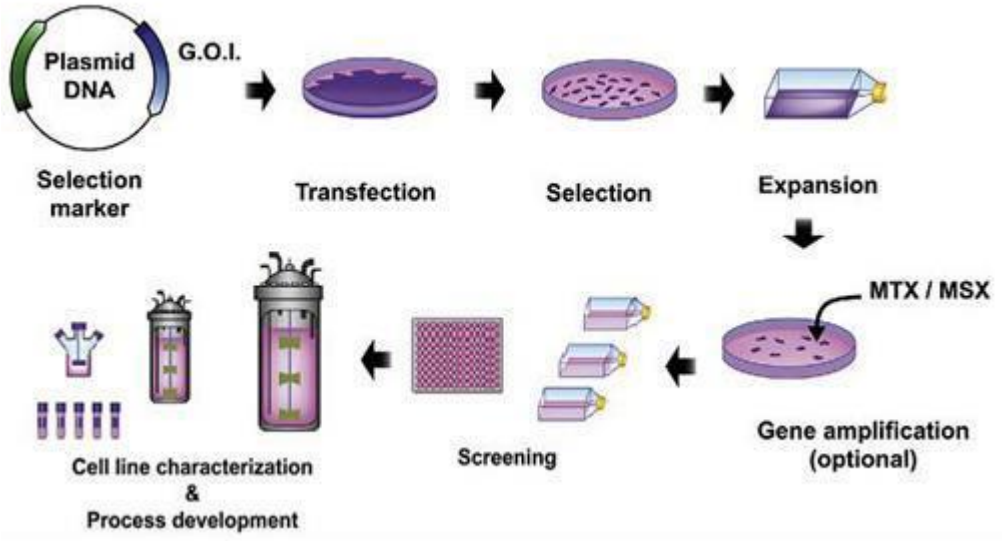

Figure 5 – Cell line development processing.

In the end, the chosen clones are expanded through a number of passages and each is evaluated and characterized in bioreactors mimicking the conditions found in large-scale production facilities. A single production cell line, the one that has the better results in the parameters evaluated, is then banked as frozen vials for future use.[10]

I.2.3. Culture Medium (Serum-based vs Serum-free)

Cell lines used for the manufacturing of protein products usually have complex nutrient requirements that are different for each type. So, finding a culture medium that suits the cells that one wants to use is a very challenging process. It is necessary for the culture conditions to mimic the ones find *in vivo* (with respect to oxygen supply, temperature and pH) and the medium must supply the culture with all nutrients required for the cells metabolism, growth and proliferation.[11]

In early days, the type of medium that was used the most to meet the requirements that cells need to grow and proliferate was serum-based and bovine serum was the supplement of choice. Serum provides all essential nutrients that cells need, including, for example, cell attachment factors, growth factors, macromolecular proteins and anti-oxidants, apart from a high concentration of albumin that helps protect cells from stress factors inherit to bioreactor culture, being thus used as a universal supplement for most type of animal cell culture

mediums. Regarding CHO cells, they have not a lot of problems with serum requirement, growing with 10% FBS (Fetal Bovine Serum), with a doubling time of 12 hours at 37^{o}C. Despite all of its benefits and meeting the requirements needed for cells to grow, there were some issues to the industrial side of therapeutical protein production brought by serum, like batch-to-batch variability, difficult and costly downstream processing in order to remove serum contaminants from the end-product and the risk of transmission of animal diseases to humans (virus-positive serum). Regulatory agents have, nowadays, a lot of constraints, orientating the biopharmaceutical industry to the development of serum and protein-free medium.[11, 12]

Currently, animal cells are being grown in serum-free medium (SFM) to work around all the disadvantages of having serum when culturing cells, maintaining proper cell growth. In this case, there is the need for the addition of several of the components present in the serum to the basal medium. Some of the supplements used in SFM includes hydrolysates, a mixture of aminoacids that provide nutritive support, which can be non-animal; albumin, a protein present in blood plasma that binds toxin components, protects cells from shear stress and provides transport to fatty and aminoacids, and is available in a recombinant form; growth factors; and hormones like insulin that are essential for cell proliferation.[12]

Nowadays, there are several types of formulated medium, and the tendency is to increase its definition and to eliminate any components with animal origin.

I.3. mAbs Purification

Downstream processing (DSP) development has been one of main focus points when trying to improve monoclonal antibody production, and is mainly directed to improve yield, productivity and purity. DSP is the key step for the development of biotherapeutics since it accounts for 50-80% of total manufacturing costs. Nowadays, the sources from which antibodies are isolated are no longer limited to body fluids of immunized animals (blood, essentially), but to engineered cell cultures as well. Traditionally, purification workflows involve an initial enrichment step (usually precipitation or filtration), an intermediary purification to 40-90% purity and a final purification to ~100% purity.[13]

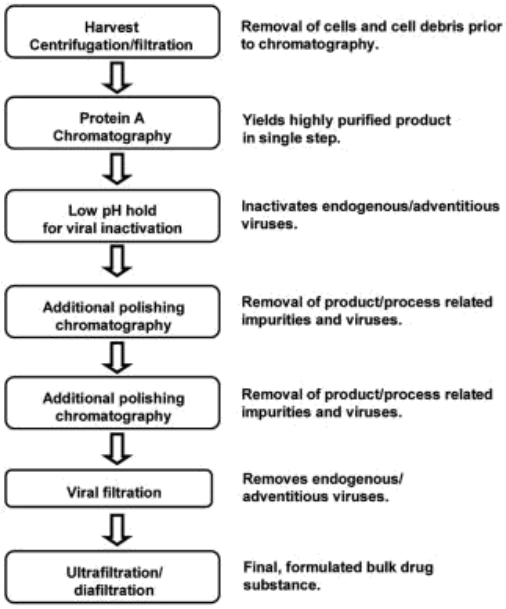

Figure 6 – Typical mAb recovery process.[13]

A generic process for mAb purification (**Figure 6**) assumes that this process can be applied to all monoclonal antibodies, the same way. However, despite all the advantages that this approach may bring to biotechnological companies, like resources and time reduction to develop and improve a new and specific process for each of the products of interest, it is not completely feasible without performing some optimizations.

I.3.1. CHO Cell Line Supernatant Impurities

Given the importance and market share of monoclonal antibodies and knowing that this molecule are secreted from the cell where they are produced and then recovered from the culture's supernatant, there is the need for several downstream operations in order to purify the end-product, by removing CHO host cell proteins (CHOPs), gDNA and lipids. This downstream processing of mAbs from cell culture supernatants usually rely on centrifugation and filtration steps to harvest the cells, followed by several chromatography steps for capture and polishing of the product (**Figure 7**).[14]

An important quality control parameter for determining the process robustness is the abundance of CHOPs in the final product. This impurities must be removed to less than 1-100ppm, in order to mitigate the risk of adverse clinical effects. This contaminant is either secreted from the host cell or released into the culture's supernatant when cell lysis occurs. Tait et al. demonstrated in 2012 that the majority of CHOPs that appear in the supernatant result from lysis and breakage of cells associated with loss of viability and not due to secretion. The important step to perform is to identify, afterwards, those CHOPs that sustain with the product of interest, in order to design easily, alternative recovery strategies to reduce the level of this problematic proteins and to improve the recovery of the product, namely by developing effective chromatographic processes.[14, 15, 16]

Apart from CHOPs, one of the regulatory issues related with the use of animal cells is the clearance of gDNA. Although not considered a risk factor for a patient, it needs to be reduced to very low levels, specifically up to 10ng of gDNA per dose (approved by the World Health Organization and the European Union). Thus, a very sensitive method of detection and quantification is needed, such as qPCR.[17, 18]

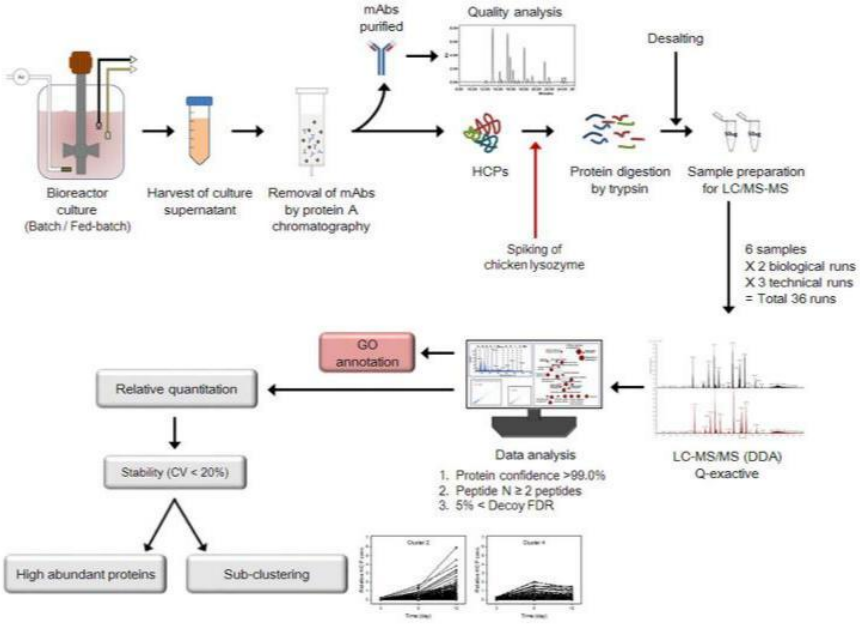

Figure 7 – Workflow for the characterization of mAbs and identification of HCPs in culture supernatants.[16]

Although all the techniques that are perform are important and have its own purpose for the purification of mAbs (mostly focused on purifying IgG, the most abundant type), below, a more profound overview about the different chromatographic approaches that can be used will be done.

I.3.2. Chromatography Techniques

I.3.2.1 Protein A Chromatography

The most selective and most used chromatography is affinity chromatography, which allows the separation based on the reversible interaction between a protein and a biospecific ligand, covalently attached to a matrix (Figure 8). The most applied affinity system for the purification of IgG involves the *Staphylococcal* protein A and small derived ligands. The affinity of protein A to IgG was one of the first interactions to be studied and developed for the development of new and improved systems for protein purification. Despite all its advantages, ligand stability has been an issue.[19]

Protein A is a protein anchored to the cell wall of *Staphylococcus aureus* bacteria and is characterized by having five homologous IgG binding domains that share 65-90% of sequence identity and a molecular mass of 54kDa. The degree of purity that can be obtained is around 95%, being removed process related impurities like host cell proteins and gDNA. Elution conditions of this initial chromatographic step vary accordingly to the next unit operations. Sorbents for protein A can be bought accordingly to the origin of the protein A ligand (recombinant, expressed in *E. coli*, or wild type) and matrix composition and bead and pore size can lead to changes in available surface area or resin compressibility, which are factors that can easily influence the performance of this initial chromatographic step and consequently the entire downstream processing.[20]

As mentioned, this step's goal is to capture the product of interest from a harvested cell culture fluid (HCCF) and the removal of impurities that can be present in it, and it also serves as a volume reduction process, resulting in a product concentration of 5-10 fold. Usually, the HCCF is loaded to the column around pH 7 and this step is the rate-limiting stage of the entire processing. After the loading step is done, a washing step is necessary for the removal of non-specifically bound impurities. However, this impurities may bind to the hydrophobic regions of the mAb, thus the pH of the intermediate wash need to be as low as possible, but not as low as to initiate mAb elution. Since small pH changes in the elution step can affect product stability as well as result in the formation of aggregates, compromising drug safety and raising the need for posterior purification steps, this step should be done at a pH value as high as possible without compromising product yield. In the end, the column is regenerated for further use, since a Protein A has a lifetime of around 200 cycles. One of the major difficulties of this type of chromatography is the co-elution of mAb and ligand that occurs by cleavage of the interdomain sequences, effect that can be diminished by the addition of EDTA to the HCCF in order to inhibit protease activity.[20, 21]

Despite all the problems related with Protein A chromatography, this is a very efficient and selective process for IgG purification and the most used by biopharmaceutical industry nowadays. The demand of mAb-based therapeutics and the requirements imposed by regulatory agencies led the path for the development of alternative strategies to Protein A, like cation exchange or multimodal resins, which will be discussed below.[21]

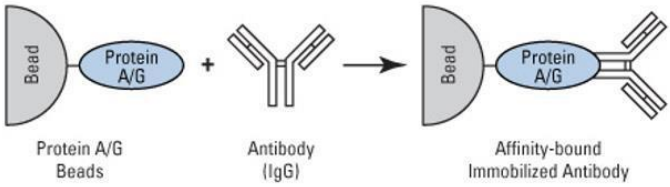

Figure 8 – Protein A chromatography principle.

I.3.2.2. Ion Exchange Chromatography

Ion Exchange Chromatography (IEX) relies on the separation of proteins accordingly to their surface charge and the charged groups that contribute to that charge have different pKa values. This type of purification technique takes advantage on this protein specific relationship of surface charge and pH. Reversible interactions are established between charged molecules and oppositely charged matrixes and are controlled having as goal binding or elution. Isoelectric point of a protein is the pH value at which the protein has no net surface charge. When the pH is above the pI, a protein has negative surface charge and will bind to a positively charged matrix or anion exchanger (AEX); when the pH is below the pI, a protein has positive surface charge and will bind to a negatively charged matrix or cation exchanger (CEX). However there are other kinds of interactions that may occur, like van der Waals' forces or non-polar interactions.[22]

Usually, pH and ionic strength of the buffer are selected making sure that the target protein binds to the matrix and the other proteins (the ones that do not have the same surface charge) will flow through and be eluted in the beginning of the process. After the undesired proteins have been eluted and the column washed, the

conditions are changed in order to allow the elution of the target/bound protein. The most used method to allow this elution step is to increase the buffer's ionic strength (increasing salt concentration), competing the salt ions with the bound molecules for the charges on the matrix, or its pH. As an alternative, the conditions can be established in order for the impurities to bind to the matrix and the target protein to flow through the column.[22]

Although IEX chromatography has the possibility of replacing Protein A as a primary capture step in mAb purification, it is normally used as a polishing step. Compared with Protein A chromatography, non-affinity processes can offer some advantages like higher binding capacity, lower resin cost (30 times less expensive) and greater resin longevity. As referred before, CHOPs and gDNA are the most important impurities and the ones that are in greater amount in CHO cell line supernatants. This proteins have a wide range of isoeletric points, so the pH at which the loading process occurs will influence which ones will flow through or bind to the matrix and the choice of buffer conditions are very important to improve capture efficiency.[23]

There are several studies comparing the level of clearance of CHOPs with using processes that have as one of its steps a Protein A chromatography process and processes without this step. Follman *et al.* tested several different resins, including a cation-exchanger (SP-Sepharose) and an anion-exchanger (Q-Sepharose) and the same CHOPs clearance (less-than-detectable) was observed with both processes.[23]

- Q-Sepharose – strong anion-exchanger containing a quaternary amine group as ion exchange group and is shown to be charged over a wide pH range.
- SP-Sepharose – strong cation-exchanger containing a sulphopropyl group as ion exchange group.

