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Margaux Vigata

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Margaux VIGATA

Née le 08 Mars 1990 à Strasbourg

Titre de thèse

**DEVELOPPEMENT ET OPTIMISATION DE PROCEDE DE
PRODUCTION D'ANTICORPS MONOCLONAUX EN
CELLULES CHO**

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Abbreviations

Ab: antibody	iCE: Isoelectric Capillary Electrophoresis
APG: Acidic peak group	IgG: Immunoglobulin G
BPG: Basic peak group	IL: InterLeukin
CHO: Chinese Hamster Ovary	INF: Interferon
CCCF: Clarified Cell Culture Fluid	IVCC: Integrated VCC
CDR: Complementary Determining Region	LMWS: Low molecular weight species
cGMP: current Good Manufacturing Practices	mAb: Monoclonal antibody
CPP: Critical Process Parameters	MTX: Methotrexate
CQA: Critical Quality Attributes	N.A: Non Applicable
DF: DiaFiltration	NBE: New Biological Entity
DNA: DeoxyriboNucleic Acid	NCE: New Chemical Entity
DO: Dissolved Oxygen	NS0 & sp2/0: murine myeloma and hybridoma cell lines
DoE: Design of Experiment	NTU: Nephelometric Turbidity Units
DSP: Downstream Process	PCV: Packed Cell Volume
Fab: Fragment antigen binding	pDADMAC: Polydiallyldimethylammonium Chloride
Fc: Fragment crystallisable	pI: Isoelectric point
FcR : Fc Receptor	PQA: Product Quality Attributes
FcRn: neonatal Fc Receptor	P/V: Volumetric Power input
FDA: Food and Drug Administration	QbD: Quality by Design
Fv: single domain variable Fragment	rpm: round per minute
HACA: Human Ab against Chimeric Ab	scFv: single chain Fv
HAHA: Human Ab against Humanised Ab	scFv-Fc: scFv with Fc domain
HAMA: Human Ab against Murine Ab	SDM: Scale-down model
HAT: Hypoxanthine, Aminopterin, Thymidine	SEC: Size Exclusion Chromatography
HCP: Host Cell Protein	TNF: Tumor Necrosis Factor
HEK293: Human Embryonic Kidney cells	TSD: Target Seeding Density
HGPRT: Hypoxanthine-Guanine-PhosphoRibosylTransferase	UF: UltraFiltration
HMWS: High molecular weight species	USP: Upstream Process
HPLC: High Performance Liquid Chromatography	VCC: Viable cell Concentration
HT-1080: Human HT-1080 cells	

Glossary

Depth filters:

A_{D1}: Depth filter 1 from supplier A. Pore diameter: 3.5 - 0.2 μm; Surface 22 cm² or 250 cm²

A_{D2}: Depth filter 2 from supplier A. Pore diameter: 0.85 - 0.1 μm; Surface 22 cm² or 250 cm²

B_{D1}: Depth filter 1 from supplier B. Pore diameter: 0.4 - 0.1 μm; Surface 23 cm²

B_{D2}: Depth filter 2 from supplier B. Pore diameter: 0.7 - 0.1 μm; Surface 23 cm²

B_{D3}: Depth filter 3 from supplier B. Pore diameter: < 0.1 μm; Surface 23 cm²

C_{D1}: Depth filter 1 from supplier C. Pore diameter: 1.0 - 0.3 μm; Surface 25 cm²

C_{D2}: Depth filter 2 from supplier C. Pore diameter: 0.3 - 0.3 μm; Surface 25 cm²

C_{D3}: Depth filter 3 from supplier C. Pore diameter: 1.2 μm; Surface 17.2 cm²

C_{D4}: Depth filter 4 from supplier C. Pore diameter: 0.65 μm; Surface 17.2 cm²

D_{D1}: Depth filter 1 from supplier D. Pore diameter: 0.4 - 0.1 μm; Surface 25 cm²

D_{D2}: Depth filter 2 from supplier D. Pore diameter: < 0.1 μm; Surface 25 cm²

D_{D3}: Depth filter 3 from supplier D. Pore diameter: < 0.1 μm; Surface 25 cm²

Membrane filters:

A_{M1}: Membrane filter 1 from supplier A. Pore diameter: 0.60 - 0.20 μm; Surface 13 cm²

A_{M2}: Membrane filter 2 from supplier A. Pore diameter: 0.20 μm; Surface 20 cm²

B_{M1}: Membrane filter 1 from supplier B. Pore diameter: 0.50 - 0.20 μm; Surface 3.5 cm²

C_{M1}: Membrane filter 1 from supplier C. Pore diameter: 0.45 - 0.2 μm; Surface 17.3 cm²

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Introduction

1. Financial stakes

For the most part of the 20th century, drugs developed and marketed were chemically synthesised and the pharmaceutical industry profited from the massive gains generated from chemical blockbuster drugs. Blockbusters are drugs which generate more than a billion USD in sales per year(1). Concrete examples of such drugs are Effexor[®], Lipitor[®] and Plavix[®].

However, these medicines lost their patent respectively in 2010, 2011 and 2012. This patent expiration resulted in a decrease in global sales. This phenomenon is called the patent cliff(1) and is the cause of the rising competition of generic drugs.

The decrease in the discovery of NCE (New Chemical Entities) has also been a difficulty for the industry, with a steady decrease of 38.6% in the number of entities approved by the FDA (Food and Drug Administration) between 1996 and 2008 in spite of the funding which had been doubled for research since 1991(2).

All these factors, in combination with others, have been major challenges the industry has had to face and it resulted in a shift towards biopharmaceuticals. This evolution had been possible thanks to recent technological innovations, among which the mastery of genetic engineering, which allowed designing and producing more effective and cheap recombinant proteins, also known as biopharmaceuticals. Big Pharmas have massively invested in biotechnologies, merging with smaller biotech companies in order to enlarge their portfolio. For example, Genzyme was acquired by Sanofi-Aventis for 20.1 Billion USD or Wyeth was bought by Pfizer for 68 Billion USD(3).

Today, all indicators are green for biotechnologies since this industry engendered a turnover of 123 billion USD and 14.85 billion USD in profits in 2014 along with a 20% rise in the R&D investment to reach 35.4 billion USD. Many biopharmaceuticals are currently in clinical trials phases IIb and III and in 2014, out of the 40 drugs approved by the FDA, more than 10 of them were recombinant proteins(4). Biopharmaceuticals are seen as providential medicines since they successfully target previous unmet medical need i.e. auto-immune disease, cancer or viral infectious disease. Moreover, among the 10 most sold drugs in 2014, 5 are biopharmaceuticals and 3 are monoclonal antibodies also known as mAbs(5) (**Appendix 1**).

mAbs are indeed a leading class of biopharmaceuticals which represents 2.5% of world market share:

Therapeutic group	Market share (%)
Anti-TNF	3.4
Human insulins	3.2
Anticholesterolemic	2.8
Antiulcera	2.7
Antipsychotic	2.5
Antineoplastic – Monoclonal antibodies	2.5
HIV Antivirals	2.5
Antivirals except anti-HIV	2.4
Antiepileptic	1.9
Traditional Chinese medicine	1.8

Table 1. Major therapeutic groups in 2014(5).

Therefore, monoclonal or fragment antibodies are among the most profitable molecules of the market. Still, such elaborated and high-potential proteins involve a highly complex production process that needs to be understood, optimised and characterised to ensure robust and safe therapeutic production.

2. New generation drugs

2.1. Recombinant proteins

A recombinant protein is a protein produced by a cell whose genes have been modified(6). There are different types of genetically modified hosts used to produce recombinant proteins:

- Prokaryotes: Gram positive and Gram negative bacteria.
- Eukaryotes: yeast, insect cell, transgenic animals, plant cells, plants, mammalian cells.

After their production, proteins are to be purified whether they are meant for therapeutic, industrial or research use. The first therapeutic recombinant protein ever approved is Humulin®, a human insulin licensed to and manufactured by Eli Lilly & Co. since 1982(7).

2.2. Focus on monoclonal antibodies

2.2.1. Market

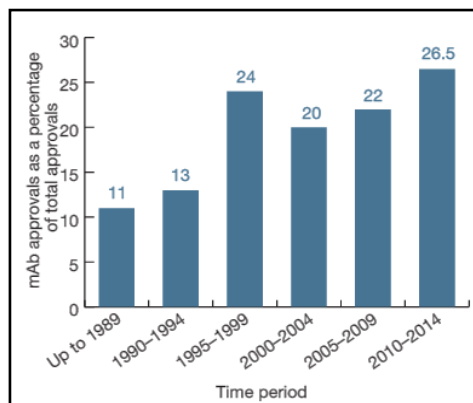


Figure 1. mAbs approvals over the years. mAbs approved expressed as a percentage of total biopharmaceutical product approvals within the same period. Fc-based fusion products are not categorised as mAbs in these data(8).

As previously stated, mAbs are a leading therapeutic class. **Figure 1** illustrates the steady rise in mAb approvals from around 10% in the end of the 80's to 26.5% of total approvals in 2014. Apart from the time period 1995-1999, this increase has been constant and the nature of the mAbs which were approved changed towards humanised antibodies as well as human forms at the expense of chimeric, and particularly murine mAbs(8).

