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

Tiago Porfírio Santos

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.

Introduction

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.

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.[1]

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).

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.[2] Usually, the method used to perform this purification is protein A affinity chromatography, followed by one or two additional polishing chromatographic steps.

There are two drivers behind the current interest and demand in continuous bioprocessing. 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.[3]

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.

Materials and Methods

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).

Cell Culture

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 $5.3x10^5$ cells/mL each and incubated under constant agitation at 37 ^oC at 5% CO_2 atmosphere. 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 $2.8x10^4$ cells/cm^2 and incubated at 37^oC at 5% CO_2 atmosphere. Passages and supernatant collection were performed each four days.

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.

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.

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. Operating in two different configurations (Filter-to-waste and DirectStack™ modes), 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.

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).

Results and Discussion

CHO Cell Culture Supernatant Analysis

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 1**.

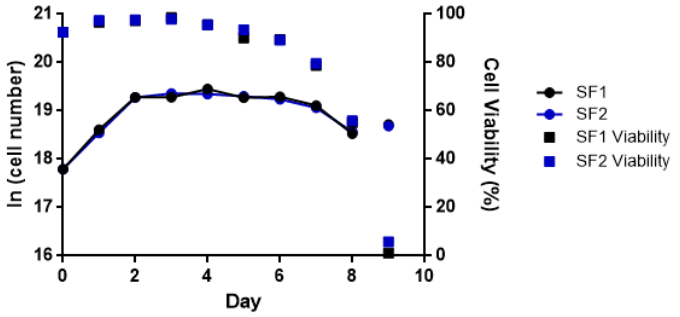

Figure 1 – CHO-S cell line growth curves and associated cell viability for SF1 and SF2 parallel cultures. The • and • correspond to the cell count (ln [cell number]) for the samples of the SF1 and SF2, respectively. The ■ and ■ correspond to cell viability (%) for the samples from the SF1 and SF2, respectively.

As expected and as shown in **Figure 1**, 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 1**, 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 1**, 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 1**.

Table 1 – 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 1**, are both similar between shaker flasks and to the results reported in the literature.[4]

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 2**).

As shown in **Figure 2**, 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.

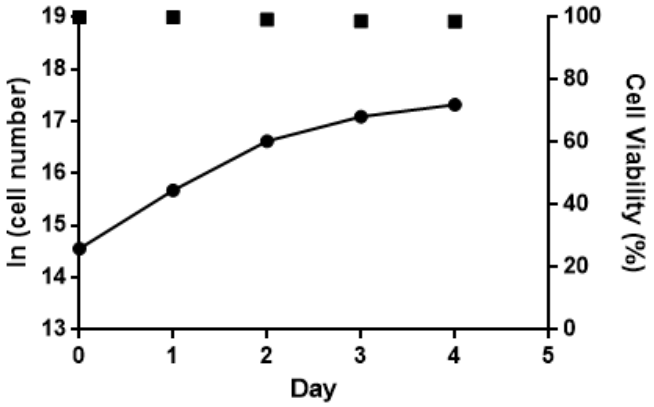

Figure 1 – CHO DP-12 cell line growth curve and associated cell viability. The • correspond to the cell count (ln [cell number]) and the ■ correspond to cell viability (%).

Based on the growth curves presented in **Figure 2**, 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 regarding the maximum growth rate (1.03 day^{-1}) and duplication time (0.671 day) 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.

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 3** and **4**, for the CHO-S and CHO DP-12 cell culture, respectively.