I.3.2.3. Multimodal Chromatography

Multimodal or mixed-mode chromatography (MMC) is becoming a popular chromatographic process in biopharmaceutical industry due to its selectivity to a specific target. MMC is a method in which solutes interact through more than one type of interaction or mechanism with the matrix, including ion exchange, hydrogen bounds or hydrophobic interactions, at the same time (**Figure 9**).[24]

In order for MMC to be established as a commercially viable option for mAb purification process there is the need for the clarification of interactions between ligands with the antibody and major impurities (like CHOPs) and for the development of an efficient method to apply this type of chromatography. Not only is MMC a more selective approach but also has salt-independent adsorption and easy elution by charge repulsion. Ligands used in this kind of process usually have an aliphatic or aromatic group as hydrophobic moiety and an amino, carboxyl or sulfonic group as ionic moiety. Heterocyclic groups are good ligands because of their hydrophobicity.[24]

Some of the developed resins have shown promising results regarding protein binding. Capto™ MMC (2-benzamido-4-mercaptobutanoic acid ligand) is a weak cation-exchanger that contains a carboxyl group for ion-exchange, an amine group for hydrogen bonding and a phenyl group for hydrophobic interactions. Elution from this matrix requires an increase in the pH value and salt concentration.[19] Results published by Toueille *et al.*, using two different types of resin (MEP Hypercel™, mercapto-ethyl-pyridine ligand, and HEA Hypercel™,

hexylamine ligand) as an alternative for mAb purification showed that they still are not able to reach as good selectivity as the one observed with affinity sorbents.[24, 25]

Other study was done using four different multimodal resins (namely, Capto adhere, PPA Hypercel, HEA HyperCel and MEP HyperCel) and showed that they can be used as capture step for mAb purification from a CHO cell culture supernatant and the authors were able to define the optimal conditions for that purification to occur. The elution fractions were afterwards analyzed by mass spectrometry to determine the proprieties of CHOPs that could impact the purification process. They demonstrated that the population of CHOPs that contaminated the mAb fraction was different for each one of the different resins and by identifying CHOPs should help on the development of a process to eliminate this impurities.[26]

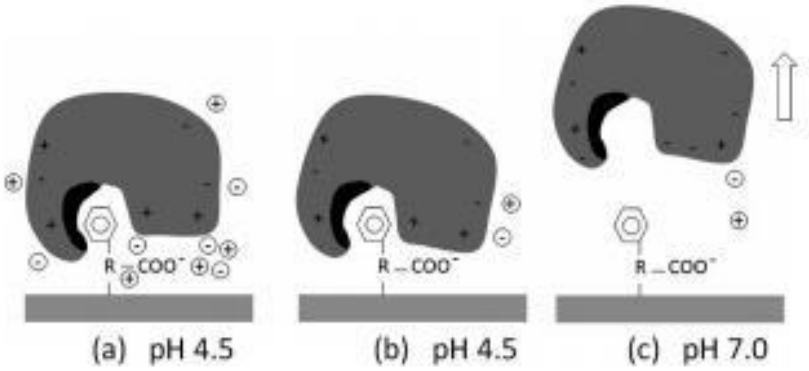

Figure 9 – Multimodal chromatography principle. The binding and elution strategy of a target protein to a matrix containing a ligand with both hydrophobic and charged groups.[26]

I.4. Future Trends in Downstream Processing – Continuous Bioprocessing

There are two drivers behind the current interest and demand in continuous bioprocessing (**Figure 10**). The first is the increasing pressure faced by the biopharmaceutical industry to reduce the price of biotherapeutics, leading to a tremendous interest in finding ways to reduce the cost of all aspects of drug discovery, clinical trials, and manufacturing. In addition to the economic factor, continuous processing also has the potential to provide significant improvements in product quality through enhanced control and uniformity of the manufacturing process.[27]

As examples of batch processes where variability is present and is harmful for the overall process is mAbs secreted by CHO cells at the start of the cell culture, which are produced in a nutrient-rich environment with few lysed cells, remaining remain within the bioreactor for multiple days before downstream processing occur. The situation is dramatically different for an antibody produced right before cell harvesting. Other example regards proteins loaded on a column at the start of a chromatography step, and that remain bound some time, while proteins near the end of the loading process remain bound for only a small fraction of that time.[28]

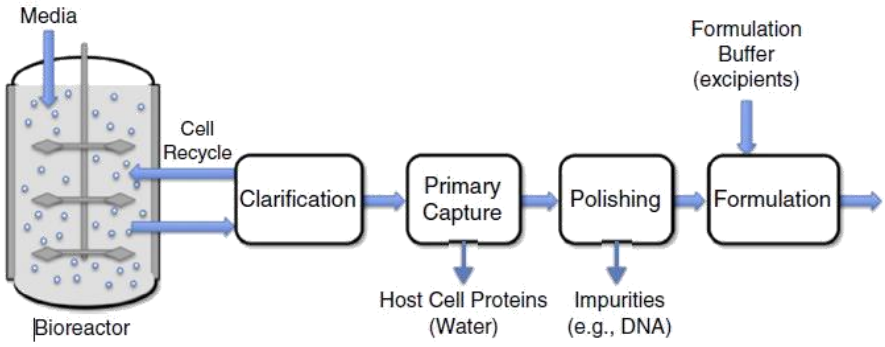

Figure 10 – Representation of a generic continuous downstream bioprocess.[28]

Regarding chromatography, there is no issue in scalability when this process is applied to biomolecules. The simplest way to operate chromatography in a continuous mode is to run several columns in parallel or simultaneously. While one column is being loaded, the next ones are washed, eluted, regenerated, and finally re-equilibrated. A number of approaches have been developed to achieve continuous chromatographic separations, not only based on multi-column systems, including periodic counter-current chromatography (PCC), simulated moving-bed chromatography (SMB), and multicolumn counter-current solvent gradient purification (MCGSP), but also rotating chromatography devices, like annular chromatography already used for the purification of mAbs from a cell culture supernatant. Within the first type of systems enumerated, which provide cyclical operation, the product concentration in the eluent stream varies with time during each cycle.[29]

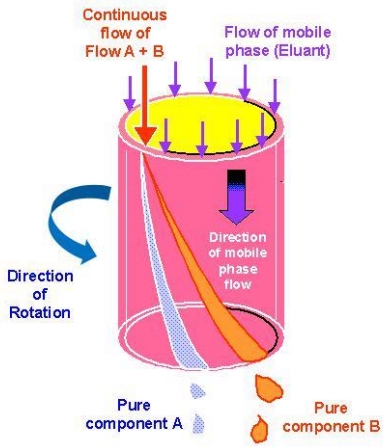

Figure 11 – Representation of the annular chromatography principle.

Continuous processes are of lower cost, but need to be balanced against the risk associated with the implementation of technologies that have yet to be proven in commercial operation. There are also questions about equipment robustness and operability, maintaining sterility over long processing times, and speed of development is absolutely crucial in bringing new therapies to market. Although unlikely for continuous processing investment to replace the one existent in large-scale batch manufacturing, there will be exciting opportunities for the implementation of fully continuous or partially continuous processes for facilities designed for production of important biotherapeutic products, like monoclonal antibodies.[27, 28]

I.5. High-Throughput Process Development

High-throughput process development (HTPD) platforms involve the combination of several features like miniaturization, automation, and parallelization and provide a systematic approach for time- and resource-efficient process development. The relevance of this platforms is high considering the strict requirements regarding time and resources faced by the biopharmaceutical industry nowadays.[30]

Regarding the upstream process development, several systems have been proposed to address the throughput requirements, including small shaker flasks, microtiter plate and spin tubes. This devices were aiming to perform medium screening, to study clone selection and develop batch/fed-batch culture.[31] Microfluidic bioreactors have also been used, providing several advantages from the HTPD perspective. They are based on small working volumes, between 5 and 700 μL, enabling parallel assembly of single bioreactors in small modules, thus providing the required experimental throughput.[32]

The development of high-throughput platforms for downstream processing, especially for early stage evaluation of chromatography conditions has been gaining increasing attention. Most of these approaches are based on 96-well microtiter filter plates (resin volume 20−200μL), prepacked pipette tips and recently, miniature columns (resin volume ∼200μL). The main disadvantage of these technologies is the lack of resemblance with conventional column chromatography operation, in the sense of having a continuous flow of a mobile phase through a stationary phase. Microfluidic platforms comprising chromatography beads inside microcolumns represent a more capable solution to address this problem, offering additional advantages such as the very low reagent consumption and faster result output.[33]

Plate and tip based methods are usually used for investigating the effect of adsorption conditions on binding capacity and/or impurity removal. Typically these experiments focus on the determination of static binding, however it has been shown that both microtiter filter plates and pipette tips filled with resin can be used to predict resin performance in a packed column under some process conditions, such as residence time, pH and conductivity. The mini/micro columns can be used not only for determination of both static and dynamic capacities, but also for elution studies and, in principle, for any type of studies that can be performed using a standard chromatographic system.[34, 35]

Pinto *et al.* reported the development of a microfluidic method to screen multiple adsorption and elution conditions early stage multimodal chromatography optimization. The technique developed can be used with any target molecule or resin, assuming a previous labeling procedure with a photostable, pH-insensitive fluorophore. The attractiveness of this high-throughput approach rely on the low amounts of resin and antibody molecules that are required to perform the screening of conditions in microscale (around a 10-fold decrease).[33]

The impact of high-throughput approaches on process development efficiency evolution and overall process knowledge is significant and will become increasingly important. The systems used for kind of approach will continue to evolve over the coming years as technology and automation supporting high-throughput analytical and process development techniques improve.[31]

I.6. Microfluidic Approach

Microfluidics is the field of study that involves fluid movement through micron-sized chamber and channels. It can be considered a mix of several areas, like fluid mechanics, chemistry or surface science, and to perform research in this field it necessary for the integration of this different disciplines.[36]

Lab-on-a-chip devices are an immense revolution in lab work, due to its benefits, namely miniaturization, integration and automation to many different industries. The increasing use of this microfluidic chips is related with the advantages over non-microfluidic devices including smaller sample volume requirements, low production costs by device, speed of analysis, portability, parallel sample processing and compatibility with other techniques. PDMS (poly(dimethylsiloxane)), glass and silicon are the most used materials when fabricating microfluidic devices. Generally, they are of low cost, able to be machined and possess a number of proprieties that make then usable for a number of different applications.[36]

The global microfluidics market witnessed a significant and consistent growth during the last decade due to the growth of biopharmaceutical research funding and by the rising demand for point-of-care (POC) testing. However, the reluctance of researchers to use microfluidic technology are inhibiting an even bigger growth of this market to a certain extent. Since the first patent was applied in 1991, the microfluidic market experienced an immense growth (550 applied patents in 2013) and is expected to be worth a total of 8.78 billion USD by 2021 (it was worth around 2 billion USD in 2015).[36]

Garza-García *et al.* studied and developed a micro-fluidic device for the production of mAb on two different substrates (PDMS and PMMA – polymehtyl methacrylate) and using an antibody-producing CHO cell line. The results suggested that using micro-channel devices the same mAb production can be achieved, using only 1% of the total process volume and of the process time. The device developed by the authors has several applications, like optimization of CHO cell production or the assessment of a cell line productivity.[37]

This type of device can also be used for protein study to perform, for example as substitute for the conventional 2D-PAGE. Li *et al.* (**Figure 12**) integrated a protein concentration/separation system that combined IEF (isoelectric focusing) and a SDS-PAGE on a microchip. In this device, proteins are electrokinetically transferred into an array and then resolved by SDS gel electrophoresis. This electrokinetic transfer of proteins is performed to introduce all the focused protein bands from the first to the second separation dimensions, allowing for a parallel size-based separation in a solution containing SDS. The work developed demonstrated potential to transform the conventional 2D-PAGE into a portable, rapid and reproducible protein analysis technology.[38]

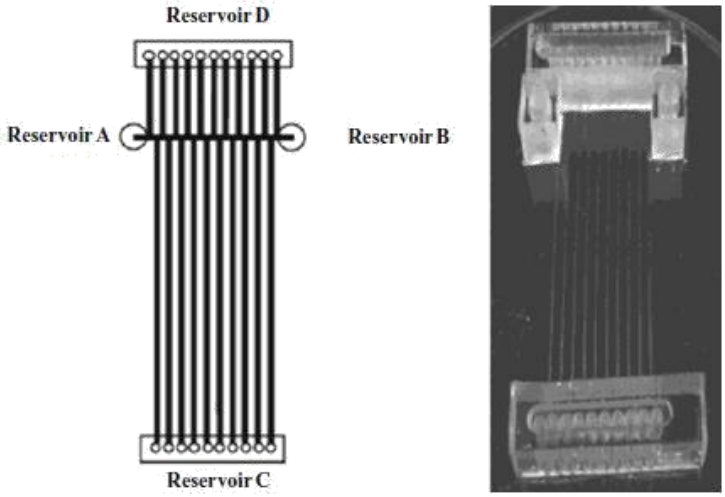

Figure 12 – Image of a 2D protein separation platform.[38]

Regarding the development of chromatography microfluidic devices, the works Shapiro *et al.*[39, 40] describing and characterizing a microfluidic packed be system, paved the way for the appearance of several work about these topic. Chromatographic techniques and microfluidics are side by side for different purposes, including proteomic analysis and detection of biomarkers, separation of nucleic acids, separation conditions optimization for biopharmaceutical products and HPLC analysis of metabolites. Pinto *et al.* described a microfluidic approach with fluorescence measurements with the goal of optimizing multimodal chromatography conditions using a mAb as a target and developed a new and integrated microfluidic platform for the improvement of chromatographic conditions for the capture of mAb from a CHO culture supernatant (**Figure 13**). The possibility of completely regenerate and reuse the device developed allows for a series of experiments to be performed sequentially in the same microcolumn, reducing the time needed for optimization but also resulting in more reliable and robust results. The architecture of the device also allows for assay parallelization.[41, 42]

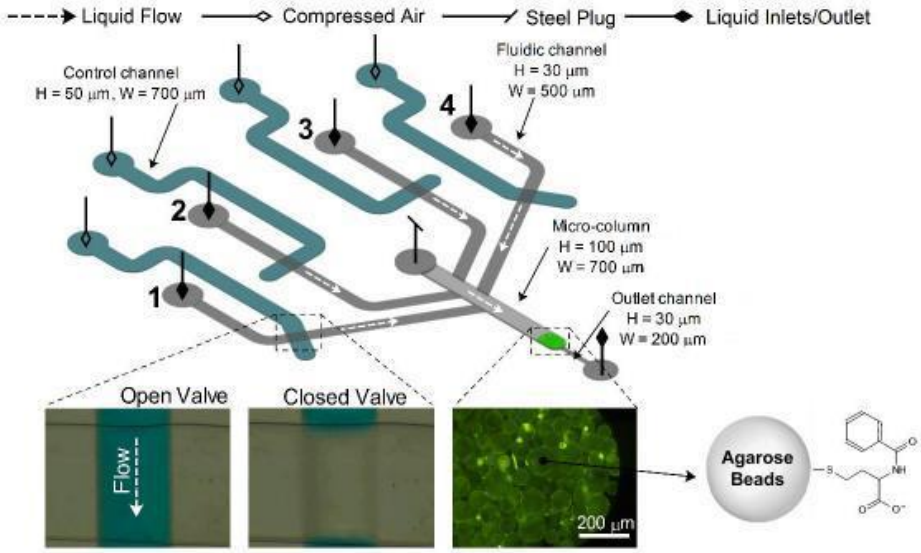

Figure 13 – Regenerable microfluidic structure comprising a main channel for bead packing (agarose beads with a multimodal ligand - Capto™ MMC) and fluidic channels for sequential liquid insertion controlled by integrated valves.[41]

II. Aim of Studies and Research Strategy

Monoclonal antibodies (mAbs), in the past few years, had a rapid increase in demand due to their many potential therapeutic applications. Because mAbs are a highly successful class of therapeutic products (that require the highest level of purity and yield) companies have made efforts to create processes that respond to this necessity. Mammalian cells, in particular CHO (Chinese Hamster Ovary) cells, are extensively used for the production of mAbs, since these cell lines possess the machinery to perform the post-translational modifications, essential for the biological activity of these proteins and, in this type of cells, these molecules are secreted to the medium, simplifying their purification process.

However, alongside mAbs, there are other molecules that are secreted to the outside of the cells, for example, host cell proteins and genomic DNA. Therefore, there is the need for the development of techniques that separate the product of interest (mAb) from the impurities (like CHOPs and gDNA). Usually, the method used to perform this purification is protein A affinity chromatography, followed by one or two additional polishing chromatographic steps. Lab-on-a-chip devices are an immense revolution in lab work, due to its benefits over non-microfluidic devices, namely miniaturization, smaller sample volume requirements, low production costs by device, and portability.

The goal of this project was to develop a lab-on-a-ship platform to purify a monoclonal antibody produced by CHO cells, through a chromatographic process.

The project began with the production procedures of two different CHO cell lines:

a. CHO-S cells, from a non-producing antibody cell line engineered to grow in suspension, were grown in a serum-free FreeStyle™ CHO Expression medium, in shaker flasks. The culture was maintained until exhaustion to collect the maximum amount of intracellular content possible. Cell density and viability were followed during the entire 9 days of culture;
b. CHO DP-12 cells, from an anti-IL-8-antibody-producing cell line, were grown in a serum-containing 25%/75% (v/v) DMEM/ProCHO5 (10% ultra-low IgG FPS) medium, in T-flasks. Passages were performed, and supernatant samples were collected each 4 days, with the exception for the cell density and viability assay (samples collected every day).

To better understand the nature of impurities, present in both cell cultures' supernatants, some analysis was done, including protein concentration assays, Isoelectric Focusing (IEF), SDS-PAGEs and 2D-PAGEs. After these procedures were done, the chromatographic conditions to begin the initial adsorption tests were chosen.