Additionally, in the last period studied (2010-2014), mAbs is the most approved therapeutic class relative to all biopharmaceuticals and, in terms of cumulative result, they are ranked first ex-aequo with growth hormones, with 22% of all biopharmaceuticals approvals (**Figure2**).

The expression system used to manufacture biopharmaceuticals which were approved shifted as well over the years (**Figure 3**). Non-mammalian systems dominated the end of the 20th century but were overtaken by mammalian systems from 2000 onwards. The use of mammalian systems rose from 33% in the 80's up to 60% in recent years (2010-July2014).

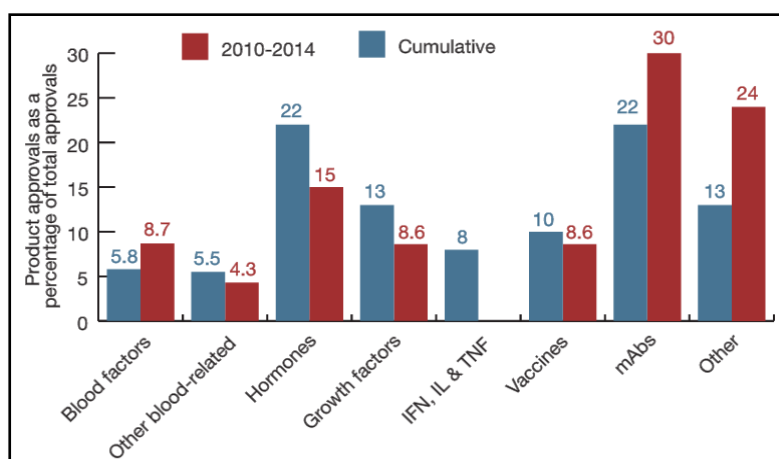


Figure 2. Biopharmaceuticals approvals over the years. Product approvals: cumulative (1982-2014) and for the period 2010-July 2014 in the context of product class. Data expressed as a percentage of a total biopharmaceutical product approvals for the period in question. IL: Interleukine; IFN: Interferon(8).

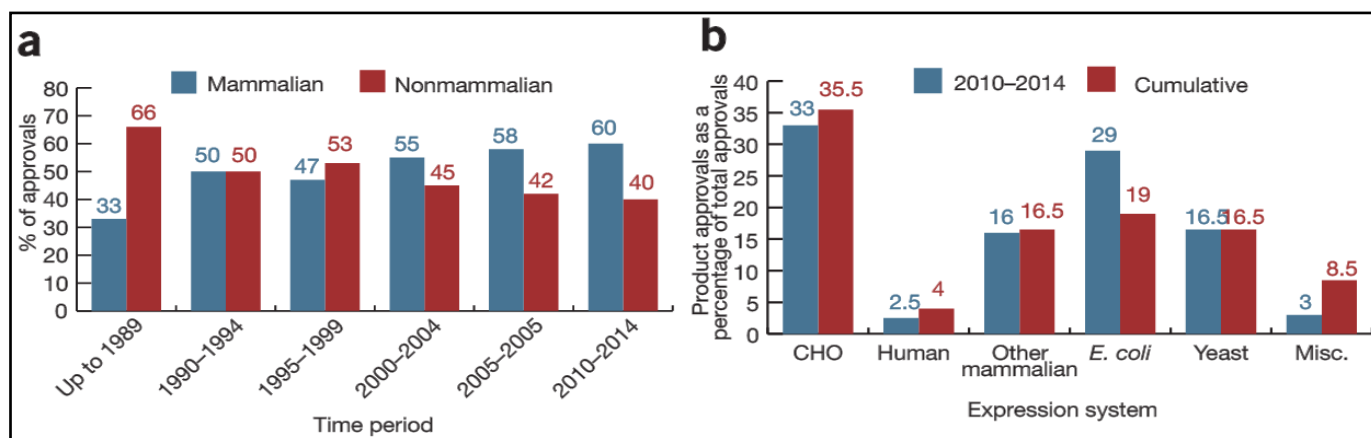


Figure 3. Expression systems used to manufacture biopharmaceutical products.

(a) Relative application of mammalian versus nonmammalian-based expression systems in the production of biopharmaceuticals approvals over the indicated periods. (b) Products approvals cumulative (1982-2014) and 2010-July 2014 in the context of expression systems employed. Each data is expressed as a percentage of total biopharmaceutical product approvals for the period in question (8).

If we analyse in detail the production system used, it is clear that mammalian prevails. The group is divided into two groups, namely CHO cells, dominating with around 33-35% of the total approval overall, and other mammalian cells which represent around 16%. Except for CHO cells, mammalian expression systems used for mAbs production include Myeloma/Hybridoma, HEK293/HT-1080, NS0 & sp2/0 (murine myeloma and hybridoma cell lines).

The dominance of mammalian systems can be explained by the rising number of approved molecules harboring glycosylation patterns which can only come out similar to humans' using mammalian cells(8). Moreover, CHO cell lines are the most used because they allow production of proteins with similar folding to those occurring in humans and it is among the least prone types to produce post-translation N-glycosylation patterns which are immunogenic to humans i.e. induce an immune response(9).

The number of mAbs currently being developed keeps rising and a new trend is evident as well: development of smaller antigen-binding fragments e.g. scFv (single chain Fv) fragments, diabodies, minibodies or nanobodies which have several advantages(8).

2.2.2. Structure of antibodies

Antibodies are glycoproteins i.e. proteins with carbohydrate groups attached to the polypeptide chain. It is a complex, large and heavy protein (150 kDa) that belongs to the Immunoglobulin (Ig) superfamily. **Figure 4** displays a schematic representation of an IgG. It is a "Y" shaped heterodimer protein which is comprised of four chains: two light chains (*in*

orange) and two heavy chains (**in blue**) maintained together by disulfide bridges. The light chain can be of two types, either κ or λ whereas the heavy chain can be α , δ , ϵ , μ or γ , which determines the isotype of the Ig. There are five different isotypes (A, D, E, M, and G). Some of these isotypes have subclasses, hence we can have an: IgA1, IgA2, IgE, IgD, IgM, IgG1, IgG2, IgG3 or an IgG4 respectively. The light chains are divided in two sections, V_L and C_L : a constant immunoglobulin region and a variable one. On the contrary, heavy chains are divided into four immunoglobulin regions: V_H which is the variable region and C_{H1} , C_{H2} and C_{H3} which form the constant part of the chain. The antibody can be dissociated into three parts according to the enzyme used: two Fab regions and one Fc region.

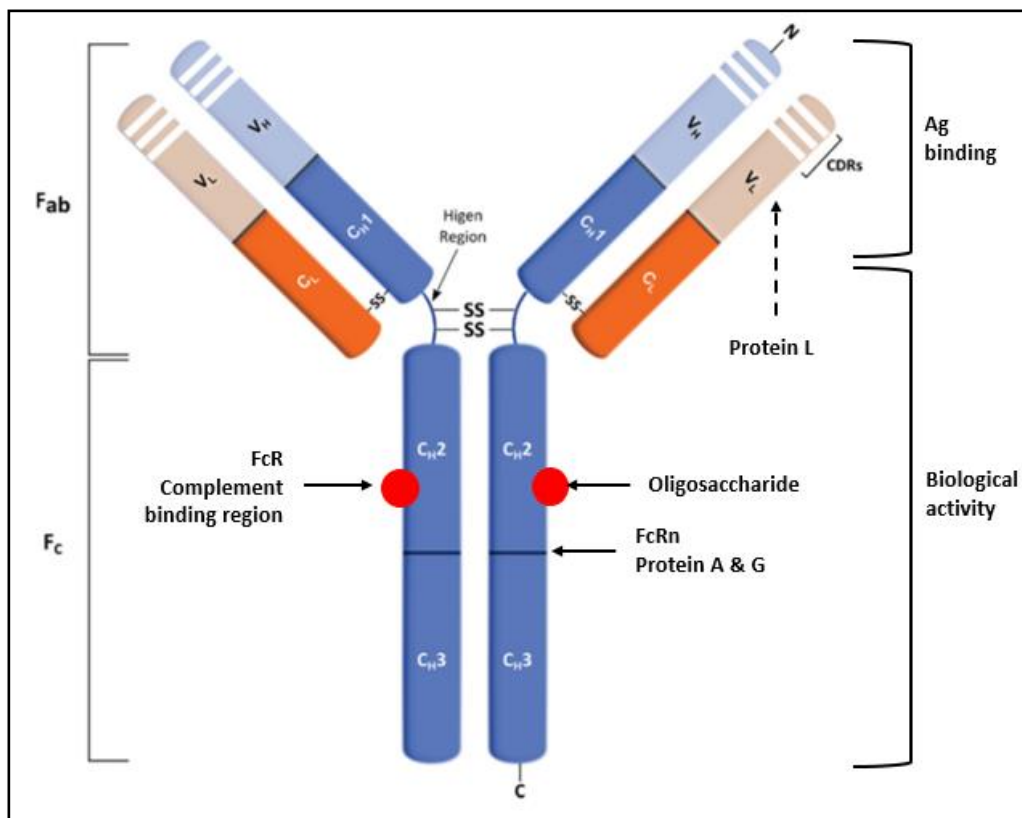


Figure 4. Schematic representation of an antibody (IgG1).
 SS: disulphide bounds; CDRs: Complementarity Determining Regions (CDR1, CDR2, CDR3); Fab: Fragment antigen binding; Fc: Fragment crystallizable; V_H : variable region of heavy chain; V_L : variable region of light chain; C_{H1} , C_{H2} , C_{H3} : constant regions of heavy chain; C_L : constant region of light chain, FcR: Fc Receptor; FcRn: neonatal Fc Receptor; the Higen region gives flexibility to the antibody for a better binding with the antigen (10).