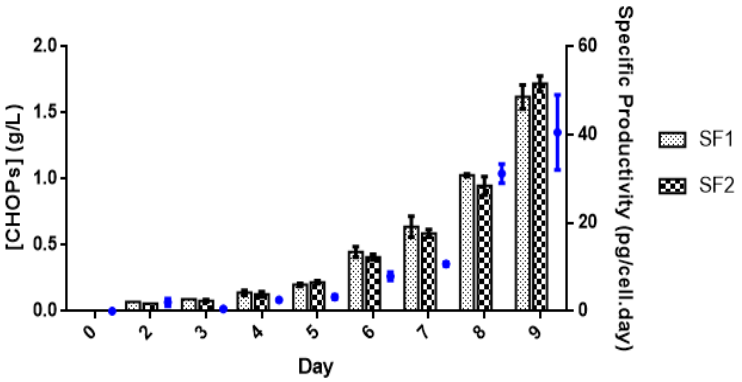

Figure 3 – CHOPs concentration, in mg/L, and specific productivity, in pg/cell·day, determined for samples collected for each day of CHO-S cell culture. The ▨ and ▩ correspond to CHOPs concentration for the SF1 and SF2 cultures, respectively, and the • correspond to an average of the specific productivity determined for each SF. There is no result for day one of culture, since the sample was discarded.

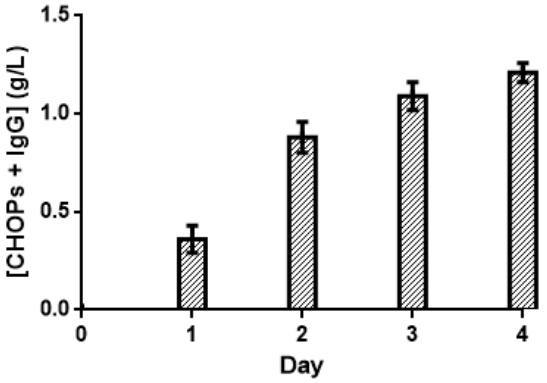

Figure 4 – CHOPs concentration, in mg/L, determined for samples collected for each day of CHO DP-12 cell culture.

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 3**) 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 (**Figure 4**), 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.

2D-PAGE

To associate the molecular weight of proteins to their pI, 2D-Electrophoresis were performed with samples from both cell culture supernatants (**Figures 5** and **6**).

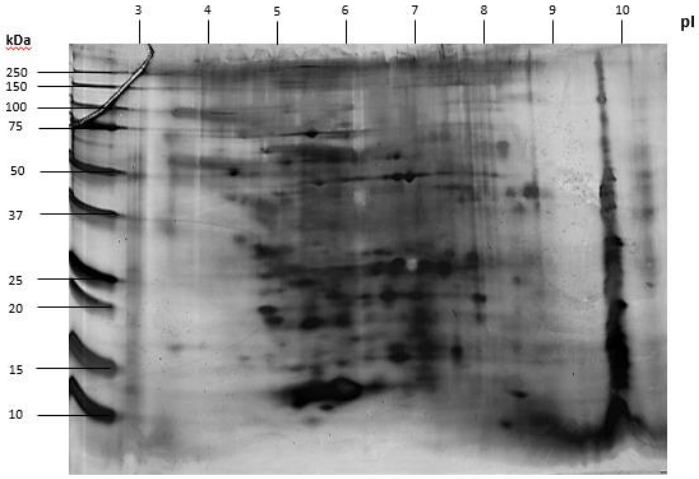

Figure 5 – Silver stained 2D-Electrophoresis a sample from the FT of the CHO-S cell culture supernatant. Proteins (500µg) were precipitated and separated based on their pI in 7cm IPG strips (pH 3-10) and then applied on an SDS-PAGE gel.