Two different anion exchanger resins (one strong and one weak) were chosen to test the binding capacity for these impurities: Q-Sepharose Fast Flow and DEAE-Sepharose Fast Flow. The procedure involved 96-well plates with a filter, where the resin solution was placed and the CHOPs solutions are tested. A difference in pressure was applied and the protein concentration of solutions recovered was determined to obtain the adsorption isotherms at different pH values (basing this choice on the results obtained in the analysis done to supernatant samples).

Afterwards, several breakthrough curves were performed to determine columns saturation and the best flow-rate for the chromatographic process to be designed. Three flow-rates were chosen (5, 3 and 1 µL/min) and the fluorescence was measured on the outlet channel, over time, under three different conditions. The assays were done flowing the BSA (0.5 g/L) and IgG (0.1 g/L) samples in three different ways: i) empty channels ("Channel" series); ii) in channels fully packed with beads and equilibrated with adsorption buffer ("Adsorption" series), and iii) in channels fully packed with beads, but equilibrated with elution buffer ("Elution" series).

Finally, adsorption/elution tests were performed, first using the BSA + IgG artificial mixture samples and then using the CHOPs. The values measured are presented in arbitrary units since the measurements was done at the end of the column and not on the liquid outlet channel, like for the breakthrough curves.

III. Materials and Methods

III.1 Reagents

Tris(hydroxylmethyl)aminomethane, phosphate buffered saline (PBS), sodium chloride (NaCl), sodium carbonate (Na2CO3), sodium thiosulphate (Na2S2O3), ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), glutaraldehyde (C5H8O2) solution 50% (v/v) in water and were obtained from Sigma-Aldrich (St. Louis, MO, USA); Sodium citrate was acquired from Merck (Kenilworth, NJ, USA); Formaldehyde (CH2O) 37% (w/w) was purchased from PanReac, AppliChem (Barcelona, Spain); DL-dithiothreitol (DTT), 1 M solution in water, was purchased from Nzytech (Lisbon, Portugal); Trichloroacetic acid (CCl3COOH), silver nitrate (AgNO3) and acetic acid (CH3COOH) Glacial were acquired from Fisher Scientific (Hampton, NH, USA), glycerol from VWR Chemicals (Radnor, PA, USA); Hydrochloric acid (HCl) 37% (v/v) was obtained from Fluka (Buchs, Switzerland) and sodium dodecyl sulfate (SDS) from Bio-Rad (Hercules, CA, USA).

Bovine serum albumin (BSA) standards (2 mg/mL) and Coomassie Plus (Bradford) Protein Assay were purchased from Thermo Scientific Pierce (Rockford, IL, USA). Myoglobin from equine skeletal muscle was purchased from Sigma-Aldrich, as a lyophilized powder. Coomassie PhastGel from GE Healthcare (Chicago, IL, USA) was used to stain the SDS-PAGE gels. Water used in all experiments was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA).

III.2 Cell Culture

III.2.1 Cell Lines

The non-anti-IL8-antibody-producing cell line used was a FreeStyle™ CHO-S cell line which grows in suspension, obtained from Life Technologies (Carlsbald, CA, USA), and cultured in a defined, serum-free medium. Initially, the cells were inoculated only in one shaker-flask (SF) until they reached a viability of around 95%; then, two shaker-flasks (100 mL of working volume each), working in parallel, were inoculated with 5.3×10^5 cells/mL each and incubated under constant agitation at 37 ^{o}C at 8% CO_2 atmosphere and 125 rpm. The cultures were led to exhaustion and the supernatant was collected for further studies. The cultures were led to exhaustion (9 days' culture), the supernatant was collected and cells counted every day.

The anti-IL-8-antibody-producing cell line was a CHO DP-12 cell line, acquired from ATCC (American Type Culture Collection), which grew adherently in a serum-containing medium. This clone was derived by transfection of CHO DP-12 cells with the vector p6G4V11N35E.choSD.10 which confer resistance to methotrexate (MTX). To select the cells that possess the plasmid, MTX was added to the culture medium and selected with methotrexate. They were inoculated with 2.8×10^4 cells/cm^2 and incubated at 37^{o}C at 8% CO_2 atmosphere. Passages and supernatant collection were performed each four days.

III.2.2 Culture Medium

CHO-S cells grew in suspension conditions and were grown in a medium developed for high-density and suspension culture named FreeStyle™ CHO Expression Medium. This medium was supplemented with 8 mM L-glutamine and 0.5% (v/v) PenStrep (Penicillin/Streptomycin), Thermo Scientific (Waltham, MA, USA).

CHO DP-12 cells were grown in adherent conditions in a serum-containing medium with a 25%/75% (v/v) mixture of DMEM (Gibco®, Thermo Scientific) and ProCHO™5 (Lonza, Basel, Switzerland), respectively. DMEM formulation (powder) contained 4.5 g/L D-glucose, 4 mM L-glutamine (Gibco®) and 1 M sodium pyruvate. After resuspension with MilliQ water, 1.5 g/L $NaHCO_3$ (Sigma-Aldrich), 200 nM MTX (Sigma-Aldrich), 2 mg/L recombinant human insulin (Sigma-Aldrich), 35 mg/L L-proline (Sigma-Aldrich), 0.1% (v/v) trace element A and B (Cellgro®, Mediatech) and 1% (v/v) antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, from Gibco®) were added. The serum used was an ultra-low IgG FBS (Gibco®), in a 10% (v/v) concentration. ProCHO™5 is a protein-free medium developed to ease the production and downstream processing of recombinant proteins expressed in CHO cells. It was formulated with 0.1% (w/v) Pluronic® F-68, without L-glutamine, phenol red, hypoxanthine and thymidine, and the supplementation was performed with 4 mM L-glutamine (Gibco®), 2.1 g/L $NaHCO_3$, 200 nM MTX, 10 mg/L recombinant human insulin (Lonza), 0.07% (v/v) lipids (Lonza) and 1% (v/v) antibiotics.

III.2.3 Cell Dissociation Reagents

TrypLE™ Select 1X (Gibco®), an animal origin free, recombinant enzyme for dissociating a wide range of adherent mammalian cells, including CHO, containing EDTA. This dissociation agent was used for the CHO-S cell line culture since the medium does not contain serum.

Trypsin (Life Technologies) 0.05% (v/v), with 0.5 M EDTA, was diluted from a stock of 2.5% Trypsin. It is an irradiated mixture of proteases derived from porcine pancreas. This dissociation reagent was used for the CHO DP-12 cell line culture since the culture medium contains 25% (v/v) serum.

III.3 Total Protein Quantification

The total protein content of the supernatants collected from both cell line cultures was determined by the Bradford method using a Coomassie assay reagent. BSA and BGG weres used as protein standards and calibration curves were prepared with concentrations ranging from 5 to 400 mg/L. In a 96 microwell plate, a sample volume of 50 µL was mixed with 200 µL of the Coomassie reagent. The plate was then mixed for 30 s and incubated for 10 min at room temperature. The absorbance was measured at 595 nm in a Spectramax 384 Plus microplate reader, from Molecular Devices, equipped with a Softmax Pro 5.3 processing software.

III.4 Isoelectric Focusing (IEF)

For the IEF of the shaker flasks supernatant samples, a PhastGel® IEF 3-9 with 50 x 46 x 0.45 mm, from GE Healthcare, was used. PhastGel® IEF media are precast homogeneous polyacrylamide gels (5%T, 3%C) containing

2-6% Pharmalyte® as carrier ampholytes. The IEF separation was performed in a multi-tasking PhastSystem™ from Pharmacia. The method contains three steps: (i) a pre-focusing step, in which the pH gradient is formed and sample applicators can be loaded; (ii) a sample application step, in which the samples are applied to the gel for 15 Vh; (iii) and a focusing step, in which the applicators are raised and the proteins migrate in the gel. On the applicator, 1 µL of a pI marker, 2 µL of the samples under analysis and 2 µL of a myoglobin sample were loaded. The myoglobin sample was used to identify of the bands, if there were any problems with the marker, and was prepared by adding 1 mL of Milli-Q water, 300 µL of 1 M NaCl and 50 µL of 1.5 M Tris-HCl (pH 8.5) to 2.5 mg of horse myoglobin, and diluted 1:4. For pI marker, the broad pI kit (pH 3-10), from GE Healthcare was selected, which includes: amyloglucosidase (pI 3.50), methyl red (pI 3.75), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myoglobin-acetic band (pI 6.85), horse myoglobin-basic band (pI 7.35), lentil lectin-acidic band (pI 8.15), lentil lectin-middle band (pI 8.45), lentil lectin-basic band (pI 8.65), and trypsinogen (pI 9.30).

The IEF gels were silver stained according to the following protocol: (i) gel's fixation with a 20% (w/v) trichloroacetic acid solution, for 5 min at 20 °C; (ii) gel's washing with a 50% (v/v) ethanol, 10% (v/v) acetic acid solution, for 2 min at 50°C; (iii) gel's washing with a 10% (v/v) ethanol, 5% (v/v) acetic acid solution, for 6 min at 50°C; (iv) gel's incubation in a sensitizer solution composed of 8.3% (w/v) glutaraldehyde, for 6 min at 50°C; (v) gel's washing with a 10% (v/v) ethanol, 5% (v/v) acetic acid solution, for 8 min at 50°C; (vi) gel's washing with Milli-Q water, for 4 min at 50°C; (vii) gel's incubation in a 0.5% (w/v) silver nitrate solution, for 10 min at 40°C; (viii) gel's washing with Milli-Q water, for 1 min at 30°C; (ix) gel's incubation in a freshly made developer solution composed of 0.015% (v/v) formaldehyde in 2.5% (w/v) sodium carbonate, at 30 °C until the desired intensity; (x) and gel's incubation in a stop solution, with 5% (v/v) acetic acid, for 5 min at 50°C. The IEF gel was scanned using a GS-800 calibrated densitometer, from Bio-Rad. All the solutions used were prepared in Milli-Q water.

III.5 Gel Electrophoresis (SDS-PAGE)

A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) allows the separation of proteins based on their ability to move within an electrical current, which is a function of their molecular weight. All samples analyzed were previously diluted in a loading buffer containing 62.5 mM Tris-HCl, pH 6.2, 2% SDS, 0.01% bromophenol blue and 10% glycerol, and denatured in reducing conditions (0.1 M DTT), at 100 °C for 5 min. For a final volume of 50 µL, 25 µL of loading buffer, 20 µL of protein sample, and 5 µL of 1 M DTT were used. Samples were applied in a 12% (v/v) acrylamide gel, prepared from a 40% (v/v) acrylamide/bisacrylamide stock solution (29:1), and ran at 90 mV using a running buffer that contained 192 mM glycine, 25 mM Tris, and 0.1% (w/v) SDS, pH 8.3. The molecular marker used in all gels was the Precision Plus Protein™ Dual Color Standards, from Bio-Rad.

Gels were stained for 1 h with Coomassie PhastGel™ Blue R, from Pharmacia, and destained by washing with a 30% (v/v) ethanol, 10% (v/v) acetic acid solution. The SDS-PAGE gels were also scanned using a GS-800 calibrated densitometer.

III.6 2D Gel Electrophoresis (2D-PAGE)

The procedure followed was adapted from the instructions of *PROTEAN® IEF Cell*, from Bio-Rad, and consisted in three steps: protein precipitation, isoelectric focusing and SDS-PAGE. Samples for 2D Polyacrilamide Gel Electrophoresis (2D-PAGE) were precipitated for 11 h using cold acetone (-20 ^{O}C). For a total of 500 µg of protein, 211 µL of protein sample were precipitated with 4 times this volume of acetone, vortexed and incubated at -20 ^{O}C. Then, the mixture was centrifuged at 14000 g for 15 min at 4 ^{O}C. The supernatant discarded and acetone was allowed to evaporate for 30 min. The pellet was ressuspended in 500 µL of Rehydration buffer (Bio-Rad) - RH (8 M Urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte® 3/10 ampholyte, 0.001% Bromophenol blue), to a final protein concentration of 1 g/L.

The IPG strips (with a linear 3-10 pH gradient) were rehydrated with 125 µL of RH buffer for 12 h at 20 ^{O}C on the focusing tray. The IEF occurred at 250 V for 15 min, followed by 4000 V for 1 h, 4000 V in linear mode until a total of 15000 Vh was reached and a holding period of 500 V.

The SDS-PAGE protocol was started with the equilibration of the IPG strip in Equilibration buffer I (Bio-Rad) – EBI (2% (w/v) DTT) for 10 min at 20^{O}C, followed by the equilibration with EBII, Bio-Rad, (2.5% (w/v) iodoacetamide) at the same conditions. Then the strip was immersed in Running buffer 1x (25 mM Tris-HCl pH 8.3, 192 mM glycine, 1% (w/v) SDS) and placed on top of an SDS-PAGE gel (*Mini-Protean TGX Gel Any kD,* Bio-Rad). The molecular weight marker (5 µL) was placed in a piece of agarose gel (1% (w/v) in loading buffer). The strip and the marker were sealed with a 0.5% (w/v) agarose solution and the electrophoresis occurred at 200 V for about 40 min. The gel was first stained with the protocol explained above for the SDS-PAGE and then silver stained, beginning with a washing step with 30% (v/v) ethanol for 10mins, followed by two washing steps of 10 min with Milli-Q water. The sensibilization step was performed with 0.02% (w/v) sodium thiosulfate during 1min, followed by three washing steps (30 sec each), with Milli-Q water. The next step was the staining with silver nitrate for 30min, followed by a washing step of 1min with Milli-Q water. The development step involved a solution with 3% (w/v) sodium carbonate and 0.05% (v/v) formaldehyde until the bands started appearing and the stopping reaction was performed by the addition of 5% (v/v) acetic acid for 15 min. The gel was silver stained to observe all the proteins in solutions regardless of their concentration.

III.7 Monoclonal Antibody Purification

A pre-packed HiTrap MabSelect SuRe 1 mL column (0.7 cm x 2.5 cm) from GE Healthcare was used to perform the purification of the anti-interleukin-8 monoclonal antibody produced by the CHO DP-12 cells. This resin is based on a highly cross-linked agarose matrix with a recombinant alkali-stabilized Protein A-derived ligand, and its hydrophilic nature ensures low levels of non-specific binding leading to low levels of host cell-derived impurities in the elution pool. The ligand used was engineered from one of the IgG-binding domains of Protein A to achieve enhanced binding capacity for IgG.

The experiment was carried out in an ÄKTA Purifier system, from Amersham Biosciences (GE Healthcare), equipped with a Unicorn 5.1 data acquisition and processing software. The adsorption was performed in 20 mM

phosphate buffer containing 150 mM NaCl at pH 7.2, and the elution with 100 mM sodium citrate buffer at pH 3-3.6. To avoid denaturation of the eluted antibody, 1 M Tris-HCl pH 8 was added to the tubes where the samples were collected. The column was stored at 4 ^{o}C in 20% ethanol after being used. The CHO cell culture supernatant was injected at a rate of 1 µL/min and the unbound proteins were washed-out with adsorption buffer.

III.8 High-Throughput Screening using 96-well Plates

A screening of the best pH condition for the binding of CHOPs (CHO Host Cell proteins) to the Q-Sepharose beads was performed using a MultiScreen®$_{HTS}$ Vacuum Manifold system from Millipore (**Figure 14**).

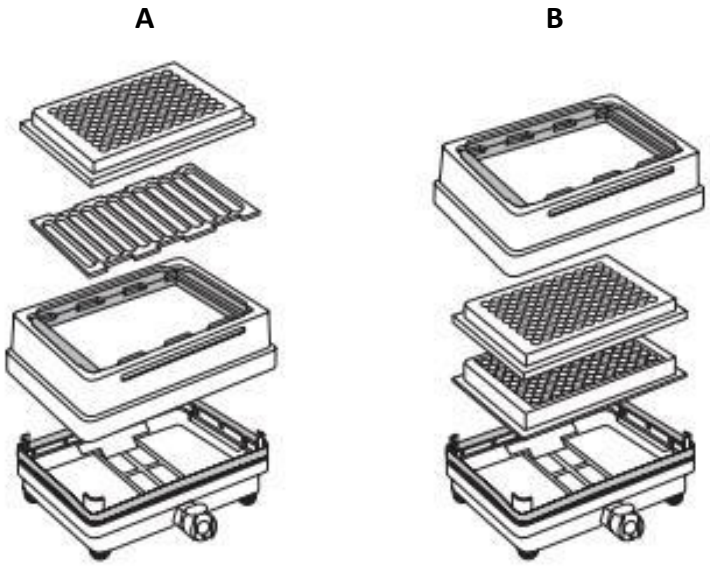

Figure 14 – MultiScreen®$_{HTS}$ Vacuum Manifold system from Millipore different configurations. A – Filter-to-waste; B - DirectStack™.