Each chain possesses three CDRs (Complementary Determining Region) at the tip of its variable region. These three regions are hypervariable and the most variable of them is CDR3. The three CDRs of a light chain along with the three CDRs of the heavy chain

alongside shape the paratope which specifically and with a specific affinity binds the epitope which is the part of the antigen recognised by the antibody. The rest of the protein, which is the constant part, is responsible and essential for the biological activity of the Ig, for it holds oligosaccharides, which are the binding sites for Fc receptors of immune effector cells, and for the complement system; the Fc region also bears sites for the neonatal Fc Receptor (FcRn) which, among other things, has the role to recycle the Ig thereby extend its half-life. Proteins A (from *Staphylococcus aureus*) and G (from *Streptococcus* strain) can also bind the Ig, for example on the Fc region, and are used to purify the mAbs. Protein L (from *Peptostreptococcus magnus*) that can bind the κ light chain is, generally, less used for mAb purification.

2.2.3. Action mechanism of antibodies

There are four ways an antibody can fight a foreign organism in the body.

- **Neutralisation:** when toxins, bacteria or viruses enter the body, antibodies bind to them until they are completely covered. As a consequence, the intruder cannot bind to any receptor nor enter a cell to pursue its actions.
- **Opsonisation:** Once the antibody has bound the antigen at a pathogen's surface, it can activate phagocytic cells thanks to its Fc region which bears Fc receptors. The activated cells will endocytose the Antibody-Antigen (Ab-Ag) complex and digest it.
- **ADCC (Antibody-Dependant Cell-mediated Cytotoxicity):** Once the Ab-Ag complex is formed on infected body cells, the antibody recruits and activates immune system killer cells thanks to its Fc region. These cells release lytic enzymes, perforins, TNF (Tumor Necrosis Factor) or granzymes leading to cell death.
- **Activating the complement system:** Once the Ab-Ag complex is formed on infected body cells, the antibody recruits the first protein (C1q) of the complement system thanks to its Fc region. This will be followed by the complement cascade that destroys the cells

via perforation.

All isotypes are not equal regarding these effector mechanisms. The most complete is IgG1, which also happens to have the longest half-

Functional activity	IgM	IgD	IgG1	IgG2	IgG3	IgG4	IgA	IgE
Neutralization	+	-	++	++	++	++	++	-
Opsonization	+	-	+++	*	++	+	+	-
ADCC	-	-	++	-	++	-	-	-
Activates complement system	+++	-	++	+	+++	-	+	-

Model used for therapeutic antibodies

Figure 5. Antibody activity according to isotype.

life. This is why a lot of therapeutic mAbs or mAb derivatives are based on it. Yet, mAbs can be and are designed based on other IgG types: this depends on the effector mechanisms needed for a specific target or application.

2.2.4. Monoclonal antibodies: definition & genesis.

Polyclonal serum is a mix of antibodies produced by different B cell clones that recognise different epitopes on the same antigen. Each B cell clone produces one antibody type only which is specific to one epitope and has its own unique affinity.

On the opposite side, an immune serum contains only one monoclonal antibody that is produced by one B cell clone and targets only one epitope on the antigen. This serum has been used in diagnostics, research as well as therapeutics. Yet, there was a major hindrance to its use: the mortality of B cells, which made this resource very limited. Fortunately, a method developed by G. Köhler and C. Milstein in 1975, earning them a Nobel Prize, addresses this issue (10).

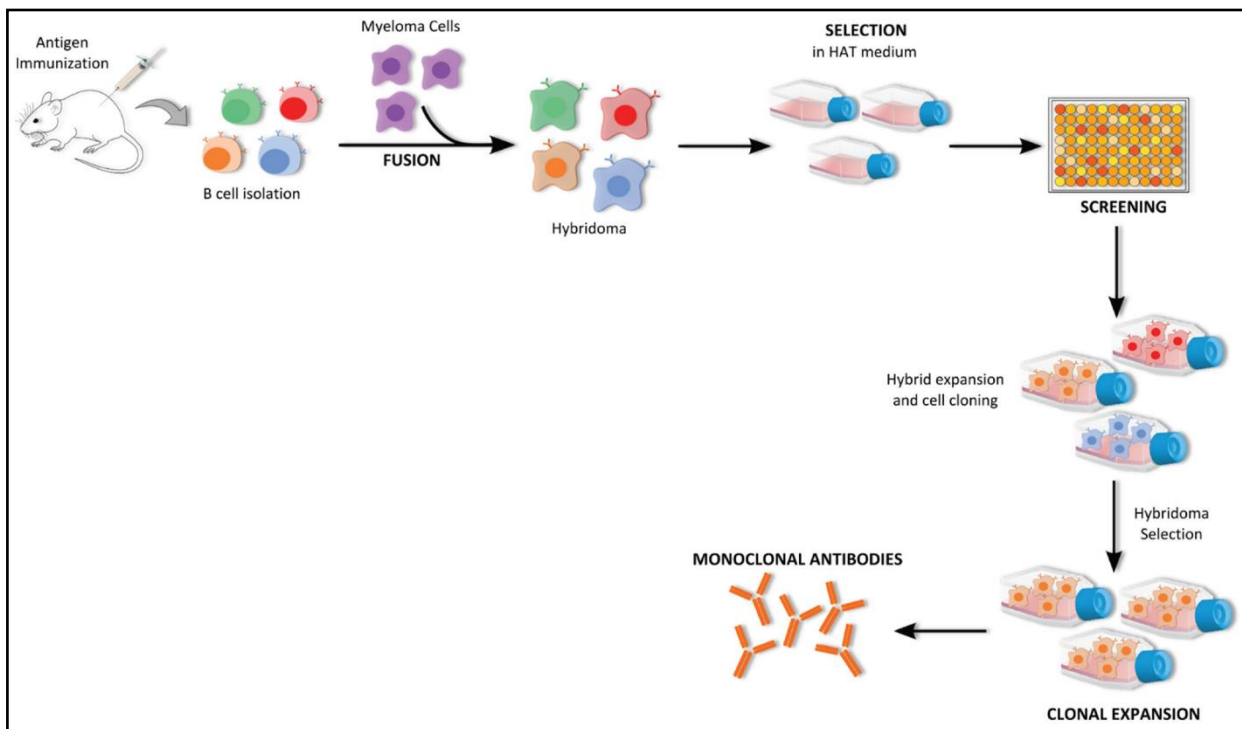


Figure 6. Schematic representation of the hybridoma technology(10).

The principle is to combine the genetic material of B cells which are able to produce a monoclonal antibody but are mortal, and myeloma cells, i.e. murine cancerous plasmocyte cells which are immortal, in order to produce a stable mAb producing cell line: hybridoma

cells. It starts with the immunisation of an animal against the targeted antigen. Then the generated B cell clones are recovered in the spleen of the animal and fused with myeloma cells that lack the HGPRT (Hypoxanthine-guanine-phosphoribosyltransferase) gene. In order to select the correctly fused cells, an HAT (Hypoxanthine, Aminopterin, and Thymidine) free medium is used. In this medium, both double fused myeloma and double fused B cells will die, respectively because of their inability to synthesise nucleotides and their mortality. As a result, only hybridoma cells will survive and undergo screening and further expansion to select the “best” hybridoma which produces the desired mAb with the best affinity. This technology is used for large scale production as it is a robust, unlimited and very specific mAb source.

2.2.5. Antibody engineering

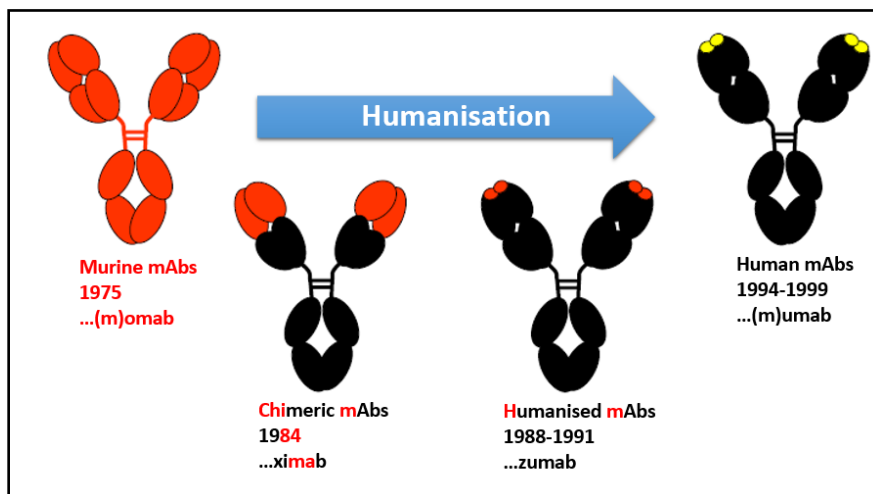


Figure 7. Monoclonal antibodies evolution over the years: mAbs humanisation(11) with international denomination associated.