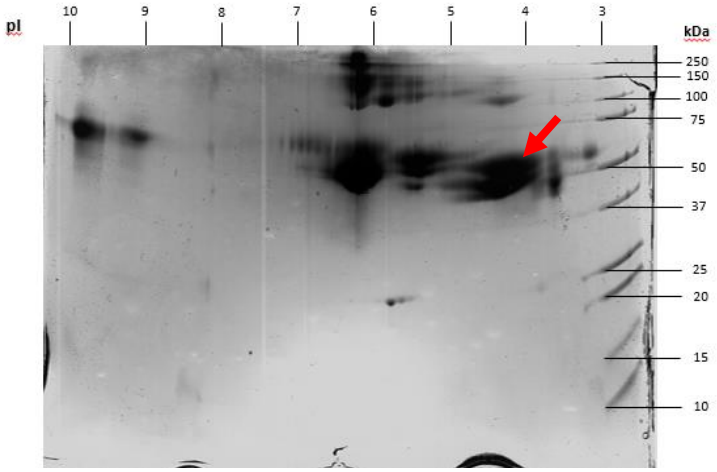

Figure 6 – Silver stained 2D-Electrophoresis a sample from the FT of the CHO DP-12 cell culture supernatant. Proteins (500µg) were precipitated and separated based on their pI in 7cm IPG strips (pH 3-10) and then applied on an SDS-PAGE gel. The red arrow points the most abundant protein present in the supernatant, BSA, which is added to the culture medium when adding the FBS.

In **Figure 5**, 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 is a promising indication that separation by charge will be possible since the mAb of interest (IgG) has a pI ≥ 9.3[5], and consequently a pH value higher than 7 should allow a selective fractionation between protein and mAb. However 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. 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. For the sample from the CHO DP-12 supernatant (**Figure 6**), the majority of proteins have a pI lower than 7.0, however there are some proteins appearing with a pI closer to the IgG and that will be harder to separate in the charge-based chromatographic process.

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 7**).

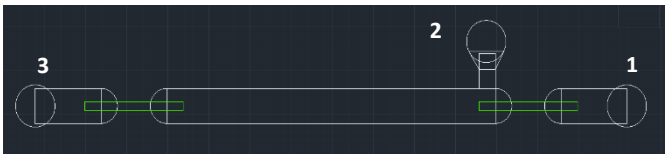

Figure 7 – AutoCAD mask designed by Master's student Pedro Monteiro. The structure has one liquid inlet (1), one beads inlet (2) and one liquid outlet (3).

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 and confirmed that the binding of the dye to the protein/antibody did not change their isoelectric point, 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.

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 8** and **9**, respectively), then with a mixture of BSA and IgG (**Figure 10**), and finally with CHOPs collected from the FT samples (**Figure 11**). The results are shown below.

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").