Operating in two different configurations (Filter-to-waste and DirectStack™ modes, **Figure 14**), 200 µL of a 20% (v/v) resin suspension were added to each well of a 96-well plate, equipped with a filter membrane (MultiScreen®-HV 96 well-plates). Resin deposition was accomplished by filtration and consequent waste removal, using the filter-to-waste configuration. Afterwards, an equilibration step was performed with the addition of 200 µL of the adsorption buffer to each of the wells. The plate was then incubated at velocity 7 (1368 rpm) in a shaker-incubator Stat Fax-2200 at room temperature. The remaining liquid was filtered to waste. To guarantee a good resin equilibration, the previous step was repeated three times. Then, a volume of 200 µL of the feed solution was added and the plate was incubated for 30 min at velocity 7 and room temperature. After the incubation, the flow-through (FT) was filtered in DirectStack™ mode to a collection plate (Greiner UV-Star® 96-well plate) and stored for further analysis. Finally, 200 µL of elution buffer were added to each well and the steps performed for adsorption, regarding incubation and filtration, were repeated. Samples were collected for further protein quantification of the flow-through and the elution pools.

III.9 Microfluidics

III.9.1 Hard Mask Fabrication

The protocol followed for the fabrication of the structures used for the microfluidics assays is summarized below, in **Figure 15**. An aluminum mask was designed using AutoCAD software (Autodesk Inc., San Rafael, CA, USA). Since the design requires two different mask levels, all the following steps described in this section were performed in duplicate. The fabrication process starts with a glass (Corning, Corning, NY, USA) substrate being cleaned, then rinsing it with acetone, deionized (DI) water, immersion in Alconox solution (for 15 min at 65 ^{O}C), and finally with DI water, followed by drying with compressed air. Subsequently, a 200 nm aluminum (Al) layer is deposited using a Nordiko 7000 magnetron sputtering system. A positive photoresist (PFR 7790G) layer of 1.5 µm is spin-coated onto the deposited aluminium layer. The AutoCAD file is then converted and transferred to a DWLii laser direct write lithography system (diode laser: 405 nm), which is then used to photolithographically transfer the pattern to the photoresist. The resist is developed exposing parts of the aluminium layer, which is later removed by wet etching with a standard aluminum etchant. Finally, the remaining photoresist is stripped away resulting in a patterned aluminium mask on the glass substrate. The previous microfabrication steps are all performed under class 100 clean-room conditions, except for the photolithography step, which is performed in class 10 conditions.

III.9.2 Mold Fabrication

The master mold was fabricated using SU-8, a negative photoresist. Firstly, a silicon substrate was cleaned by sequential with acetone, isopropanol (IPA) and DI water, sequentially, to remove any residues of photoresist (used to protect the substrate during the dicing process) on the surface. Then, the wafer was immersed in a heated Alconox™ detergent bath (65 ^{O}C) for 15 min, followed by a rigorous cleaning with DI water and drying with compressed air. The substrate was then placed in a UVO cleaner for 15 min to remove any remaining organic contaminants.

To fabricate the mold, SU-8 2015 was spin-coated onto the previously cleaned substrate for 10 s at 500 rpm with 100 rpm/s of acceleration, followed by 34 s at 1700 rpm with an acceleration of 300 rpm/s, resulting in a 20 µm thick layer. After a 4 min pre-exposure bake at 95 ^{O}C using a hot plate, the substrate was cooled down for 1 min and the hard mask with the design for the channels was placed over the SU-8 layer with the aluminum surface facing down, to prevent a loss in resolution due to scattering effects. The stack is exposed to a 400 W UV light (with an energy per unit area of 178 mJ/cm^2), baked for 5 min at 95 ^{O}C, and cooled down to room temperature for 2 min.

The development of the non-exposed photoresist is achieved by immersion of the SU-8 in a propylene glycol monomethyl ether acetate (PGMEA) solution for 2 min with manual orbital agitation.

After the development, the substrate is cleaned with IPA and dried with compressed air. The second layer with 100 µm height is defined by spin-coating a SU-8 50 film on top of the previous layer at 10 s at 500 rpm with an

acceleration of 100 rpm/s, followed by 30 s at 1000 rpm with an acceleration of 300 rpm/s. A pre-exposure bake process is then performed, comprising baking at 65 ^{o}C for 10 min followed by a gradual ramping-up to 95 ^{o}C, where it is baked for 30 min and then cooled for 1 min. Then, the second hard mask for the 100 µm features is manually aligned to the previous layer using a stereomicroscope and placed on the SU-8, again with the aluminum surface facing down, and exposed to the UV light (with an energy per unit area of 416 mJ/cm^{2}). A post-exposure bake is performed at 65 ^{o}C for 1 min, followed by 10 min at 95 ^{o}C and 2 min of cooling down. The second photoresist layer is developed in PGMEA for 10 min with manual orbital agitation, cleaned with IPA, and dried. Finally, the mold is hard baked for 15 min at 150 ^{o}C and left to cool on top of the hot plate until below 50 ^{o}C.

III.9.3 PDMS Micro-columns Fabrication and Sealing

To fabricate the structures, the PDMS elastomer, which is a silicon-based organic polymer, was prepared with a 10:1 ratio of PDMS to curing agent, degassed, poured on top of the mold and baked at 70 °C for 1.5 h. After baking, the structure was peeled off from the mold, access holes were punched using syringe tips and sealed against a glass slide after, treating both sides using an expanded oxygen plasma cleaner (Harrick Plasma).

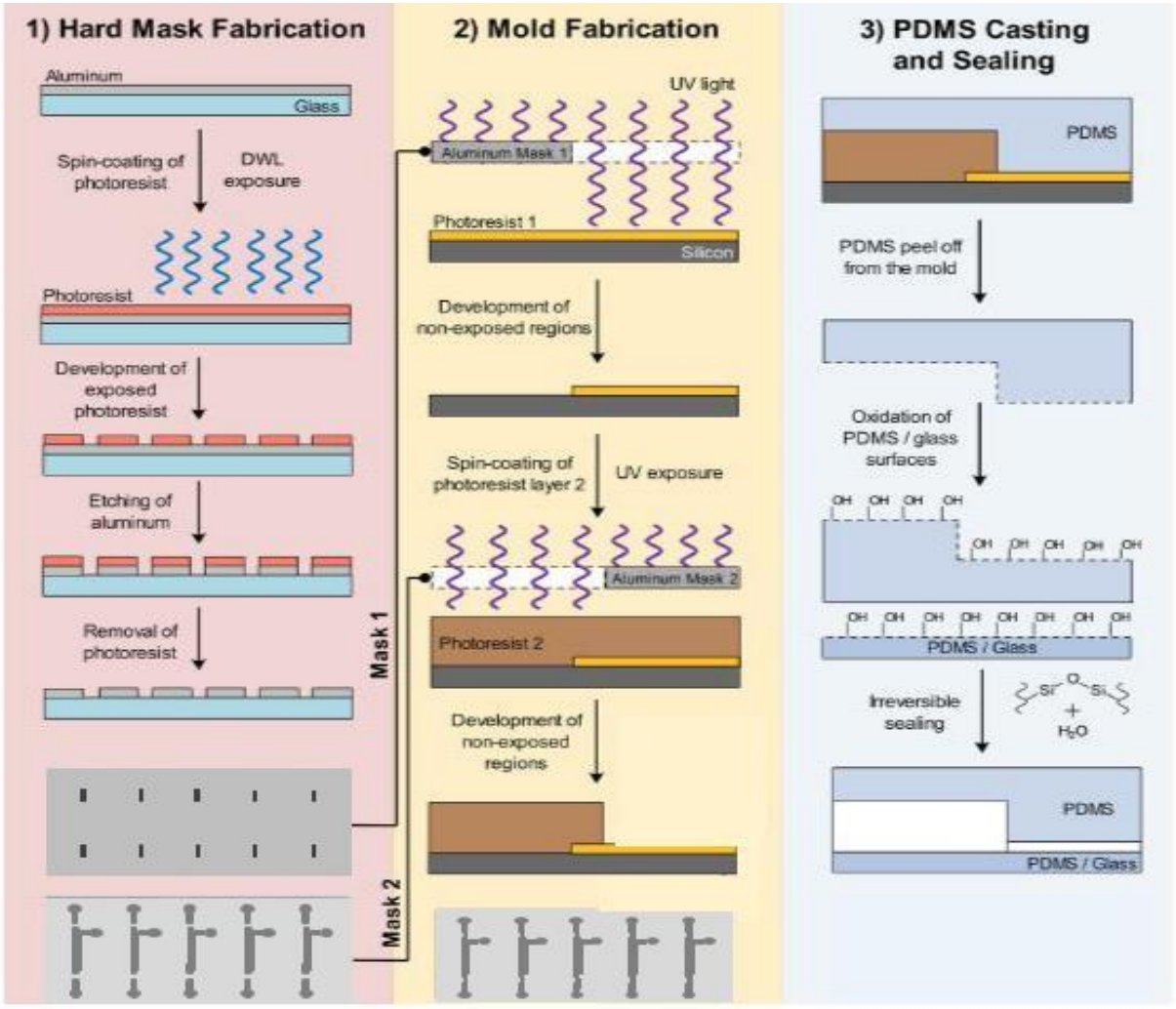

Figure 15 – Sequence of steps involved in the structures fabrication: 1 – aluminium hard mask; 2 – SU-8 negative photoresist mold; and 3- PDMS structures. Adapted from [43]

III.9.4 Chromatography Assays, Fluorescence Measurements and Analysis

Chromatography anion exchange bulk resin (Q-Sepharose Fast Flow) were suspended in a 10% (v/v) slurry in a poly(ethylene glycol) (PEG) 8000 20% (w/w) solution (Sigma-Aldrich). Beads were then packed inside the microcolumns by pushing the liquid from the beads inlet at 90 µL/min, using a syringe pump (Model NE-1002X, New Era Pump System, Inc.). After the packing was complete, the beads inlet was closed using a metallic plug and all the solutions flew through the column inlet, by pushing. After the packing step, the micro-columns were equilibrated with 40 µL of the adsorption buffer (20 mM Tris-HCl pH 8). The adsorption was performed by flowing the samples at 5 µL/min and the elution was performed by flowing the elution buffer (20 mM Tris-HCl, 1 M NaCl pH 8) at 5 µL/min.

Chromatographic assays were continuously monitored and recorded using an inverted fluorescence microscope (Olympus CKX41) coupled to a CCD color camera (Olympus XC30). A fluorescence signal from the beads inside the micro-columns was acquired with an exposure time of 200 ms, a gain of 8x and a magnification of 4x. Images were analyzed using ImageJ software and the fluorescence values were normalized to the maximum value obtained (end of adsorption step).

IV. Results and Discussion

IV.1 CHO Cell Culture Supernatant Analysis

IV.1.1 Growth Curves

A cell culture process of a CHO-S cell line (a non-producing antibody cell line engineered to grow in suspension), was performed in parallel, in two shaker-flasks. The cell line used was grown in FreeStyle™ CHO Expression medium, which is a defined, serum-free medium. It is specifically developed for the high-density and suspension culture of CHO cells and should be supplemented with 8 mM of L-glutamine and 0.05% PenStrep. The shaker flasks (SF) were inoculated with $5.3x10^5$ cells/mL each, and the cultures were maintained until exhaustion with the goal of collecting the maximum amount of intracellular content possible after total cell lysis had occurred. Cell density and viability were there followed during 9 days, until exhaustion was obtained. The results are shown in **Figure 16**.

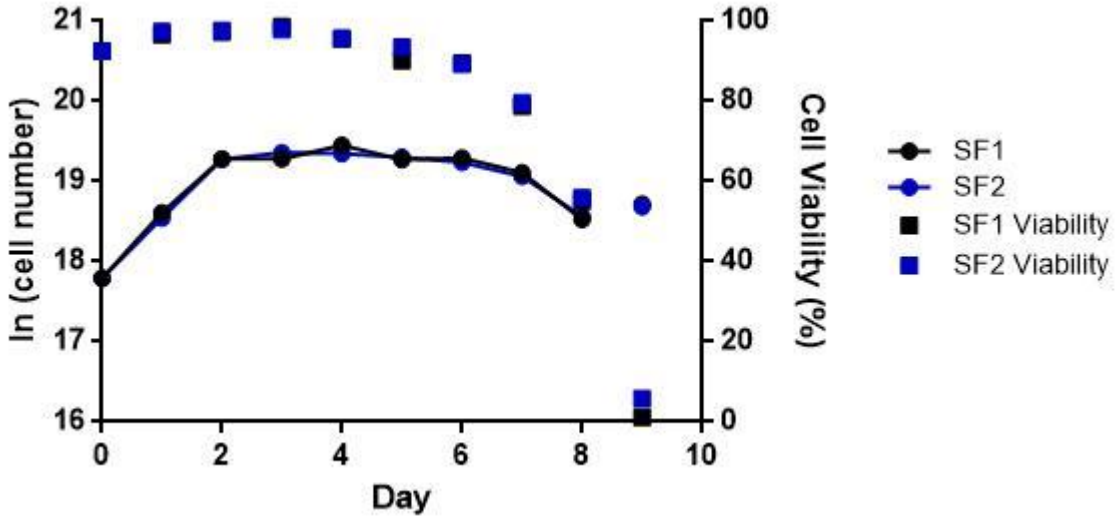

Figure 16 – CHO-S cell line growth curves and associated cell viability for SF1 and SF2 parallel cultures. The • and • correspond to the cell count (ln [cell number]) for the samples of the SF1 and SF2, respectively. The ■ and ■ correspond to cell viability (%) for the samples from the SF1 and SF2, respectively.

As expected and as shown in **Figure 16**, cell growth is observed at the exponential phase between days 0 and 2 until a plateau phase is reached. Cell viability is observed to be at around 95% until a decrease is observed after day 6, which corresponds to a decrease in total cell count (cells began to lyse). Cultures were ended when cell viability reached (almost, for SF2) 0%, at day 9. Not only was it possible to observe, by **Figure 16**, that the cultures from both SFs were growing at a similar pace, but that the values obtained for the maximum growth rate and duplication time for each one of the SF's cultures were also similar to each other.

Based on the growth curves presented in **Figure 16**, it was possible, by adjusting a linear trendline to the experimental points of the exponential growth phase of both shaker flasks' cultures (which corresponds to days 0-2), to determine the maximum growth rates (μ_{max}) and duplication times (t_d). The results are presented in **Table 2**.

Table 2 – Resume of the maximum growth rates and duplication times determined for the cultures of both SFs.

	Maximum Growth Rate – μ_{max} (day^{-1})	Duplication Time – t_d (day)
SF1	0.744	0.931
SF2	0.742	0.934

The results shown in **Table 2**, are both similar between shaker flasks and to the results reported in the literature.[44]

A cell culture of CHO DP-12 cell line was also performed. This is an antibody producing cell line that grows adherently, in a serum-containing medium. The T-flasks were incubated with $2.8x10^4$ cells/cm^2 and passages were done each four days. As for the CHO-S cell line, cell density and viability were followed during the days of culture (**Figure 17**).

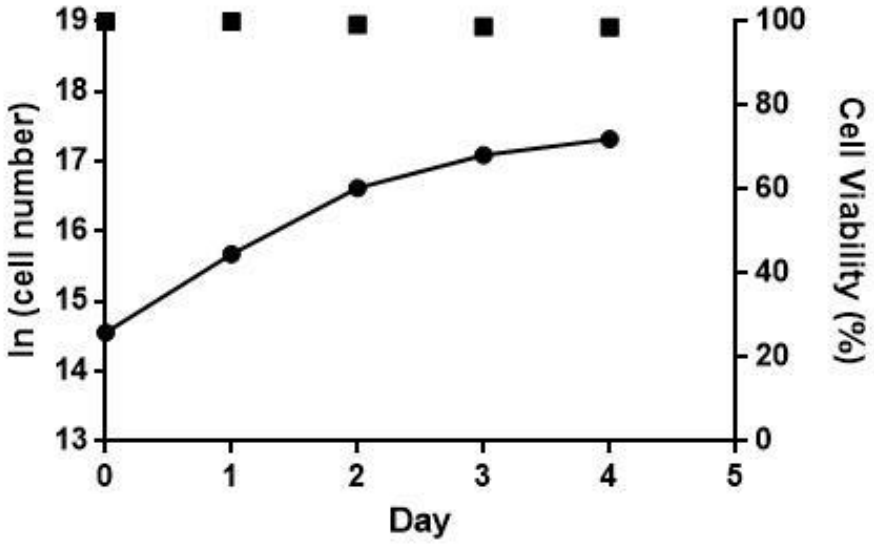

Figure 17 – CHO DP-12 cell line growth curve and associated cell viability. The • correspond to the cell count (ln [cell number]) and the ■ correspond to cell viability (%).