The first mAbs obtained with the hybridoma technology are 100% murine (Figure 7.(11)). They are only used in research or immunochemistry techniques as they

have an 84% chance to induce high immune response when injected in humans: HAMA (Human Ab against Murine Ab), and they have a very limited range of action due to a very limited half-life in the body. In 1984, Genetic engineering advances enabled producing chimeric mAbs (70% human and 30% murine) by fusing murine variable regions with human constant regions. This substitution significantly reduces the immune response against the mAb but not entirely: HACA (Human Ab against Chimeric Ab) limits therapeutic efficiency and half-life of chimeric mAbs. At the end of the 80’s CDR “grafting” made it possible to further decrease the murine part in mAbs down to 5%. These humanised mAbs induce immune response in only 9% of cases (HAHA: Human Ab against Humanised Ab)(12). Nonetheless, this technique is challenging as the grafted Ab must keep the same affinity for

the target antigen. Totally human mAbs appeared in the end of the 90's thanks to techniques such as human memory B-cell immortalisation, or using transgenic mice that have a human IgG repertoire in the hybridoma method(13).

Nevertheless, mAbs present important limitations, i.e. a high molecular weight that prevents them from penetrating solid tissue or tumors and high production costs (mammalian cell culture). To tackle these difficulties, a multitude of new formats are being

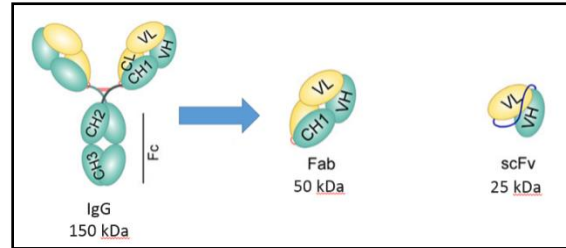


Figure 8. Schematic representation of engineered fragment Ab n°1.

developed: they are smaller and not glycosylated, hence may be more straightforward to manufacture using prokaryote systems. They bind to immune targets which are inaccessible to conventional larger mAbs and penetrate more easily through solid tumors (in other words have a better efficiency). They are less risky regarding unwanted immune reactions as they cannot induce immune mechanisms which require the Fc part when they do not have it. The absence of glycosylation means a shorter half-life, which is why, overall, they are less toxic. A few examples of such formats are displayed in **Figure 8 & 9**(14), see more in **appendix 2**.(15).

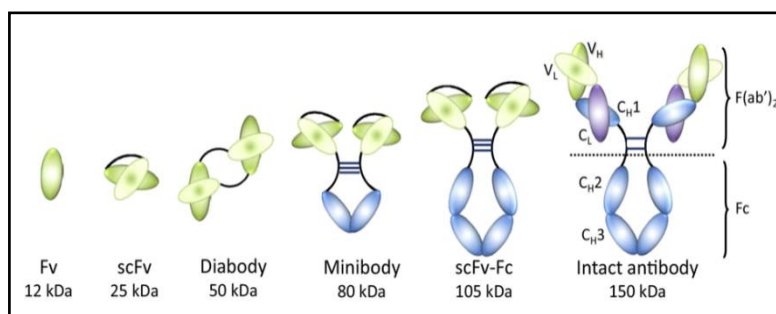


Figure 9. Schematic representation of engineered fragment Ab n°2.
 Fv: single domain variable Fragment; scFv: single chain Fv; scFv-Fc: scFv with Fc domain.

Section 1. Process development

1. Monoclonal antibodies production process

As represented in **Figure 10**, after expansion and a bioreactor production phase, the cell culture medium is harvested which is the primary recovery step. The material is then transferred to DSP (DownStream Process) where it undergoes a first capture step which aims to concentrate the mAb and eliminate HCPs (Host Cell Proteins) and other impurities.

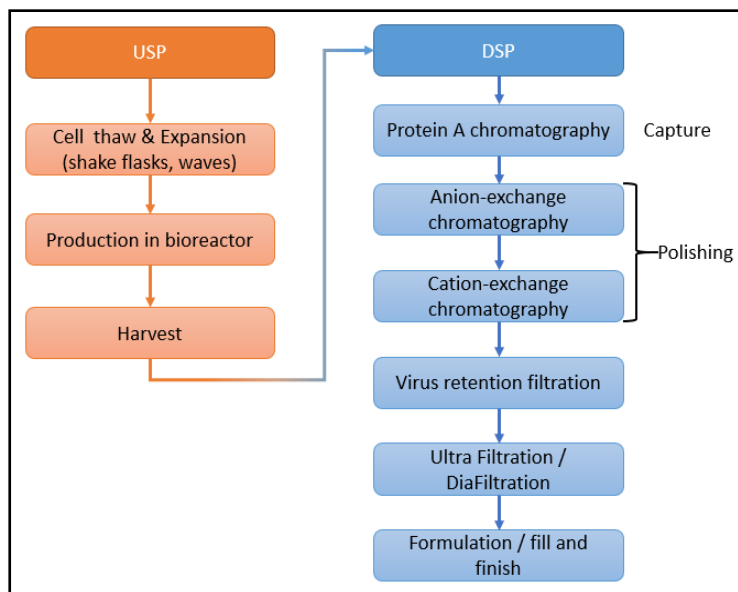


Figure 10. USP (UpStream Process) and DSP (DownStream Process) general steps for mAb production(16).

Polishing steps are used to eliminate the rest of the HCPs, DNA with aggregates and degraded forms of the mAb. Next, the protein solution is filtered in order to eradicate any viral infection risk for the patient. Then, several steps of UF (UltraFiltration) ensure the purity of the protein solution as well as achieving a target mAb final concentration, and

the DF (DiaFiltration) places the protein in adapted “physiologic like” buffer for protein stability. Finally, the product undergoes a final sterile filtration and is formulated and dispensed in its final packaging(16).

Whether it is for USP or DSP, all process steps must be well understood to achieve the development of robust production process which minimises risk of failure. Particularly for USP, since the production process is biological, there is a natural variability in the product. Furthermore, local environments within the culture impact tertiary protein structure e.g. formation of disulfide bridges, glycosylation patterns. It is important to determine and identify the underlying impact and effect of parameters on product quality and process performance, involving both critical process parameters (CPP) and critical quality attributes (CQA) determination.

1.1. Mammalian suspension cell culture: different modes

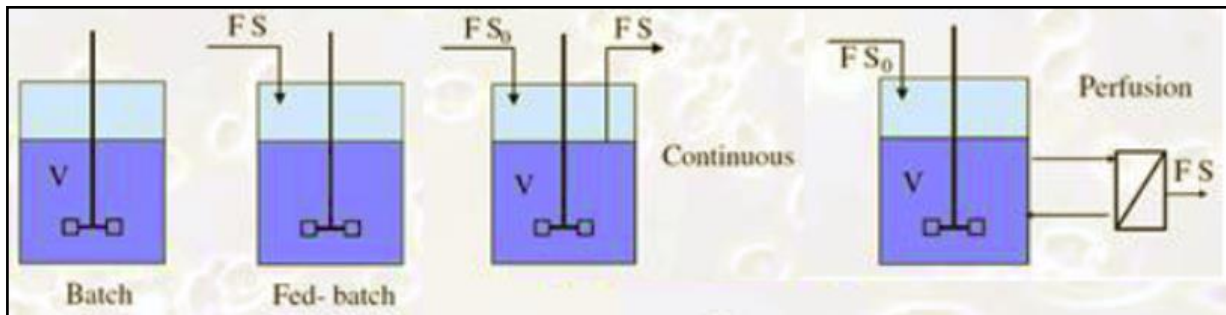


Figure 11. Schematic representation of different production methods for suspension cell culture.

Figure 11 provides schematic representation of the four production methods for suspension cell culture. The main difference is on the feeding regime:

- **Batch:**

The batch mode is characterised by a constant volume where there is no feed or medium addition nor removal. It is the simplest and cheapest process to be used. However when the cells run out of nutrient, which also means there is byproduct accumulation, they start dying. Depending on which medium and cell line is used, cells survive 3 to 7 days(17). mAb titers provided are low: about 50 mg/L(18). The different growth phases are described in **Appendix 3**.

- **Fed-batch:**

With a fed batch mode, the cell culture volume is not constant. Indeed, there is a daily addition of nutrients e.g. amino acids, nucleic acids, lipids or glucose. This systematic addition enables a significant improvement in the maximum viable cell concentration i.e. VCC (up to 19×10^6 cells/mL(19)), culture life-span (up to 14 days or more) and mAb titers: routinely 3-5 g/L but some companies have achieved 10-13 g/L(20). It is the predominant process for mAb production in the industry as it is relatively inexpensive but it calls for a lot of development on feeding strategies in order to avoid detrimental effects on the cells and therefore on the product of interest. In addition, the multiple additions raise the risk of culture contamination hence extra attention is needed.

- **Continuous:**

For this mode, there are both addition of medium and feed as well as removal of cell culture medium, i.e. medium and cells, at the same flow rate hence a steady culture volume is maintained. It has the advantage of removing any toxic metabolite from the culture,

preserving product quality as it is also removed, when controlled, ensuring a steady and defined culture environment and extending culture time since, theoretically, the bioreactor does not need to be emptied. Despite higher titers, it is not really used by the industry as the process is more challenging regarding technique and sterility(20).