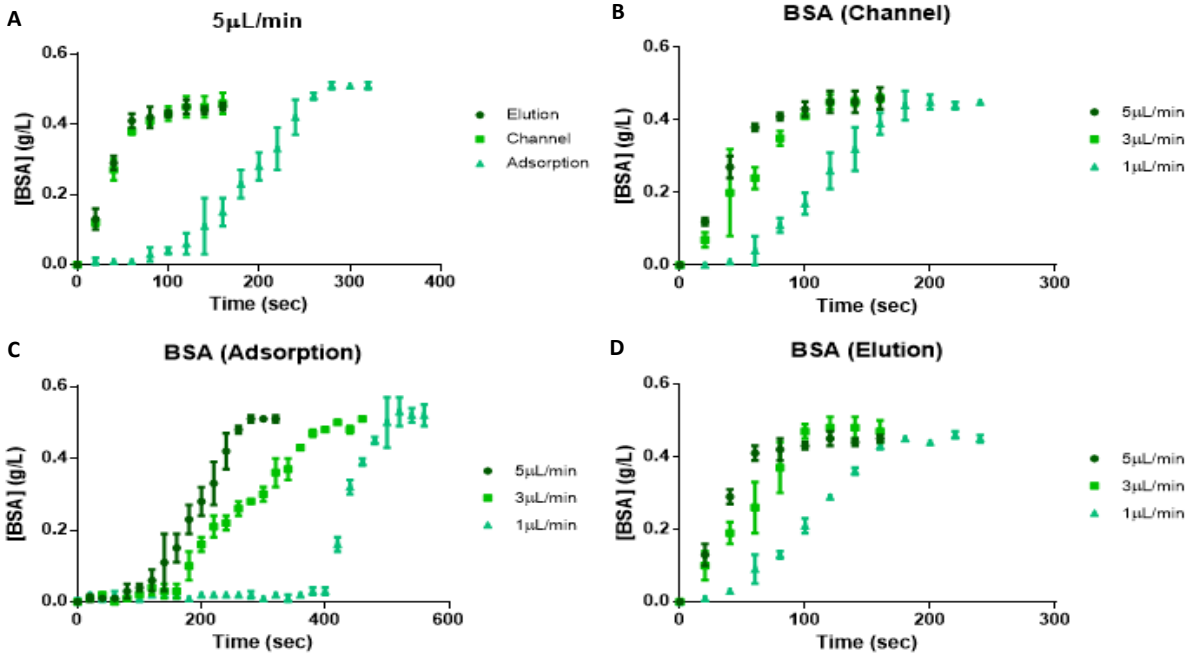

Figure 8 – Breakthrough curves obtained for BSA samples (0.5 g/L). BSA concentration was measured in the liquid outlet channel over time under three different conditions (Channel, Adsorption and Elution). A – Three conditions using a flow-rate of 5 µL/min; B – Channel condition using the three chosen flow-rates; C – Adsorption condition using the three chosen flow-rates; D – Elution condition using the three chosen flow-rates.

The 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

In every condition tested it is possible to observe that the lower the flow rate, the wider the resulting breakthrough curve (**Figure 8 – 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