As shown in **Figure 17**, cell growth is observed at the exponential phase between days 0 and 2, however a plateau phase is never reached, due to the interruption of the culture at day 4 to perform the passage and to keep cells viability, which is observed to be around 100%, during the entire culture time.

Based on the growth curves presented in **Figure 17**, it was possible, by adjusting a linear trendline to the experimental points of the exponential growth phase of the culture (which corresponds to days 0-2), to determine the maximum growth rates (μ_{max}) and duplication times (t_d). The results are presented in **Table 3**.

Table 3 – Resume of the maximum growth rate and duplication time determined for the culture.

Maximum Growth Rate – μ_{max} (day^{-1})	Duplication Time – t_d (day)
1.03	0.671

The results regarding the maximum growth rate and duplication time for the CHO DP-12 cell culture are also in accordance to the ones reported in the literature, however the cultures were maintained for a longer period than the one shown above.[45]

IV.1.2 Protein Quantification

Throughout the days of culture, the CHO host cell protein (CHOPs) content and its specific productivity (in pg/cell·day) were determined for both parallel cultures using the Bradford method. Results are shown in **Figures 18** and **19**, for the CHO-S and CHO DP-12 cell culture, respectively.

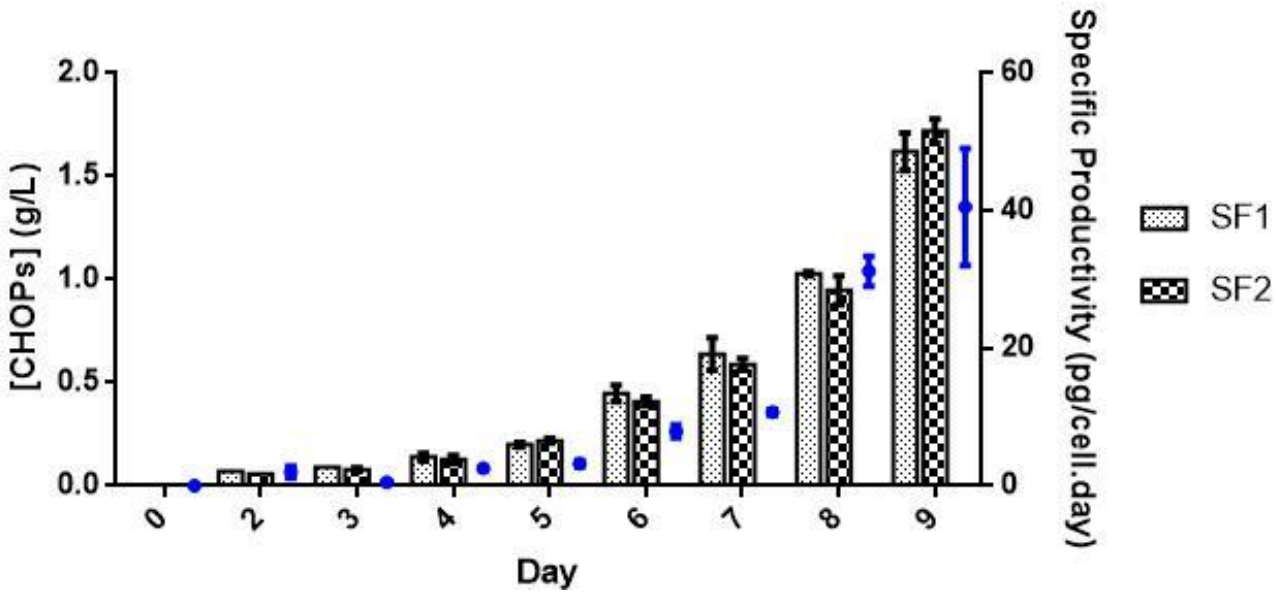

Figure 18 – CHOPs concentration, in mg/L, and specific productivity, in pg/cell·day, determined for samples collected for each day of CHO-S cell culture. The ▨ and ▩ correspond to CHOPs concentration for the SF1 and SF2 cultures, respectively, and the • correspond to an average of the specific productivity determined for each SF. There is no result for day one of culture, since the sample was discarded.

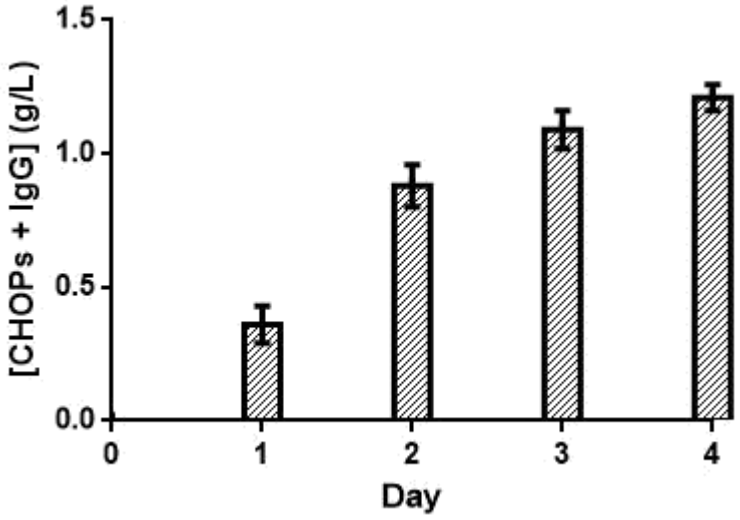

Figure 19 – CHOPs concentration, in mg/L, determined for samples collected for each day of CHO DP-12 cell culture.

Regarding the CHOPs concentration, an expected increase was observed during cell culture until a final CHOPs concentration of 1.62±0.09 and 1.72±0.06 g/L, for SF1 and SF2, for the CHO-S culture, and 1.21±0.05 g/L, for the CHO DP-12 cell culture.

The results obtained for the CHO-S cell culture (**Figure 18**) are the expected ones, with CHOPs concentration steady increasing until day 6. After 7 days in culture, cell viability starts to decrease leading cells to lyse, and consequently, the intracellular content is released to the supernatant, causing a pronounced increase in the concentration of CHOPs and in the specific productivity as well. In the case of the CHO DP-12 culture, the concentration of protein measured includes both host cell proteins, the recombinant antibody and the proteins added to the culture medium (2.5% of FBS). This culture was not followed until exhaustion and consequently the final concentration of CHOPs is lower. Nonetheless, comparing just the 4 initial days of culture it is possible to observe that the concentration of proteins is considerably higher in the case of the antibody producing cell line CHO DP-12 grown in the presence of serum.

IV.1.3 Isoelectric Focusing

To further analyze the protein content of the collected CHO cell lines supernatants, isoelectric focusing was performed to samples collected from both cultures (**Figure 20 – A** and **B**).

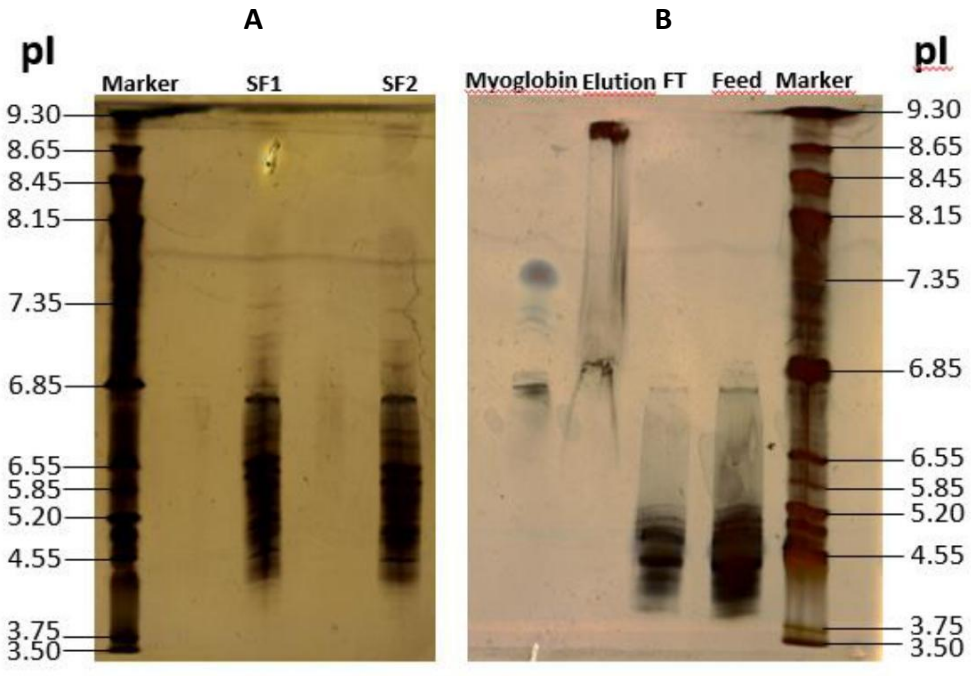

Figure 20 – Silver stained IEF gels of supernatant samples collected from both CHO-S and CHO DP-12 cell cultures (A and B, respectively).

In the case of the CHO-S culture supernatant (**Figure 20 – A**), the samples run were collected at the end of the culture (after reaching exhaustion) and it was possible to observe that, almost all the proteins present in this supernatant had a pI below 7. This is a promising indication that separation by charge will be possible since the mAb of interest (IgG) has a pI ≥ 9.3[46], and consequently a pH value higher than 7 should allow a selective fractionation between protein and mAb. However, as can be observed in **Figure 20 – A**, there are still some proteins with an isoelectric point close to the one of IgG and these will be more difficult to separate in a charge-based process like ion-exchange chromatography.

In **Figure 20 – B**, samples from CHO DP-12 supernatant previous (Feed) and after Äkta chromatographic purification (Elution and FT pools) were run and, as for the CHO-S supernatant, the majority of proteins have a pI lower than 7. However, in this case, there are no proteins with a pI close to the one of IgG, which indicates that the separation process could be easier than the one for the CHO-S supernatant.

IV.1.4 SDS-PAGE

To obtain a better characterization of the CHOPs content of the supernatants from both cell cultures, protein gel electrophoresis (SDS-PAGE) were performed (**Figures 21 – A** and **B**):

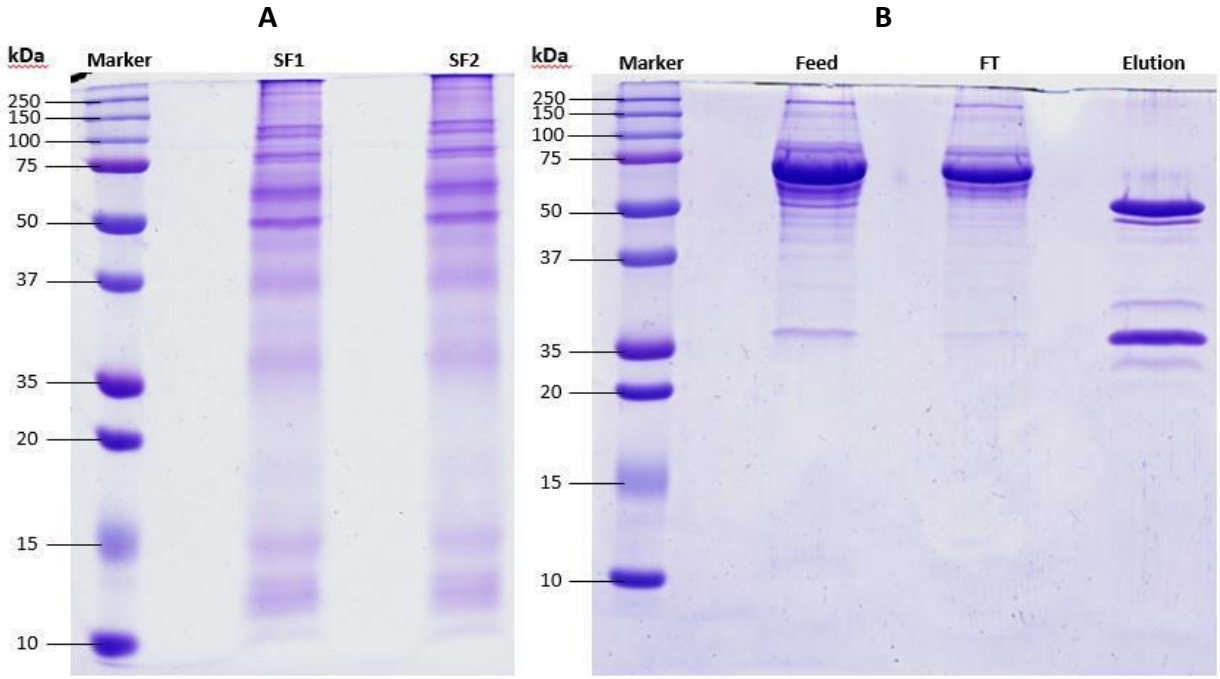

Figure 21 – Coommasie blue stained SDS-PAGE gels analysis of the samples collected from both cell culture supernatants (A - CHO-S; B - CHO DP-12). The samples run in both gels were reduced by the presence of DTT.

The two SDS-PAGEs (**Figure 21 – A** and **B**) were performed in order to know the molecular weight profile of the proteins present in each supernatant. As shown in **Figure 21 – A**, the proteins present in the CHO-S cell culture supernatant are of a wider molecular weight range than the ones in the CHO DP-12 supernatant (**Figure 21 – B**). It includes some with high molecular weight values, which could be hard to separate in the chromatographic process to be designed.

IV.1.5 2D-PAGE

Finally, to better associate the molecular weight of proteins to their pI, 2D-Electrophoresis were performed with samples from both cell culture supernatants (**Figures 22** and **23**).

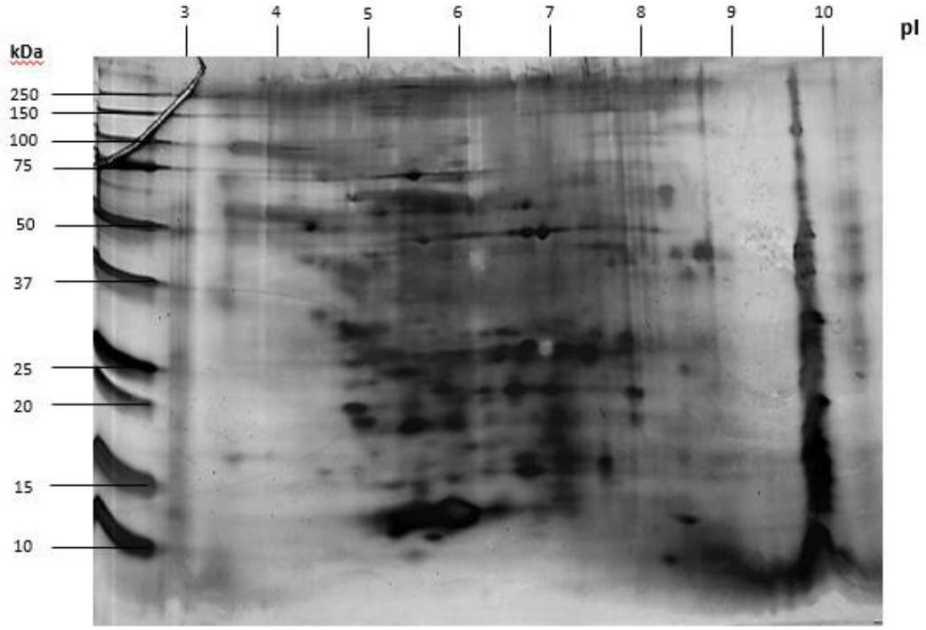

Figure 22 – Silver stained 2D-Electrophoresis a sample from the FT of the CHO-S cell culture supernatant. Proteins (500µg) were precipitated and separated based on their pI in 7cm IPG strips (pH 3-10) and then applied on an SDS-PAGE gel.