- **Perfusion:**

The principle is the same as continuous processes with one key difference: cells are not removed but recycled thanks to a cell-retention device(20). It allows the same benefits as with continuous i.e. control of the environment, detoxification of the bioreactor and product quality preservation and, most of all, a high cell density that enables a productivity 10 to 50 fold higher than in other production modes and this on small scale(17). The industry tends to develop such processes yet technical challenges regarding the cell retention device have to be addressed. These challenges can lead to long and difficult process validation, and potentially block progression(17)(19). To this regard, disposable technologies may allow to alleviate some of these constraints and are a trend investigated in the industry.

1.2. Important physical and chemical process parameters

1.2.1. Cell culture

Nowadays, mAb production platforms are mainly based on fed-batch cell culture. Some key input factors e.g. nutrients, pH, temperature, agitation, aeration and output variables such as osmolality have a high impact on process performance and product quality. Therefore,

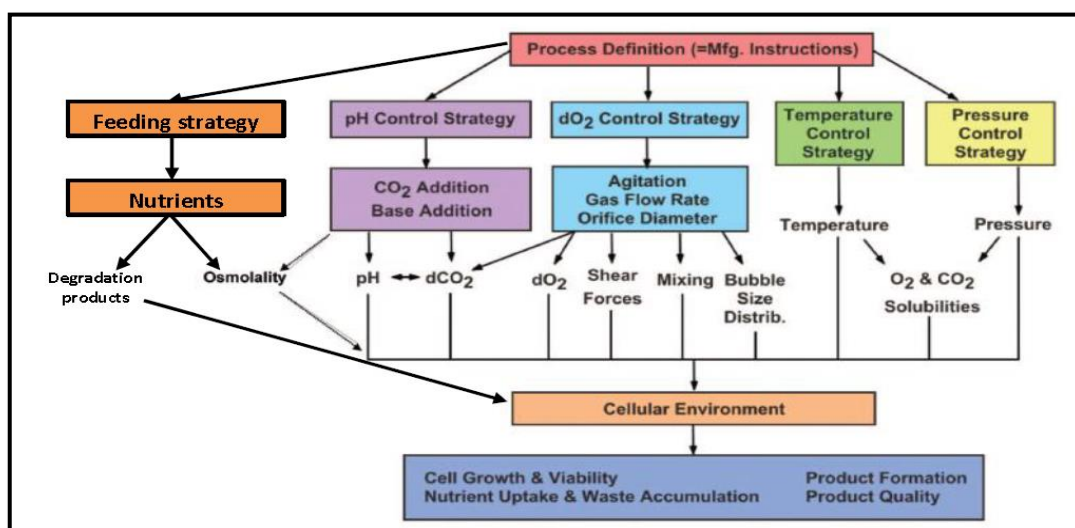


Figure 12. Cell culture process operating parameters that affect performance and product quality(20).

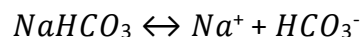
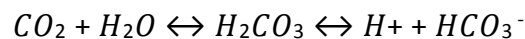
they must be closely and consistently controlled and optimised in order to ensure good cell growth, cell viability, protein production and product quality. **Figure 12** summarises cell culture process parameters that affect performance and product quality.

a. Media and feeding

mAbs are produced in CHO cells using chemically-defined media usually consisting of amino acids, vitamins, trace elements, inorganic salts, lipids and insulin or insulin-like growth factors, and exempt from animal proteins in order to reduce the risk of viral or bacterial contamination(21). These media usually contain a certain amount of nutrients, sufficient to meet cell needs only for a few days of culture. Thus, further into the run, fed-bath mode is adopted: bolus and continuous addition are freshly supplied. These feeds mainly contain amino-acids and glucose, both of which are essential for maintaining cell growth and product synthesis(22). When it comes to feeding strategy, the challenge is finding the balance between promoting good productivity and limiting degradation product accumulation.

b. pH and temperature control

pH is an essential parameter to master in bioreactor culture. Indeed, for animal cell lines, a small deviation of 0.1 units from the optimal pH value can significantly impact cell growth and metabolism, in particular glucose consumption and lactate production(21). Moreover, pH can affect protein properties and stability(23). Most transformed mammalian cells have an optimal growth at a pH varying from 7.0 to 7.4. Consequently, cell culture media contains sodium bicarbonate as a buffering agent, which is in equilibrium with CO₂ coming from the gaseous phase. This is described by the following equilibrium reaction(24).



If needed, base addition can also be of use to control the pH by increasing it.

Temperature is also important in cell culture because it has a critical influence on cell growth and the solubility of various medium components such as CO₂ and O₂, which are poorly soluble. Temperature for mammalian cell culture ranges usually between 35–37°C with an optimum at 36.5–36.8°C(24).

c. Agitation and aeration

Suspension cultures of animal cells are performed with agitation and cells need oxygen to grow which implies aeration.

As previously mentioned, oxygen has a low solubility in aqueous media. Hence, in order to prevent oxygen limitation, dissolved oxygen is typically controlled at a specific set point usually between 20-50% of air saturation. Anything below could lead to excessive and detrimental lactate synthesis, whereas higher percentages might cause cytotoxicity. Bubbling aeration through sparging and surface aeration (overlay) is an efficient way to supply oxygen inside media while agitation enhances mixing and oxygen mass transfer. As for dissolved CO₂, it is also an important parameter because it can accumulate to inhibitory values which can affect product quality(21).

Yet, both agitation and aeration present a big risk of cell damage as animal cells do not possess a cell wall. Generally, cell injury can happen during the ascension of air bubbles in the medium and during the accumulation of foam on the surface provoking cell-cell, cell-vessel wall or cell-impeller collision. Therefore, the aim is to have a stirring speed that is sufficient for aeration without causing damage to the cells(24). Thus, most of the chemically-defined media contain Pluronic F-68, a surfactant which protects mammalian cells from shear stress(25). Furthermore new cell lines developed in the industry are very resistant to shear.

Thus, in bioreactor culture, air flow and stirring speed should be modified depending on the amount of viable cells, their oxygen intake and the pressure inside the tank and cell sensitivity to shear stress.

d. Osmolality

Osmolality is defined as the number of osmoles of a compound that contributes to the osmotic pressure, per kilogram of solvent. Unlike the input parameters cited above, osmolality is an output parameter and is an important variable to be taken into account. The osmolality of human plasma is 290 mOsm/kg which is the middle of the optimal range for mammalian cells in culture ranges usually from 260 to 320 mOsm/kg(24). Osmolality is influenced by multiple factors such as metabolic transformations, byproduct synthesis, base addition and evaporation but its impact is considered to be cell line specific.

Despite slightly improving specific productivity, a high value of osmolality has been proven to induce apoptosis, resulting in growth inhibition and negative repercussions on viability. For instance, it has been shown that an osmolality reaching 450 mOsm/kg reduced specific growth rate by about 50% through apoptosis induction(26,27). Likewise, under hypo-osmolality condition, some cell lines seem also to reduce their growth rate(24).

Osmolality profile must therefore be carefully followed, especially towards the later days of the cell culture process to keep high cell densities.

In addition, other output variables like cell viability, viable cell density and the concentration of metabolites e.g. glutamate, glutamine, glucose, ammonium, lactate, sodium and potassium are commonly measured to maintain substrate levels above critical levels through feeding strategies and for process optimisation in order to reduce byproducts (24).

1.2.2. Harvest

The overall objective of harvest is to remove impurities from cell culture medium in order to promote effectiveness of further downstream purification.

mAbs are generally secreted and thus extracellular hence a cell separation step is needed at the end of culture. Commonly, the cell culture fluid is separated from the cells through centrifugation. At small scale, dead-end centrifugation is used. This type of centrifugation is usually simple to control as the cells are not exposed to great forces of shear and the obtained pellet is quite solid. On the contrary, at larger scale, a disc-stack centrifuge is

necessary. It offers continuous separation based on density difference between solids and liquids and on particle size. This system implies an involvement of a lot of important physical parameters that need to be optimised in order to achieve efficient separation. These factors include inlet flow rate, centrifugation speed (or bowl

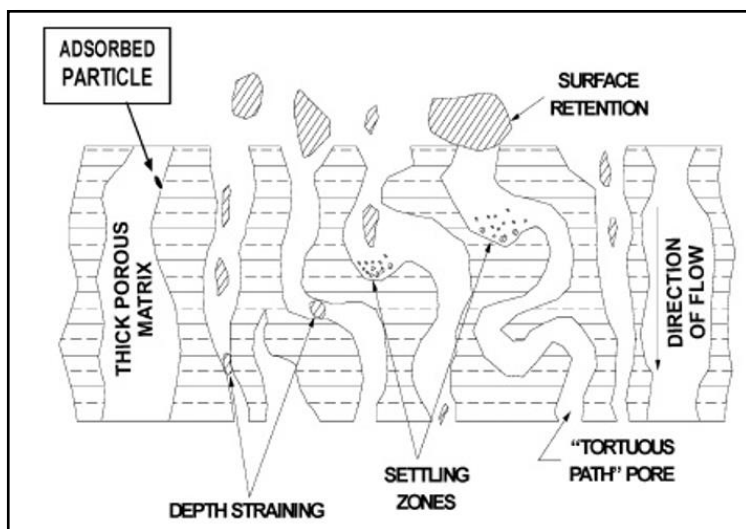


Figure 13. Mechanisms of particle retention by depth filters.

speed), solid discharge intervals and back pressure, which is the force that maintains the feed inside the bowl. Due to its complexity, disc-stack centrifugation comes with a high risk of overflow and exposure of the cells to high forces of shear which generates small particles that cannot be removed from liquid using centrifugation. This could have a negative effect on yield, separation and clarification efficiency(28).