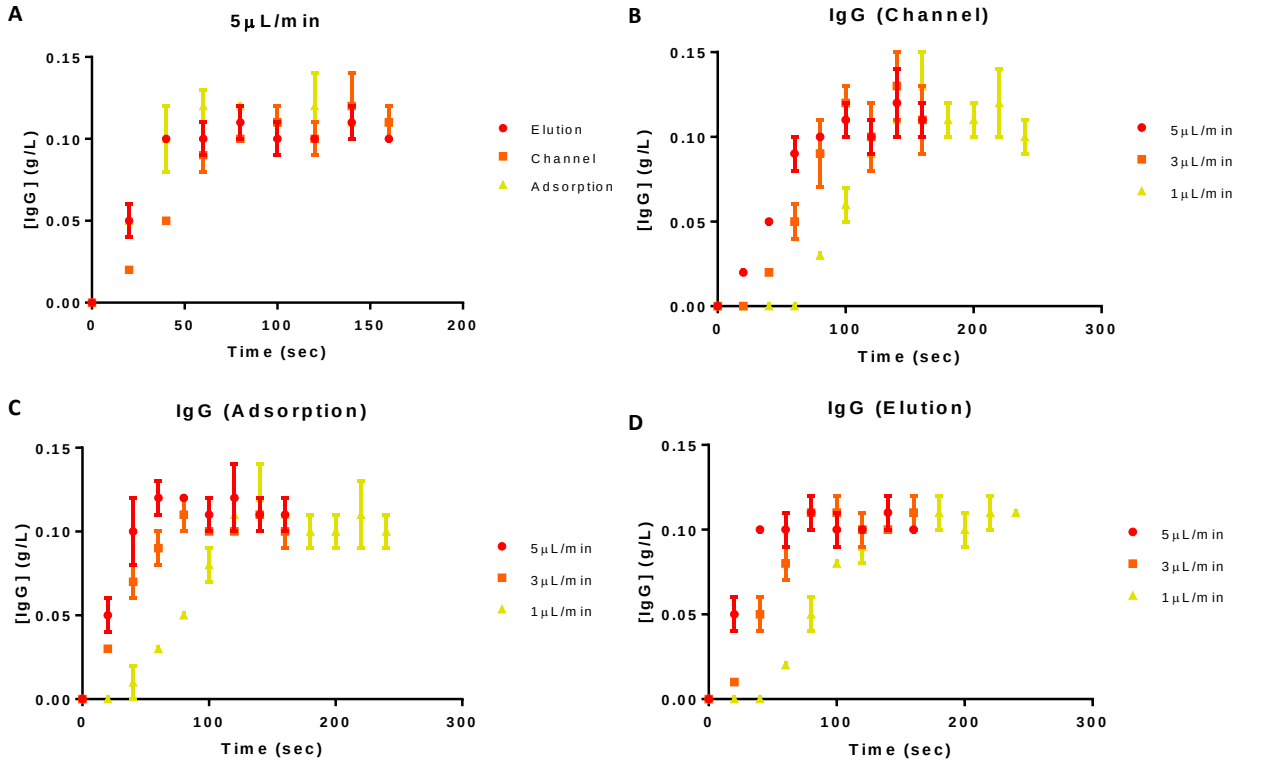

Figure 9 – Breakthrough curves obtained for IgG samples (0.1 g/L). IgG concentration was measured in the liquid outlet channel over time under three different conditions (Channel, Adsorption and Elution). A – Three conditions using a flow-rate of 5 µL/min; B – Channel condition using the three chosen flow rates; C – Adsorption condition using the three chosen flow rates; D – Elution condition using the three chosen flow rates.

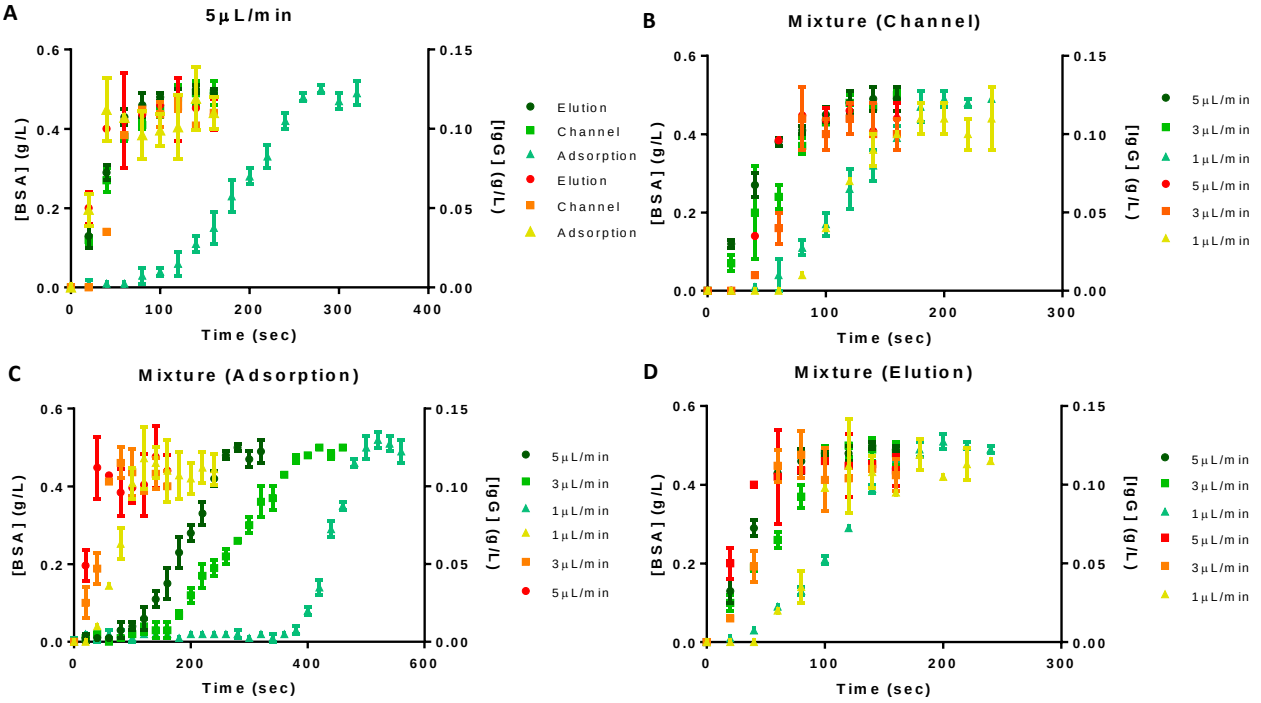

Figure 10 – Breakthrough curves obtained for mixture (BSA + IgG) samples (0.5 and 0.1 g/L respectively). BSA and IgG concentrations were measured in the liquid outlet channel over time under three different conditions (Channel, Adsorption and Elution). The measurements regarding BSA are represented in 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.

s because the molecules take more time to flow through the column and thus to interact with the ligand.