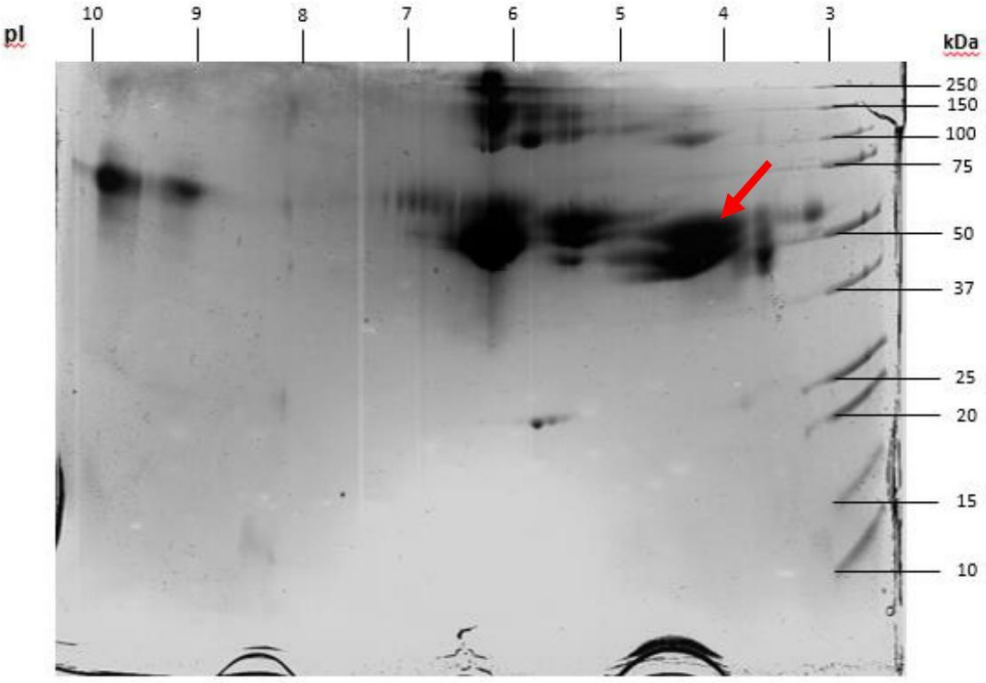

Figure 23 – Silver stained 2D-Electrophoresis a sample from the FT of the CHO DP-12 cell culture supernatant. Proteins (500µg) were precipitated and separated based on their pI in 7cm IPG strips (pH 3-10) and then applied on an SDS-PAGE gel. The red arrow points the most abundant protein present in the supernatant, BSA, which is added to the culture medium when adding the FBS.

The goal was to try to connect the proteins with a specific isoelectric point to their molecular weight. In **Figure 22**, gel run with a sample of the FT from the CHO-S cell culture supernatant, it is possible to observe a large amount of proteins with pI lower than 7.0, which confirmed the results observed in the IEF (**Figure 20 – A**). As said before, in the IEF gel, it was possible to observe proteins with a pI near the one of IgG (9.3)[46], and that was confirmed by this 2D procedure. Moreover, some of these proteins have a high molecular weight, turning them a significant challenge. Still, most of the proteins with higher pI values have low molecular weights and, therefore will enable its separation in a size-based process, either by size-exclusion chromatography or by tangential flow filtration. The results obtained for the CHO DP-12 supernatant sample (**Figure 23**) confirmed partially the ones obtained for the isoelectric focusing, however some proteins with pI closer to the one of IgG appear in this gel.

IV.2 96-well Plates Assays

As shown by the isoelectric focusing and the 2D-PAGEs represented above, the majority of proteins present in the CHO-S cell culture supernatant have pI values lower than 7.0. Thus, two different anion exchanger resins (one strong and one weak) were chosen to test the binding capacity for these impurities: Q-Sepharose Fast Flow and DEAE-Sepharose Fast Flow (**Figure 24** and **25**, respectively).

The procedure followed involved the use of 96-well plates with a filter, where the resin solution is placed, and equilibrated using 20 mM Tris-HCl buffers with pH values of 7.0 and 9.0. This choice was made to keep the impurities negatively charged, so that they could bind to the positively charged beads. The elution step was performed using a buffer of 20 mM Tris-HCl with 1 M of NaCl, which could compete for the binding places in the beads and allow the proteins to elute.

To obtain the adsorption isotherms for both resins and knowing that the CHOPs concentration on the final day of culture was of around 2.0 g/L, three different dilutions were made and tested, 1:1; 1:2 and 1:4.

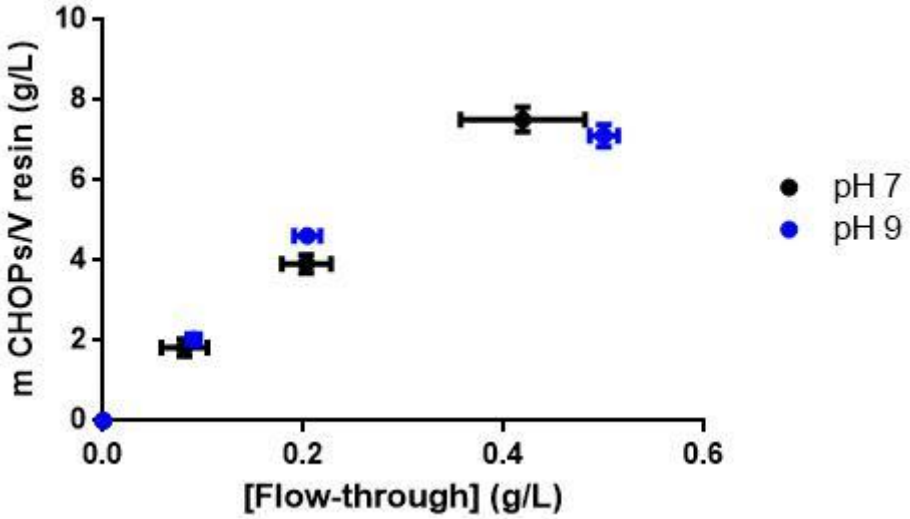

Figure 24 – Adsorption isotherm obtained, with Q-Sepharose beads, for two different pH values (7.0 and 9.0) and three successive dilutions from an initial concentration of CHOPs.

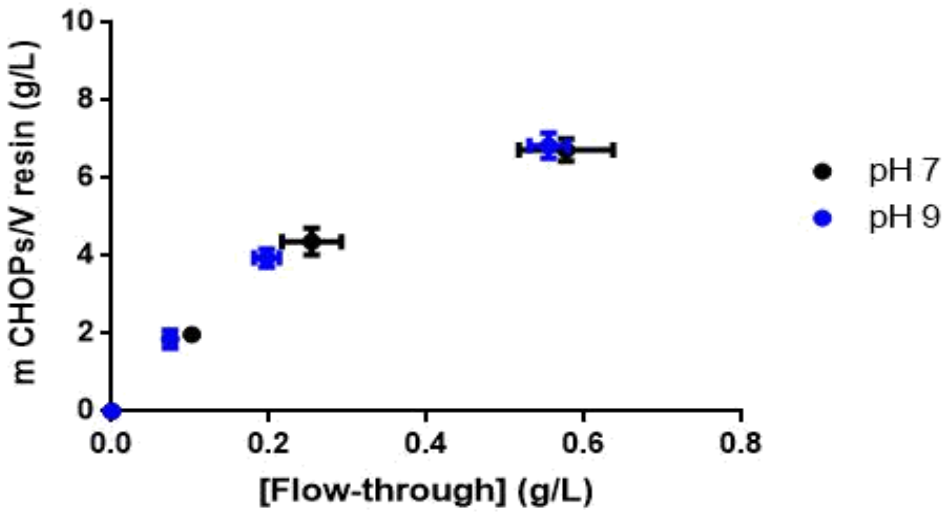

Figure 25 – Adsorption isotherm obtained, with DEAE-Sepharose beads, for two different pH values (7.0 and 9.0) and three successive dilutions from an initial concentration of CHOPs.

Represented in the graphs above is the mass of CHOPs adsorbed by the volume of resin, as a function of the protein concentration in the supernatant. The maximum value reached in both assays is supposed to be the binding capacity for the resins tested. As it can be observed, the maximum value reached corresponds to the sample which was not diluted, and this value is higher for the Q-Sepharose resin (7.12 and 7.52 g/L, **Figure 24**) than for the DEAE-Sepharose one (6.84 and 6.73 g/L, **Figure 25**). This was expected since Q-Sepharose has a higher ionic capacity (0.18-0.25 mmol Cl^-/mL resin) than DEAE-Sepharose (0.11-0.16 mmol Cl^-/mL resin).

Moreover, despite being determined for BSA samples, the binding capacity for these resins are approximately 100 g/L, which is about 10 times the values obtained in these experiments. These discrepancies may result from the variable resin volume put in each of the plate's wells, the unideal packing of the beads or the fact that the values obtained can still be in the linear zone of the adsorption isotherm. To overcome this problem, a higher initial protein concentration had to be tested.

IV.3 CHO Cell Culture Supernatant Purification

In order to separate and label the proteins present in the cell cultures and the monoclonal antibody of interest, and to perform the microfluidic assays, a protein A affinity chromatography was done (**Figure 26**).

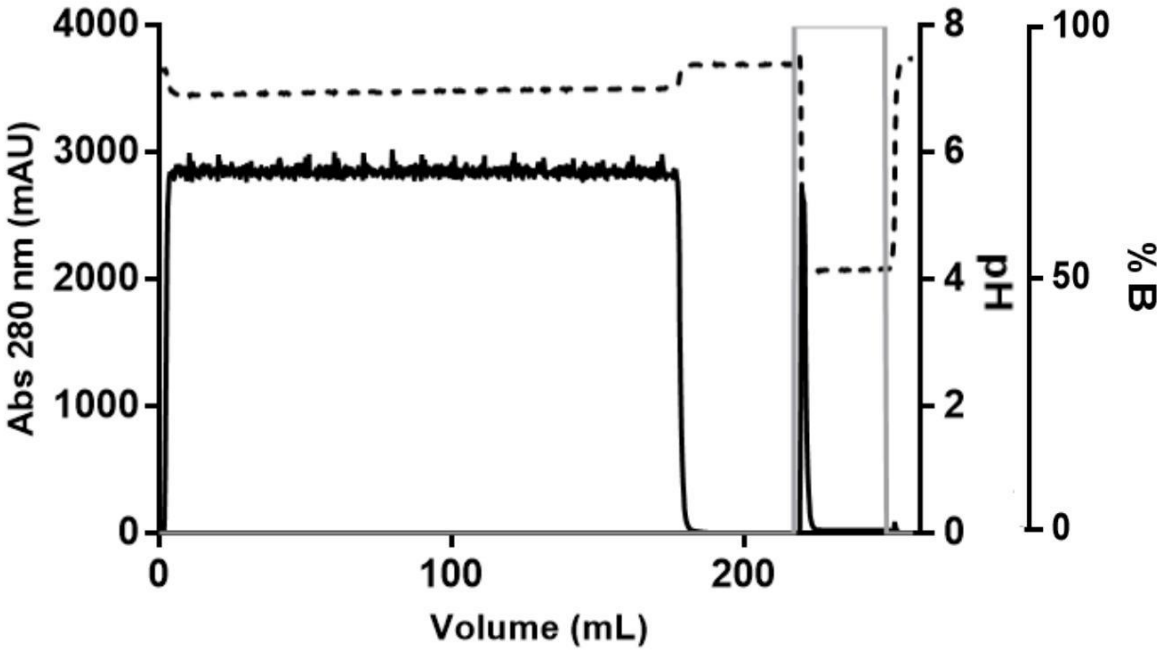

Figure 26 – Chromatographic profile of the CHO DP-12 supernatant loaded onto a Protein A resin. The adsorption was performed using 20 mM Phosphate, 150 mM Sodium Chloride, pH 7.2 buffer, and the elution with 100 mM Sodium Citrate, pH 3-3.6. Absorbance at 280 nm (mAU) ; Concentration of elution buffer (%B) and pH .

As mention, protein A resins are widely used for the capturing step of monoclonal antibodies, during the downstream processing. The chromatographic conditions, for both adsorption and elution, were already established and the approach applied for the elution step was the decrease in pH. By running a buffer with a relatively low pH (3-3.6), the highly ionizable amino acid residues on the Protein A and the Fc region of the antibody, gain positive charges thus repelling each other and separating the ligand from the molecule of interest. Although it is one of the downsides of using Protein A chromatography, to avoid the degradation of the fractions (1 mL each) of pure IgG collected by the low pH value, there is the need to add 100µL of 1M Tris-HCl, pH 8 to each tube. To obtain more concentrate and narrower peaks, the purifications were performed using single step elutions. The results obtained for the protein purity, purity factor and yield are presented in **Table 4**.

Table 4 – Resume of the protein purity (%), purity factor and yield (%) results determined for the FT and Elution pools collected from the purification process.

	Protein Purity (%)	Purity Factor	Yield (%)
FT	0.00	0.00	0.00
Elution	96.98±3.65	42.90±1.61	86.39±3.68

IV.4. Microfluidic Assays

For the microfluidic assays, a microfluidic structure was designed by a Master's student at INESC MN in the AutoCAD software. The features of the microcolumn are presented below (**Figure 27**).

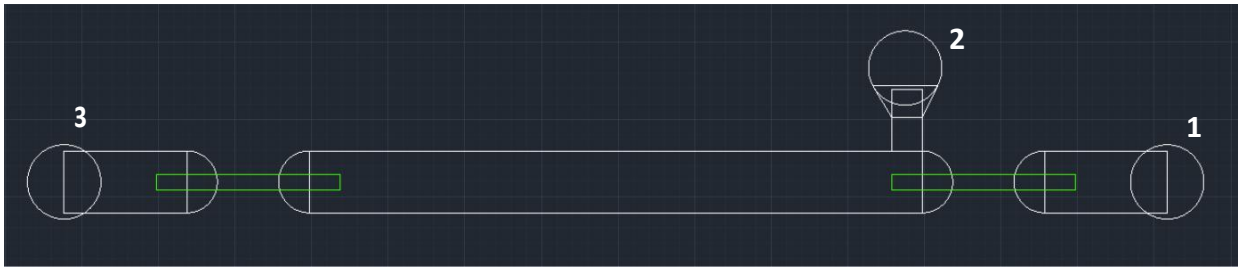

Figure 27 – AutoCAD mask designed by Master's student Pedro Monteiro. The structure has one liquid inlet (1), one beads inlet (2) and one liquid outlet (3).

The beads are packed through the beads inlet until the column is fully packed and they are trapped because of the height difference between the beads chamber (100 µm) and the green channels (20 µm) that connect the chamber where the beads are trapped and the liquid inlet and outlet. The column is 1 cm long and 1 mm wide.

Since the major impurity present in the CHO DP-12 cell culture supernatant is BSA, this protein will be used as model molecule to test the microfluidic structure and chromatographic conditions on the assays to be performed, prior to the assays using real samples (the protein impurities present in the supernatant and collected in the FT of the protein A affinity chromatography). The concentrations used for the BSA samples were of 0.5 g/L, which is the concentration determined for the FT of the CHO DP-12 cell culture supernatant. For the tests with IgG a concentration of 0.1 g/L was selected, which correspond to the maximum concentration of antibody that can be produced using these cells.

In order to allow the monitorization of the miniaturized chromatographic process, both BSA and IgG were labelled using two different fluorophore dyes. For the BSA, the dye used was Bodipy™ FL Maleimide, which is a thiol-reactive green fluorescent dye (producing electronically neutral conjugates), while for IgG, the dye chosen was Bodipy™ TMR C_5-Maleimide, which is a thiol-reactive red fluorescent dye.

The degree of labelling obtained for BSA was of 0.86 mol dye/mol BSA and for IgG a value of 0.22 mol dye/mol IgG.

After performing the labelling protocol for both species, an IEF gel was run to confirm that the binding of the dye to the protein/antibody did not change their isoelectric point (**Figure 28**).

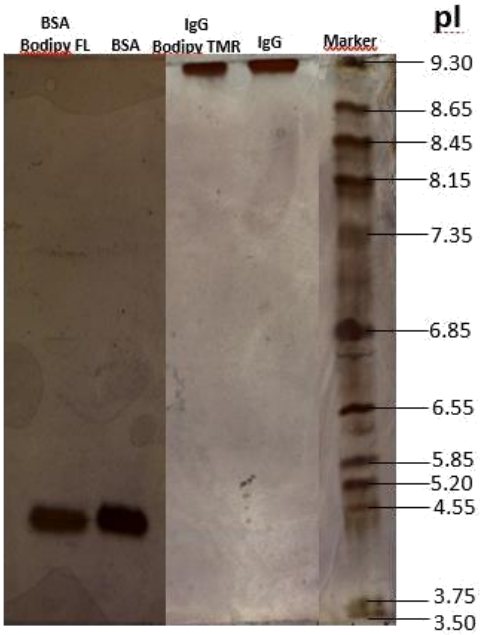

Figure 28 – Silver stained IEF gel of BSA and IgG labelled and non-labelled samples.