After centrifugation, the cell culture fluid usually undergoes additional clarification using depth filters. These filters are composed of fibrous bed of cellulose or polypropylene fibers along with an inorganic filter aid such as diatomaceous earth or perlite and with a wet-strength resin. Perlite (volcanic ash) and diatomaceous earth (fossilised remains of plankton) are available in various particle sizes to provide a range of porosity. Wet-strength resin holds all components together and confers a positive charge to the internal surfaces of the filter sheet. Filter performance relies on the ratio of all these components(29–31). Thanks to their porous matrix, depth filters are able to retain particles or colloids of different size through retention mechanisms such as sieve retention, adsorption, Brownian motion and inertial impaction (**Figure 13**).

The main mechanism is **sieving**: particles are retained because they are too large to fit through the filter mesh. This retention mechanism is not dependent on the number of particles or pores nor affected by filtration conditions. The differential pressure does therefore not affect the particle removal unless this pressure is high enough to misshape the suspended particle. Nevertheless, some filtration conditions could enlarge the pore size and reduce the filter performance. In addition, the thicker the sheet will be, the more likely the retention will be reliable(29–31). **Adsorption** is the bonding of particulates to the surfaces of filter pore walls caused by any of several electrical charge phenomena. In most cases, this interaction is linked to the presence of positively charged sites on the wet-strength resin. This enables removal of particles smaller than the filter pores(29–31). **Brownian motion** corresponds to the random motion of particles also occurring during the filtration step. Only the smaller and lighter particles are influenced by this motion which induces their random and erratic movement, the particles have more chance of encountering pore surfaces and being retained(29–31). **Inertial impaction** occurs when a particle is so large that it is unable to quickly adjust its path to the direction of the flow. Because of its inertia, the particle will

continue along its original course and will collide with the filter surface in settling zones. Adsorptive forces can then cause its arrest(29–31).

A big range of particle sizes can be covered by properly choosing filter pore diameter and membrane surface. The greatest challenge is to define the right filter for each clarification process. Wrong filter sizing can lead to saturation of pores (breakthrough) which can be identified by an increase of clarified cell culture fluid (CCCF) turbidity. Another possibility is filter blockage, detectable through a pressure increase and a decreased flow rate.

Depth filtration is followed by membrane pre-filtration (when needed) and membrane-filtration using filters with a smaller diameter pores (0.8-0.45 μm or up to 0.1 μm) to reduce bioburden(28,32). Filtration through absolute pore size membranes is typically the final clarification step before capture chromatography for the protein purification.

1.2.3. mAb titer and product quality attributes (PQA)

Once the cell culture fluid is clarified, the mAb is characterised to define the impact of the upstream process. Protein titer is measured and the protein of interest purified. Standard techniques for mAb purification and titer measurements are HPLC and other measurements based on protein A binding because of the high affinity of antibodies for this protein. Quality attributes of the antibody can be characterised on Protein A eluates. Typically, isoelectric capillary electrophoresis and size exclusion chromatography are used, respectively, to define charge variant distribution and aggregates & fragment levels present in the protein mixture. Glycosylation profile can also be determined(33).

2. Development

2.1. Drug life cycle

From the 10,000 molecules screened, only ten of them will be considered for a patent application and just one will successfully undergo the clinical trials and be approved by the authorities to be marketed. Innovation, being 12 years on average, is long, complex and expensive. Investments required to develop a new therapeutic molecule are around 1 billion Euro. In ten years, major costs, which include pre-clinical and clinical trials, more than doubled due to the legitimate increases of requirements from authorities. The patent is essential to financing research as it allows to protect the innovation for 20 years and can be extended up to 25 years in some countries, if it meets the criteria. Patent starts as soon as

the molecule is identified. The molecule will then be tested in pre-clinical and clinical trials which takes about 10 years. Upon success in those trials, the molecule is then presented to the authorities for approval to be marketed. Generally, the drug is protected for around 10 years after this approval which allows the company to repay the amount spent for developing the drug and make some profits(34). **Figure 14**(35) displays the major drug development steps:

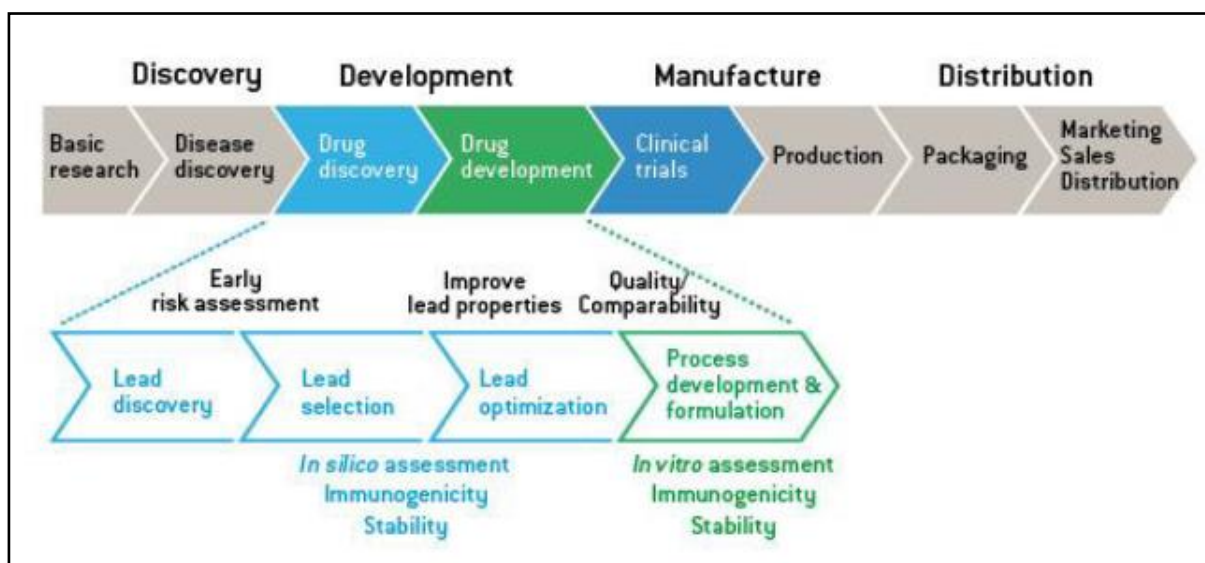


Figure 14. Drug life cycle.

Key steps in drug development are the trials. They are designed to assess the efficiency of the molecule and detect any side effect. Only the molecules with a positive balance benefit / risk are kept. Before any testing on humans **pre-clinical trials** occur. They are based on molecular, culture cellular and then animal models. They consist of pharmacology studies to prove the drug works, toxicology studies to evaluate the risk of adverse events and pharmacokinetics studies to understand how the drug is absorbed, distributed, metabolised and eliminated.

If successful, drug candidates can be tested on humans. In order for them to be commercialised they would have to proceed successfully through three phases, the fourth occurring post approval:

- **Phase I, safety and tolerance:** increasing doses are administered to healthy volunteers (up to tens) in order to assess their tolerance to the drug as well as its pharmacological properties.

- **Phase II, Drug efficiency and dose:** this phase involves one to a few hundreds of patients who volunteered. The aim is to define the optimal dose which means the best therapeutic effect with the less adverse effects possible.
- **Phase III, randomised double blind trials:** The sample used for this phase is bigger i.e. up to thousands of volunteer patients. The candidate drug is compared to a reference treatment which is either a placebo or the current treatment used, to verify its therapeutic advantage over the reference. Adverse effects are monitored for long term use.
- **Phase IV, post-marketing studies:** is the long-term monitoring of the drug and its use. It enables collecting information on treatment benefit or risk, optimal use and any previously undetected adverse events or effects or a therapeutic potential for another target(34).

2.2. Process development

Drug development is actually not limited to pre-clinical and clinical trials. It is also coupled and chronologically intertwined with process development. **Figure 15** shows the major steps of process development and how it is correlated with toxicological i.e. pre-clinical and clinical development.

Before any development starts it is important to determine the quality attributes of the molecule. Indeed, in 2002, the FDA initiated the concept of quality by design (QbD) for pharmaceuticals which is a scientific, risk-based, holistic and proactive approach to pharmaceutical development. One of the fundamentals is that products and processes have to be sufficiently understood so that those elements most critical for product quality can be identified and controlled(36). Thus, concerning bioprocess development, it is essential to understand the link between mammalian cell culture conditions and the glycoprotein i.e. identifying CQA and CPP. Throughout process development, each process step must meet those product quality attribute criteria, in other words quality is built in the process design. The implementation of QbD shall lead to the development of more robust and efficient manufacturing processes of mAbs with increased clinical efficacy(20).