In the case of IgG (**Figure 9**), 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 9 – 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 (ε =1) when compared to the packed channels (ε <1).

The results shown in **Figure 9 – 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).

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 8** and **9**).

In **Figure 10**, 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 8** and **9**), 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.

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 11** 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 11 – 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.

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 12**), 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,

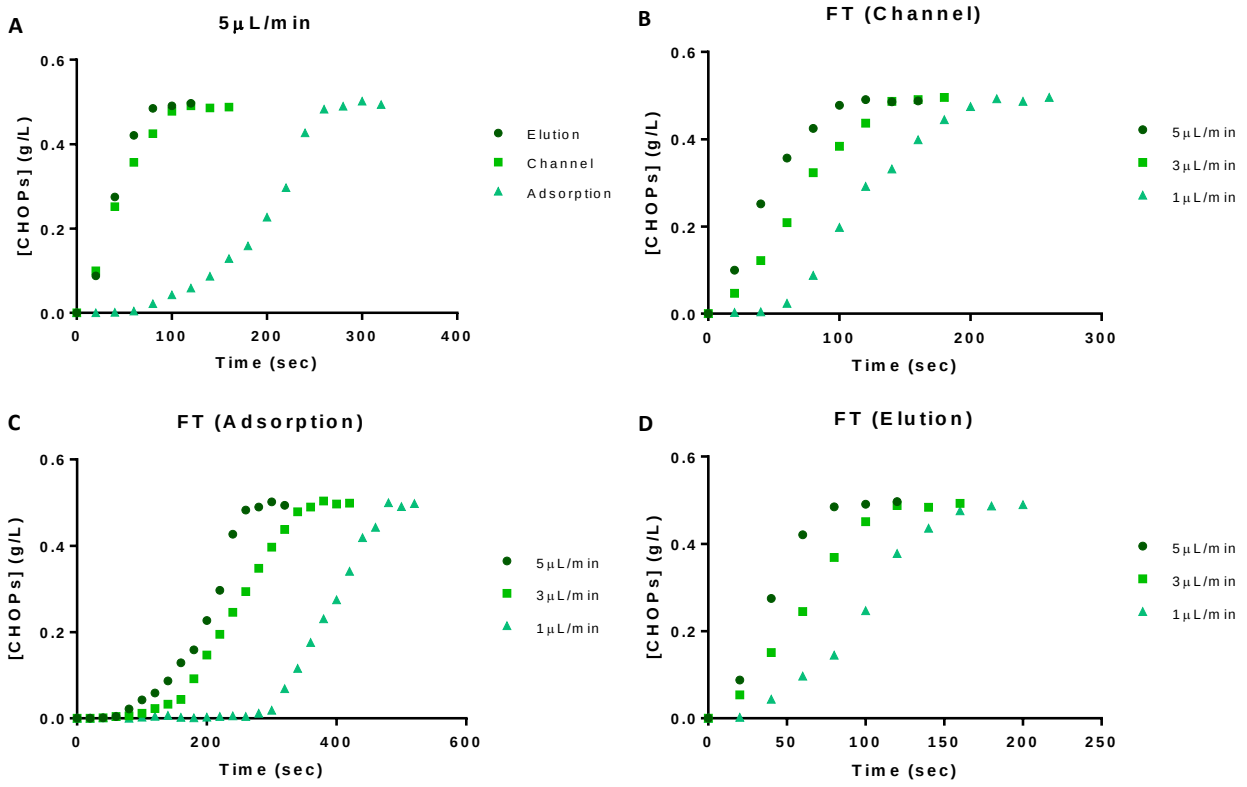

Figure 112 – Breakthrough curves obtained for FT samples (0.5 g/L). CHOPs concentration was measured in the liquid outlet channel over time under three different conditions (Channel, Adsorption and Elution). A – Three conditions using a flow-rate of 5 µL/min; B – Channel condition using the three chosen flow-rates; C – Adsorption condition using the three chosen flow-rates; D – Elution condition using the three chosen flow-rates.