As can be observed in **Figure 28**, the binding of both dyes did not change the isoelectric point of either molecule they were conjugated with, allowing the chromatographic conditions to be unchanged after the labelling procedure.

To establish a correlation between the fluorescent intensity and the concentration of each molecule, two calibration curves were calculated using four different concentrations of proteins (1.0, 0.75, 0.5 and 0.25 g/L) and the fluorescence intensity measured in the outlet channel. The results are shown below, in **Figure 29**.

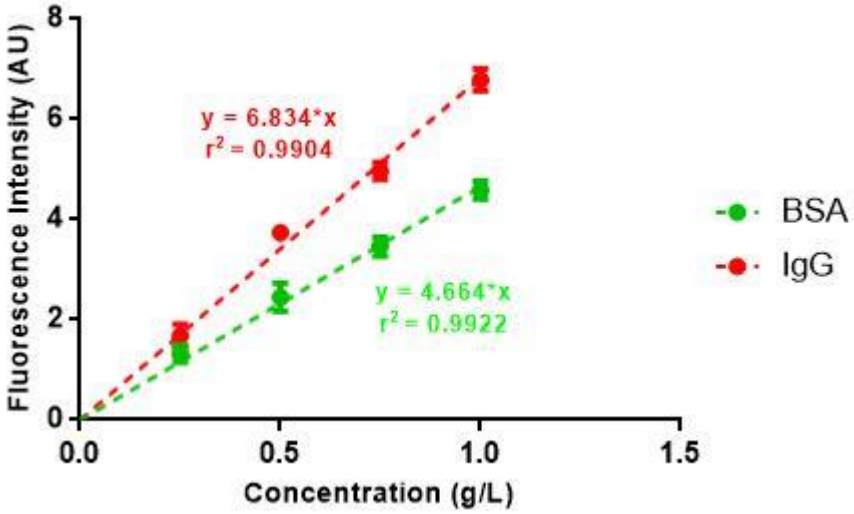

Figure 29 – Calibration curves obtained for the fluorescence intensity as a function of concentration for both BSA (in green) and IgG (in red). The values presented are an average of three independent assays.

From the graph above is possible to observe that, for the same concentration, the fluorescence intensity of the IgG labelled is higher than the one obtained for the BSA. This means that less molecules of the antibody are needed for its presence to be detected in the microfluidic channel.

IV.4.1. Breakthrough Curves

To try to determine when the column becomes saturated and which flow-rate is better for the chromatographic process to be designed, several breakthrough curves were performed. Three flow-rates were chosen (5, 3 and 1 µL/min) and the fluorescence was measured on the outlet channel, over time, under three different conditions. The assays were done flowing the BSA (0.5 g/L) and IgG (0.1 g/L) samples in three different ways: i) empty channels ("Channel" series); ii) in channels fully packed with beads and equilibrated with adsorption buffer ("Adsorption" series), and iii) in channels fully packed with beads, but equilibrated with elution buffer ("Elution" series).

These assays were performed firstly with BSA and IgG samples flowed separately through the column (**Figures 30** and **31**, respectively), then with a mixture of BSA and IgG (**Figure 32**), and finally with CHOPs collected from the FT samples (**Figure 33**). The results are shown below.

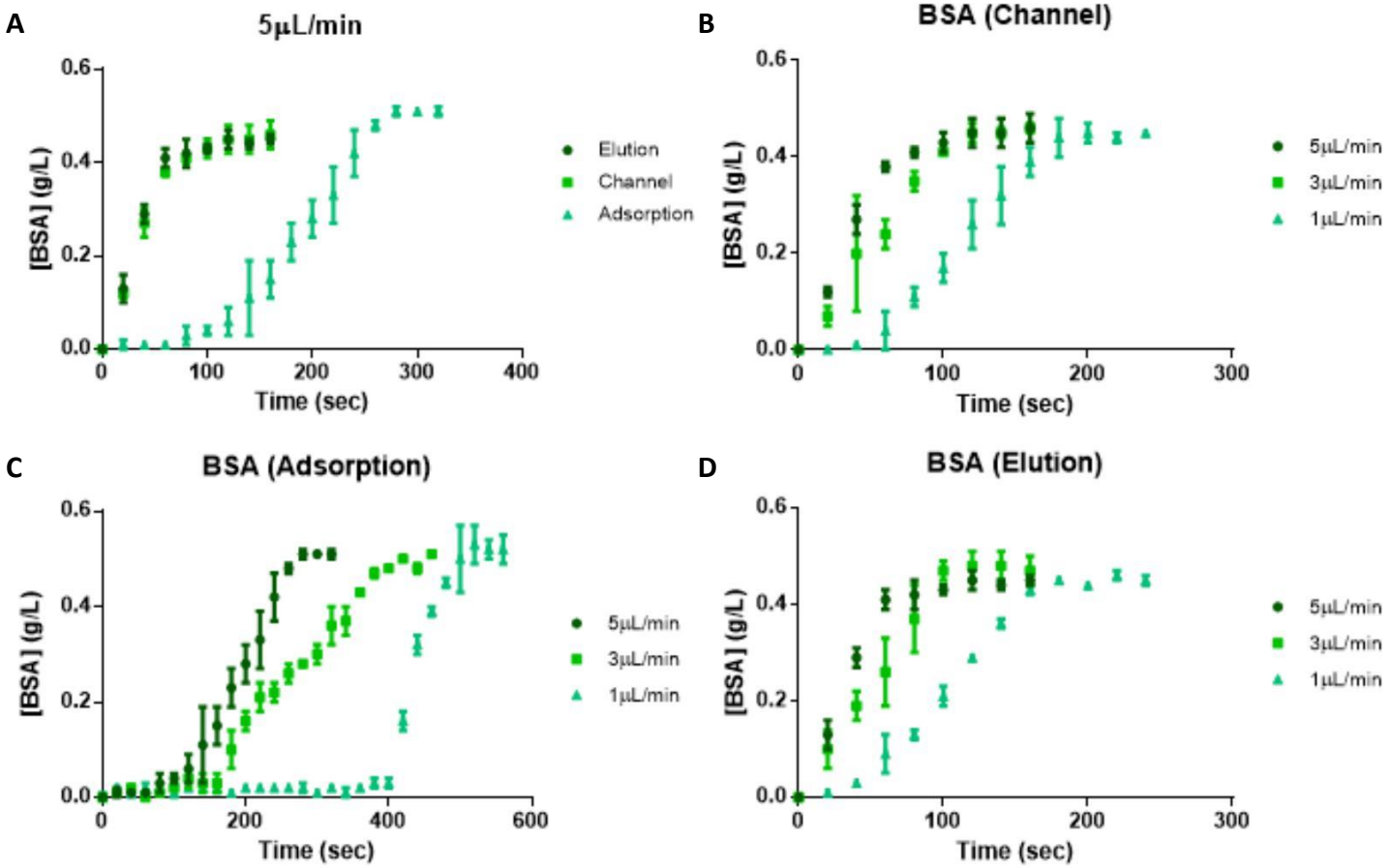

Figure 30 – Breakthrough curves obtained for BSA samples (0.5 g/L). BSA concentration was measured in the liquid outlet channel over time under three different conditions (Channel, Adsorption and Elution). A – Three conditions using a flow-rate of 5 µL/min; B – Channel condition using the three chosen flow-rates; C – Adsorption condition using the three chosen flow-rates; D – Elution condition using the three chosen flow-rates.

The results obtained from flowing BSA samples, were the expected ones. When comparing the different conditions tested, at the same flow-rate - 5 µL/min - it is possible to observe that there is almost no difference between the "Elution" and the "Channel" assay. However, when looking at the "Adsorption" condition, the curve is much wider. These results are due to the binding of the BSA molecules to the beads, leaving the column much

later than when there is nothing to interact with ("Channel") or when the conditions are not proper for the interaction with the beads to happen ("Elution"). The similarity between the "Elution" and the "Channel" assays implies that the volume occupied by the beads is not significant to cause a decrease in the residence time of a protein under non-binding conditions. In theory, the breakthrough from the empty channel should have been observed at a slightly higher time since the void volume is higher in the empty column (porosity, =1) than in the packed column (<1), and consequently the residence time under non-binding conditions should have been higher.

In every condition tested it is possible to observe that the lower the flow rate, the wider the resulting breakthrough curve (**Figure 30 – B, C** and **D**). This is due to the fact that the lower the flow rate, the higher the residence time, and consequently the more time the samples take to flow through the column. When there is adsorption of the BSA molecules to the beads (**Figure 30 – C**), the breakthrough starts much earlier, around 150 s, at the fastest flow-rate (5 µL/min) than for the slowest one (1 µL/min), at around 400 s because the molecules take more time to flow through the column and thus to interact with the ligand.

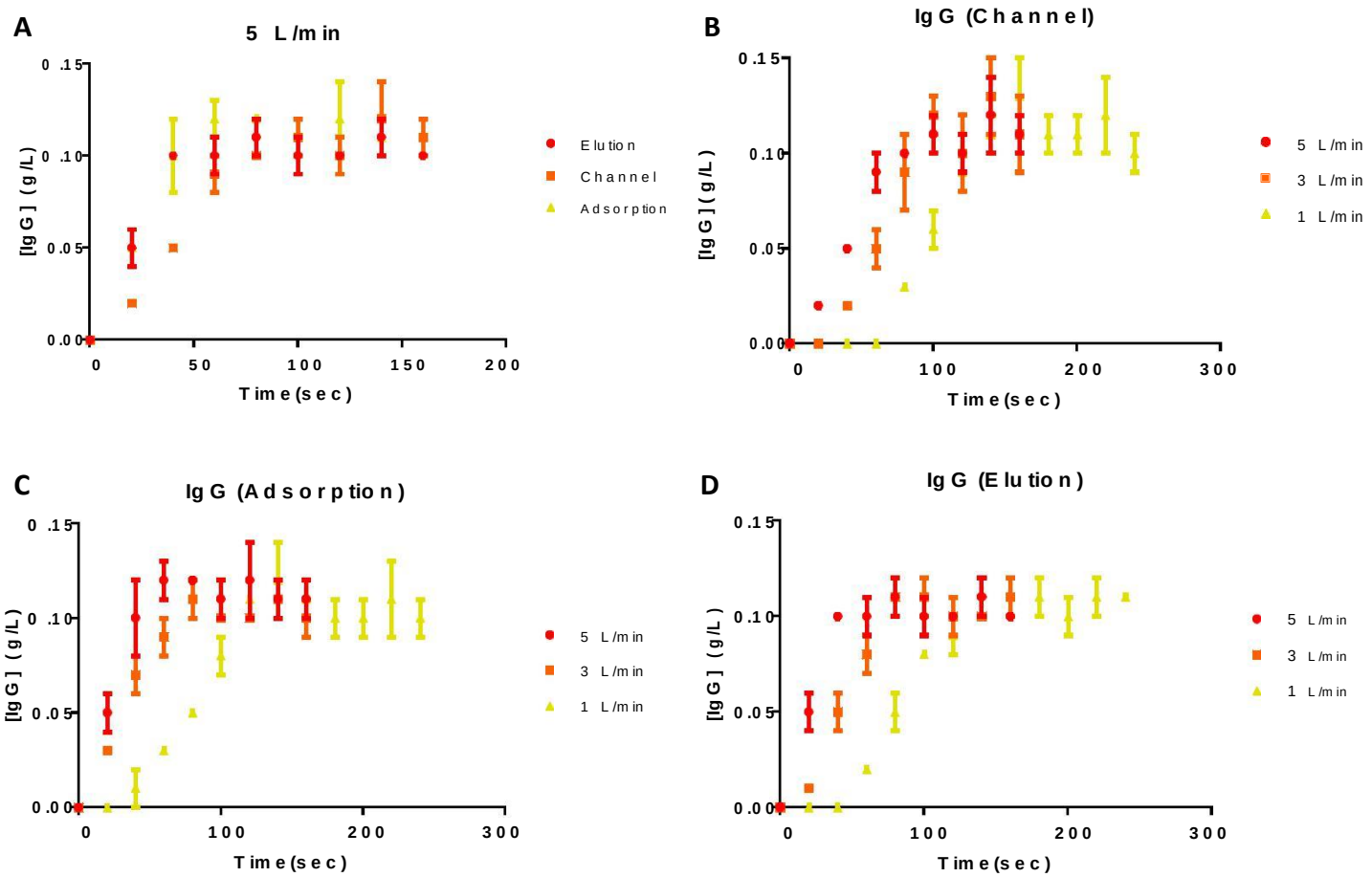

Figure 31 – Breakthrough curves obtained for IgG samples (0.1 g/L). IgG concentration was measured in the liquid outlet channel over time under three different conditions (Channel, Adsorption and Elution). A – Three conditions using a flow-rate of 5 µL/min; B – Channel condition using the three chosen flow rates; C – Adsorption condition using the three chosen flow rates; D – Elution condition using the three chosen flow rates.

In the case of IgG (**Figure 31**), as both protein and resin are positively charged there should be no binding under both adsorption and elution conditions. As said before, the goal is to capture the impurity proteins and let the antibody flow through the column unretained. Therefore, a similar behavior was expected for this molecule under all the conditions tested. In **Figure 31 – A**, where different conditions are compared for the same flow-rate, no difference can be observed between flowing IgG under "Adsorption" or "Elution" conditions. However, when IgG was flowed in an empty channel the breakthrough occurred a little bit later due to the higher residence time in the empty channel (=1) when compared to the packed channels (<1).

The results shown in **Figure 31 – B, C** and **D** confirm what was the expected behavior under different flow rates, with IgG breakthrough observed at a higher time point for higher residence times (lower flow rates).

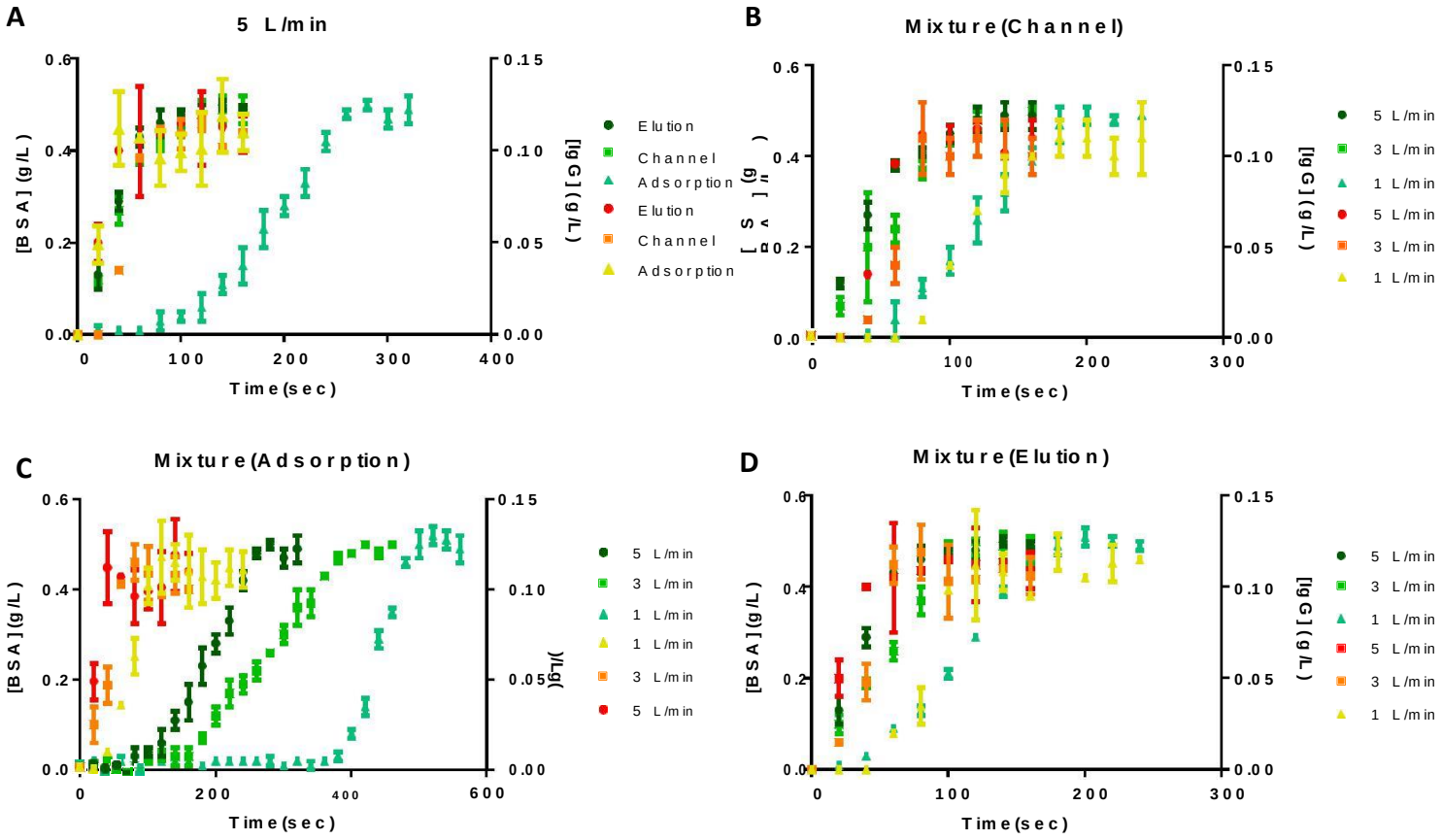

Figure 32 – Breakthrough curves obtained for mixture (BSA + IgG) samples (0.5 and 0.1 g/L respectively). BSA and IgG concentrations were measured in the liquid outlet channel over time under three different conditions (Channel, Adsorption and Elution). The measurements regarding BSA are represented in green tones and the ones of IgG are represented in red, orange and yellow. A – Three conditions using a flow-rate of 5 µL/min; B – Channel condition using the three chosen flow-rates; C – Adsorption condition using the three chosen flow-rates; D – Elution condition using the three chosen flow-rates.