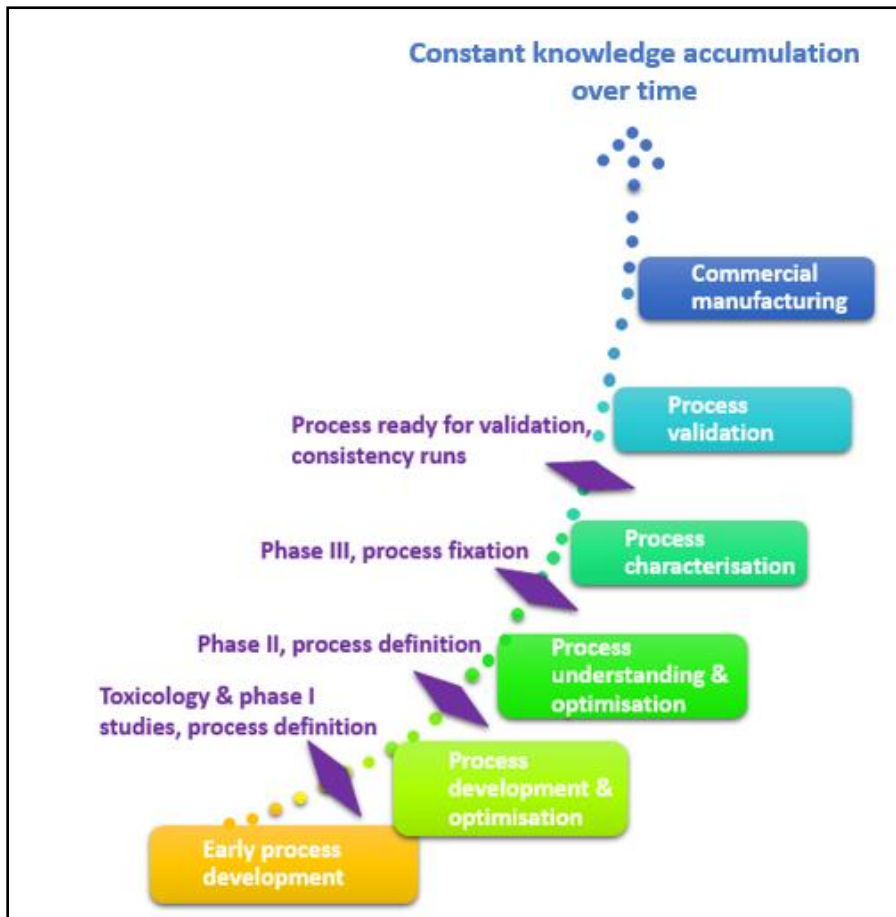


Figure 15. Process development major steps timeline coupled with preclinical & clinical development.

Before process development, cell line development and engineering, clone selection and media screening must occur. A manufacturing process is then rapidly developed at bench scale (2L-10L bioreactors). This process has to be robust and scalable. Potentials for titer, yield and product quality challenges are

identified. This corresponds to the early-stage of basic process development which is scaled-up to pilot / manufacturing scale (cGMP facility) to produce the material needed for toxicology studies and for phase I trials.

The process is developed and further optimised at bench scale using a suitable scale-down model (SDM) of the process as it is far less expensive. Process optimisation is an integrated activity involving medium development and bioreactor parameter optimisation, which is followed by scale-up considerations to an appropriate manufacturing facility. The goal is to resolve any issues encountered in phase I scale-up e.g. product quality or impurities difficulties. Addressing them and exploring potential for titer and yield increases should be done before phase II clinical trial. If changes are implemented to the manufacturing process, comparability between phase I and phase II must be proven.

The next stage focuses on understanding the impact of the process on the product and is used to explore operating ranges, to push the process to its limits, and to implement any changes

that improve process robustness. Product quality and comparability needs to be closely monitored when process changes occur to ensure patient safety. At the end of that stage, the process is fixed.

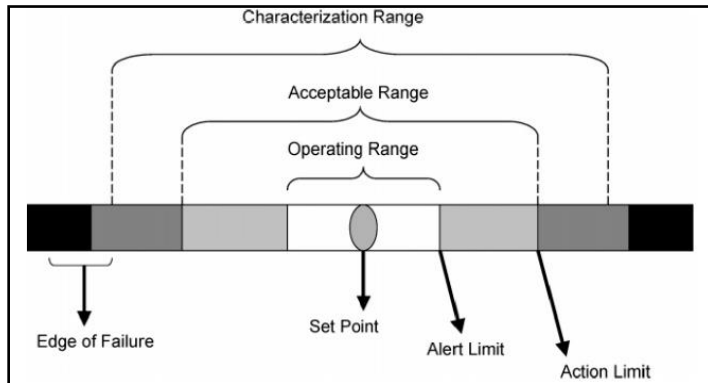


Figure 16. Ranges of process parameters.

Each operation is characterised using suitable scale-down model: potential CPPs are screened over a wide range to determine the Normal Operating Ranges (NOR) and the Proven Acceptable Ranges (PAR) (**Figure 16**)(37).

The fixed process is then validated at commercial scale (e.g. 20,000L). Validation requires process robustness and focuses on 2 aspects: robust PQA and process outputs e.g. titer. Generally, that takes place after the completion of the process understanding and characterisation phase, when the manufacturing process is locked and no further significant changes are expected.

After validation, the process enters the commercial manufacturing phase which is its final purpose. Contrary to what is believed, this phase is not the end and has its own challenges. Sometimes, process troubleshooting can be needed and it is performed using a qualified SDM which has continuously been developed and fine-tuned alongside the scale up of the process and verified using commercial scale data. Similarly to the phase IV, during the commercial phase all runs are monitored and the gathered data used in the framework of continuous process validation.

Section 2. Materials & Methods

1. Cell culture and cell expansion

Data shown in this document are from experiments that were performed using a CHO cell line. Cryogenically frozen vials of this cell line were thawed into a chemically-defined expansion medium supplemented with glutamine and methotrexate (MTX) as a selection agent. This medium was animal protein free. The cells were sub-cultured every 3 or 4 days in shake flasks for amplification. This cell culture was kept in humidified shaking incubators controlled at 36.5°C with 5% carbon dioxide. Subject to the cells exhibiting good growth characteristics and high viability, they were scaled up for production using either two rocking bioreactors followed by a 400L stirred bioreactor for 2000L scale, one rocking bioreactor for 80L or 3L shake flasks for 2L scale. Production medium is also chemically defined and supplemented with glutamine only. Gas flows, temperature, rocking or stirrer speed were controlled. **Figure 17** describes examples of expansion train for different scales.

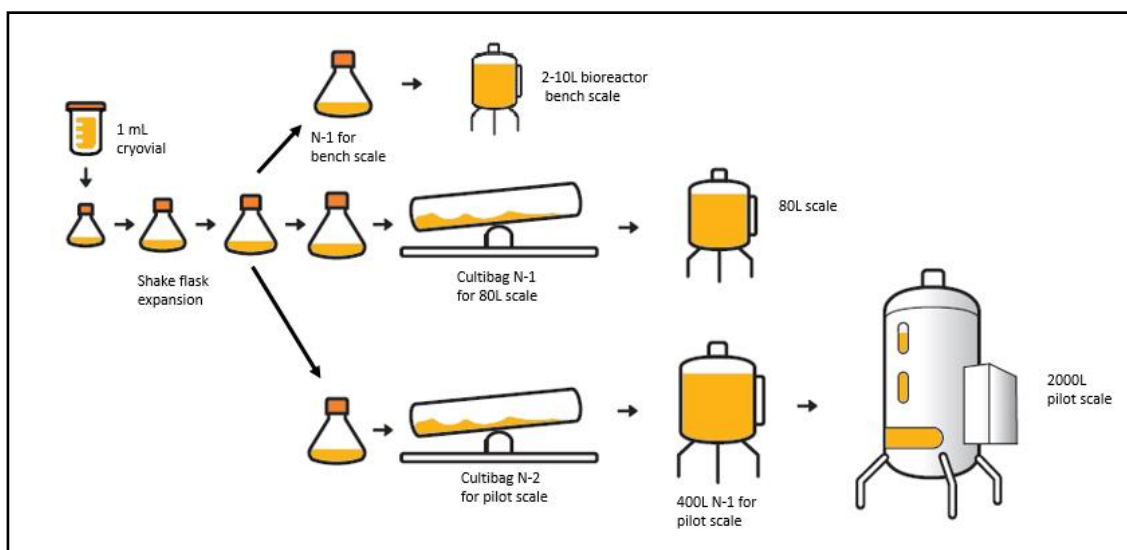


Figure 17. Cell culture expansion train for different scales.

2. Fed batch process

Figure 19 displays the common features of a bioreactor. Fed batch production cultures were seeded from expansion culture at a given target seeding density (TSD) in production medium with glutamine. Cells were cultivated for 14 days at 36.8°C under stirring and sampled daily via a sampling port. Air was injected through sparging and surface aeration for both 2000L and 80L scale whereas 2L scale has no overlay. Other gases (O₂, N₂) were

only injected through sparging. Gas flows were controlled according to an aeration cascade targeting 40% dissolved oxygen. Two feeds were added to support the expansion of the cells. Feed 1 was continuously added from day 3 to day 13. Feed 2 was added daily using a semi-continuous mode from day 3 to day 12. Glucose was supplemented separately from the rest of the feed solutions when the concentration decreased to below 5.6 g/L between day 6 and day 13. Glucose was then supplemented targeting 6 g/L. Anti-foam was added on demand, based on a regular visual check, to minimise foam appearing with stirring.

If we exclude disposable bioreactors, up to 2 and 10L, bioreactors are made of borosilicate glass whereas larger scale bioreactors are made of stainless steel. Different impeller types can be used (see **Figure 18**). Standard impellers for the experiments presented are axial flow impellers: 3-blade for 2L bioreactors and elephant ear for 80 and 2000L. The Rushton impeller creates a radial mixing flow.