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).

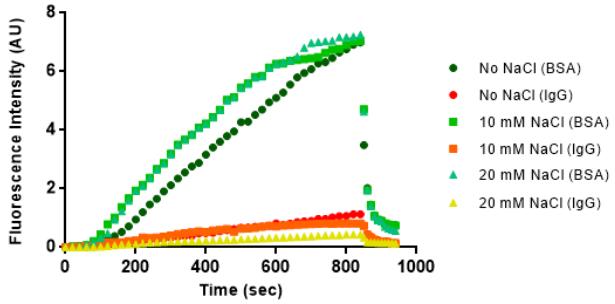

Figure 12 – Adsorption assays done with mixture samples (BSA + IgG), tested for three different adsorption buffer's salt concentration (0, 10 and 20 mM NaCl in 20 mM Tris-HCl pH 8.0), followed by elution using 1 M NaCl in 20 mM Tris-HCl pH 8.0.

In **Figure 12**, 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 2.**

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

Table 2 – 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 2**, the values obtained for the BSA measurements in the assays with three different NaCl concentration are similar to one and other. Regarding the assays performed using CHOPs, the result shown in **Figure 13** confirm that the chromatographic behavior of the FT samples is analogous to the one of BSA.

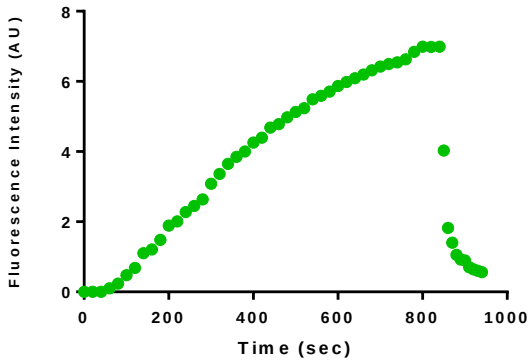

Figure 13 – Adsorption assay done with FT samples, tested with an adsorption buffer's salt concentration of 20 mM.

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 2**. Moreover, the value obtained for the first derivative at the mid-point (0.0089) is similar to the ones showed also in **Table 2**.

Conclusion 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.

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, indicating 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.

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. 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.

References

[1] Li, F., Vijayasankaran, N., Shen, A., Kiss, R., & Amanullah, A. (2010). Cell culture processes for monoclonal antibody production. *mAbs*, *2*(5), 466–479.

[2] Park, J. H., Jin, J. H., Lim, M. S., An, H. J., Kim, J. W., & Lee, G. M. (2017). Proteomic analysis of host cell protein dynamics in the culture supernatants of antibody-producing CHO cells. *Scientific Reports*, *7*(March), 1–13.

[3] Zydney, A. L. (2015). Perspectives on integrated continuous bioprocessing - opportunities and challenges. *Current Opinion in Chemical Engineering*, *10*, 8–13.

[4] López-Meza, J., Araíz-Hernández, D., Carrillo-Cocom, L. M., López-Pacheco, F., Rocha-Pizaña, M. del R., & Alvarez, M. M. (2016). Using simple models to describe the kinetic of growth, glucose consumption, and monoclonal antibody formation in naïve and infliximab producer CHO cells. *Cytotechnology, 68(4)*, 1287–1300.

[5] Rosa, S. A. S. L., Dos Santos, R., Aires-Barros, M. R., & Azevedo, A. M. (2016). Phenylboronic acid chromatography provides a rapid, reproducible and easy scalable multimodal process for the capture of monoclonal antibodies. *Separation and Purification Technology*, *160*, 43–50.