After doing the assays flowing samples of only BSA or IgG, a mixture of both molecules was prepared and samples were flowed in the same conditions and flow rates to understand if the behavior was the same as the shown above (**Figures 30** and **31**).

In **Figure 32**, the IgG measurements are represented in red, orange and yellow and the BSA ones are represented in different tones of green and by comparing the profile of the curves of the mixture samples with one represented for the samples flowed alone (**Figures 30** and **31**), it is possible to conclude that the behavior observed for the samples of the mixture solution is similar to the one obtained when the molecules were flowed alone in the columns. This indicates that there is no significant effect of one molecule on the behavior of the other.

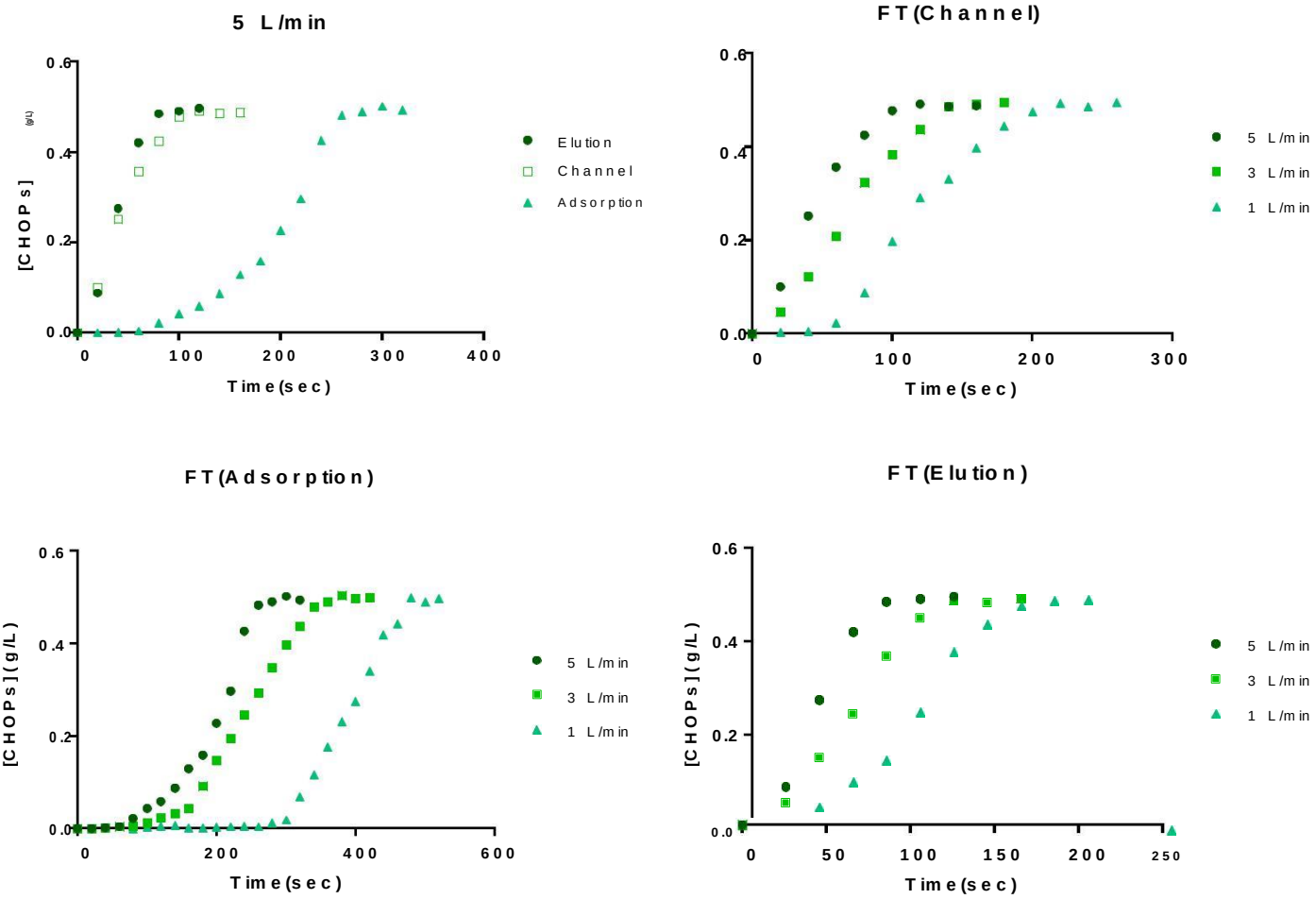

Figure 33 – Breakthrough curves obtained for FT samples (0.5 g/L). CHOPs concentration was measured in the liquid outlet channel over time under three different conditions (Channel, Adsorption and Elution). A – Three conditions using a flow-rate of 5 µL/min; B – Channel condition using the three chosen flow-rates; C – Adsorption condition using the three chosen flow-rates; D – Elution condition using the three chosen flow-rates.

After determining the breakthrough curves using a model protein (BSA), the CHOPs present in the FT samples were used. The results are presented in **Figure 33** and it is possible to observe that the profile of the curves obtained was similar to the ones obtained for the BSA measurements (both alone and in the mixture).

From the curves shown in **Figure 33 – A**, resulting from the three different conditions at 5 µL/min, was possible to observe that, like for the BSA assays, the "Elution" and "Channel" curves are similar and the Adsorption condition, when the CHOPs bind to beads, results in the proteins leaving the column later and in a wider curve.

IV.4.2. Adsorption Assays

After doing the breakthrough curves assays, several adsorption/elution tests were performed firstly using the BSA + IgG artificial mixture and afterwards using the CHOPs. The values measured are presented in arbitrary units since the measurements are done at the end of the column and not on the liquid outlet channel, like for the breakthrough curves.

For the mixture samples assay (**Figure 34**), it was observed that when the adsorption buffer (20 mM Tris-HCl pH 8.0) had no salt (NaCl) in its composition, there was an increase in IgG binding in the column. This may result from the unspecific binding of the antibody molecules to the BSA, adsorbed to the beads. This is a problem when the main goal is to develop a process to continuous purify the IgG. To overcome this problem, two different concentrations of NaCl were added to the adsorption buffer (10 and 20 mM).

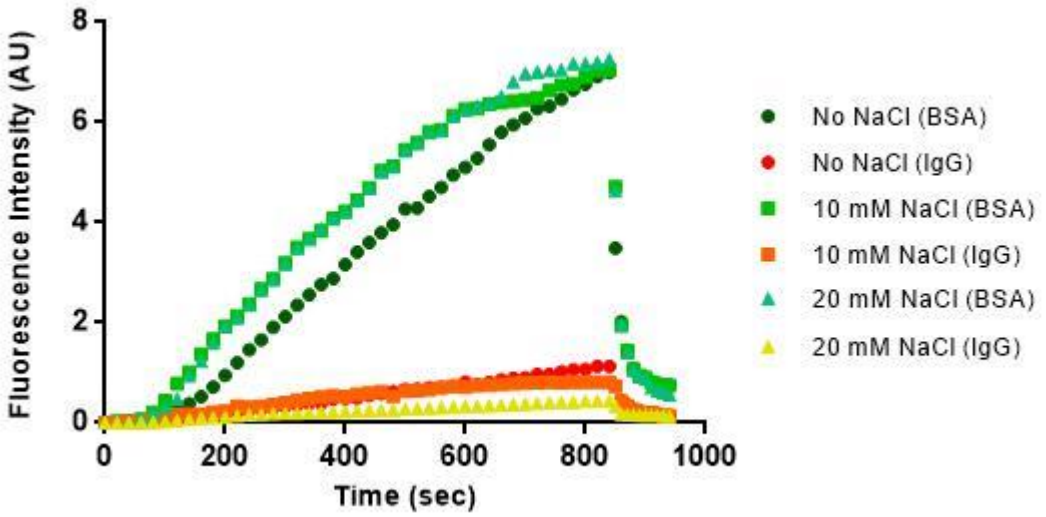

Figure 34 – Adsorption assays done with mixture samples (BSA + IgG), tested for three different adsorption buffer's salt concentration (0, 10 and 20 mM NaCl in 20 mM Tris-HCl pH 8.0), followed by elution using 1 M NaCl in 20 mM Tris-HCl pH 8.0.

In **Figure 34**, is possible to see an increase in the fluorescence intensity over time for BSA (which is similar in all conditions) and IgG, until the eluent buffer was changed to the elution buffer (20 mM Tris-HCl pH 8.0, 1 M NaCl). Although there is no major difference in BSA adsorption in all the conditions tested, the IgG unspecific binding decreases with the increase in the buffer's salt concentration, thus being the use of adsorption buffer containing 20 mM NaCl the best condition for the chromatographic process to be performed.

To compare the different adsorption assays, regarding the BSA measurements, the parameters $k_{1/2}$ (first derivative at the mid-point) and maximum fluorescence measurement were chosen. To determine the $k_{1/2}$ values, **Equation 1** was used, where F_0 is the background fluorescence, F_{max} is the maximum fluorescence, and k_s is the sigmoidal slope. The results are resumed in below, in **Table 5.**

$$k_{1/2} = \frac{k_s . ln10 . (F_{max} - F_0)}{4}$$ **Equation 1**

Table 5 – Resume of the $k_{1/2}$ and maximum fluorescence values obtained for the BSA measurements on the assays done with different NaCl concentrations.

	$k_{1/2}$	Maximum Fluorescence Measurement (AU)
No NaCl	0.0080	6.997
10 mM NaCl	0.0103	7.045
20 mM NaCl	0.0096	6.987

As can be observed in **Table 5**, the values obtained for the BSA measurements in the assays with three different NaCl concentration are similar to one and other.

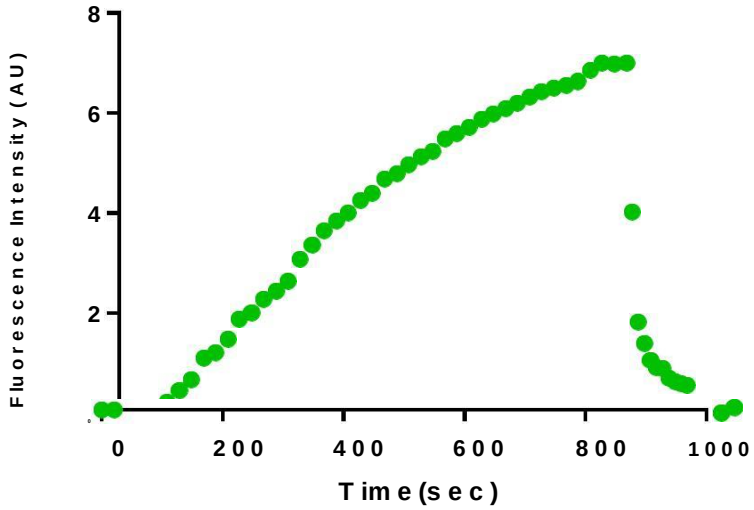

Figure 35 – Adsorption assay done with FT samples, tested with an adsorption buffer's salt concentration of 20 mM.

Regarding the assays performed using CHOPs, the result shown in **Figure 35** confirm that the chromatographic behavior of the FT samples is analogous to the one of BSA. The fluorescence intensity increases over time, to a value analogous (6.987 AU) to the ones obtained for the BSA measurements and presented in **Table 5**. Moreover, the value obtained for the first derivative at the mid-point (0.0089) is similar to the ones showed also in **Table 5**.

V. Conclusions and Future Work

The main goal of this master's thesis project was to develop a lab-on-a-chip platform for the purification of monoclonal antibodies, having as basis a chromatographic process in negative mode. That is, designing a microfluidic structure capable of successfully performing the chosen process and determining the best conditions to bind the impurities to the selected beads, and to let the antibody flow through the columns, without being retained.

With this in mind, two different CHO cell lines were successfully expanded in culture, namely a non-producing antibody cell line that grows in suspension, in serum-free medium (CHO-S) and an anti-IL-8-antibody-producing cell line that was grown adherently, in a serum-containing medium (CHO DP-12). It was observed that, during the first 4 days of culture, the maximum growth rate and protein concentration was higher for culture grown in serum-containing medium, indicating that the culture was growing at a faster pace.

The proteins present in the cell culture supernatant were analyzed by 2D-PAGE and it was possible to conclude that the CHOPs present in the CHO-S supernatant are of a wider range of isoelectric points and molecular weights. This indicates that the impurities can be harder to separate in the chromatographic process to be developed, than the ones present in the CHO DP-12 supernatant.

Regarding the determination of the binding capacity for the two anion exchanger resins selected (Q-Sepharose Fast Flow and DEAE-Sepharose Fast Flow), the results obtained confirmed what was previously known. Despite, the maximum values obtained for both resins being about 10 times lower than the described, it is higher for the Q-Sepharose one, since it is a stronger anion exchanger and has a higher ionic capacity than DEAE-Sepharose. However, it was not possible to adjust a Langmuir adsorption isotherm curve to the results because they were still in its linear zone. To adjust this curve and to determine the maximum capacity (Q_{max}) and dissociation (K_d) parameters, a higher initial protein concentration needed to be tested.

For the breakthrough curves assays, and after confirming that the labelling of the IgG and BSA did not change their isoelectric point, the results for both molecules in the mixture samples were similar to when they were flowed alone in the columns. Concerning BSA, when the conditions were proper for this molecule to bind to the beads ("Adsorption"), the curves obtained were wider than the ones for the "Elution" and "Channel" conditions. Although it was expected for BSA to leave the column later when under "Channel" conditions than under "Elution" conditions due to the void volume difference, the results do not show that. In the case of IgG, both protein and resin are positively charged and no difference was observed when flowing IgG under "Adsorption" or "Elution" conditions. However, when IgG was flowed in an empty channel, the breakthrough occurred a little bit later due to the higher residence time in the empty channel. In every condition tested it is possible to observe that the lower the flow rate, the wider the resulting breakthrough curve. This is due to the increasing residence time, and consequently the increasing time the samples take to flow through the column. The results obtained for the FT samples are similar to the ones of BSA.

The adsorption/elution assays done with the artificial mixture and CHOPs samples showed that the behavior for BSA and CHOPs is similar not only in the profile obtained (increasing fluorescence intensity over time until the eluent buffer was changed to the elution one) but also in the values of $k_{1/2}$ and maximum fluorescence determined, and that the increase in the buffer's salt concentration reduces IgG's unspecific binding.

For future work, assays for a mixture of CHOPs and IgG should be done to simulate the real supernatant and to figure out if the behavior is similar to tests performed before. Afterwards, different resins, like multimodal ones, and different buffer conditions, should be tested to evaluate if the possibility of having different types of interactions would increase the binding and removal of CHOPs from supernatant samples. Antibody production should also be tested in a serum-free medium since impurity concentration would be lower than the one tested in this project, making the process sharper.

The concentration of gDNA should also be determined, to understand if there is the need for a gDNA removal step, or if by using an anion exchanger resin it has the same behavior as the impurities and is removed in the first chromatographic step.

VI. References

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