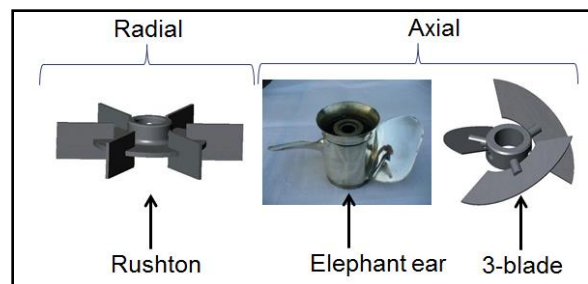


Figure 18. Different impeller types inducing radial or axial mixing flow.

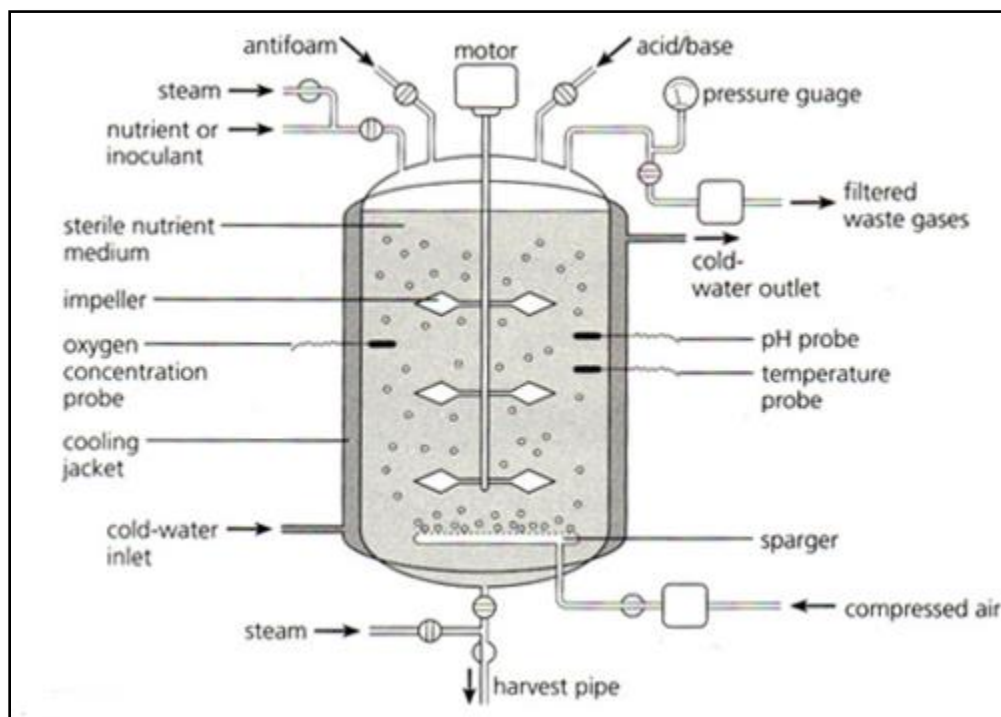


Figure 19. Bioreactor general features.

3. In process and offline measurements

Online pH and DO (Dissolved Oxygen) readings were taken continuously during the run from the corresponding probes. Other parameters such as the bioreactor weight, amounts of feed added, stirring speed, temperature, pressure and gas flow rates were monitored.

Regarding offline measurements, they were executed daily as follows:

- Offline pCO₂, pO₂ and pH were measured using the Nova Stat Profile[®] pHox[®] analyzer.
- Offline glutamine, glutamate, glucose, ammonium, lactate, sodium and potassium were measured using the Nova Bioprofile 400[®] nutrient analyzer.
- Offline osmolality was measured using the Osmometer Advanced[®] 2020.
- Viable cell density, viability and cell diameter were measured with the Vi-Cell XR Cell Viability Analyzer.

4. Harvest

The cell culture was harvested after 14 days of fed-batch production. The bulk was cooled down to 20°C before the harvest. Clarification was performed by centrifugation and filtration steps.

4.1. Flocculation

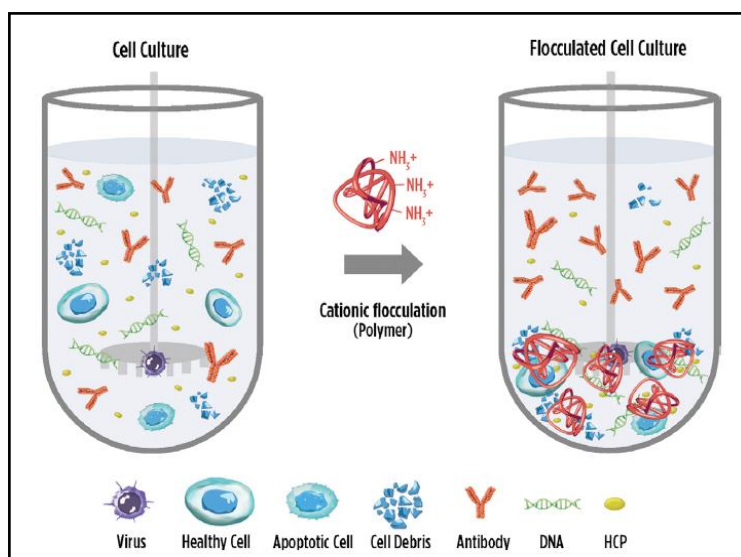


Figure 20. Outline of the flocculation mechanism for cationic flocculants. Negatively charged particles such as cells, cell debris, nucleic acids, and HCP bind to the positively charged polymers, leaving the protein of interest in solution(38).

Flocculation was performed inside the cooled 80L bioreactor using a cationic polymer. The flocculating agent was added directly to the cell culture in the bioreactor at day 14. After 30 minutes of incubation with a 50 rpm agitation, the flocculated cell culture fluid was centrifuged. Flocculating agents trigger the destabilisation of cell culture

broth suspension by causing the adhesion of dispersed particles into large size clusters. Flocculants can be anionic, cationic or both. The flocculating agent selected for the experiments is cationic and **Figure 20**(38) shows the mechanism of action. Flocculating agents mostly bind to impurity particles through electrostatic interaction yet others e.g. hydrogen bonding or hydrophobic interaction can be involved. The cationic polymer binds and flocculates negatively charged impurities which creates larger sized clusters resulting in an increase in the average particle size distribution, which improves the post centrifugation filterability, while the protein of interest remains soluble(38).

4.2. Acid precipitation

Acid precipitation was performed inside the 80L bioreactor once it was cooled down. The acid was added directly to the cell culture bioreactor at day 14. After an incubation with agitation, base was added for neutralisation of the culture broth. The acid precipitated cell culture fluid was then centrifuged. **Figure 21** displays the steps of acid precipitation.

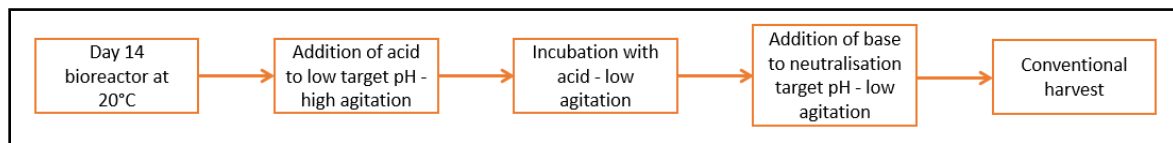


Figure 21. Acid precipitation steps.

The effectiveness of acid precipitation at removing impurities in CHO cell culture has been demonstrated since 1994. Acidification of cell culture broth results in the transition across the pI of process related impurities e.g. HCPs and DNA making them insoluble while the target therapeutic protein remains soluble. The pI of HCPs mostly ranges from pH 4.0 to 6.5, hence a pH between 4.5 and 5.0 is often the most effective(38).

4.3. Centrifugation

Centrifugation was carried out using a CSC-20 disc-stack centrifuge at 2000L scale and CSC-6 disk-stack centrifuge at 80L scale (Westfalia Separator). CSC-6 centrifuge is a scale-down model of the CSC-20 disc-stack centrifuge which allows more robust scalability. Centrifuge feed rate, bowl speed, operating pressures and discharge frequency were controlled to have suitable separation performance. At 80L scale, the cell culture fluid was processed at a flow rate of 100 L/h, a speed bowl of 9070 rpm and the discharge interval was calculated according to the following formula:

$$t = \frac{\text{Solids holding space}}{Q * PCV} * 60 * 100$$

t = Discharge interval (min)
 Solids holding space = 0.7 L for the CSC-6
 Q = Feed flow rate (L/h)
 PCV = Packed cell volume (%)

Online turbidity measurement was performed for process monitoring (in C.U i.e. concentration Units). In case of too high turbidity before the calculated discharge interval, a discharge was performed manually and the discharge interval was adjusted accordingly.

4.4. Filtration

Supernatant was collected after the centrifugation step and loaded onto depth and membrane filters. Filter performance, in removing impurities, was assessed based on the permeate flow rate, the pressure across the filter, the post-filtration turbidity (measured in nephelometric turbidity units (NTU)) and the filtered volume. The feed flow rate was kept constant. Both depth and membrane filtration setups are presented in **Figures 22 and 23**.

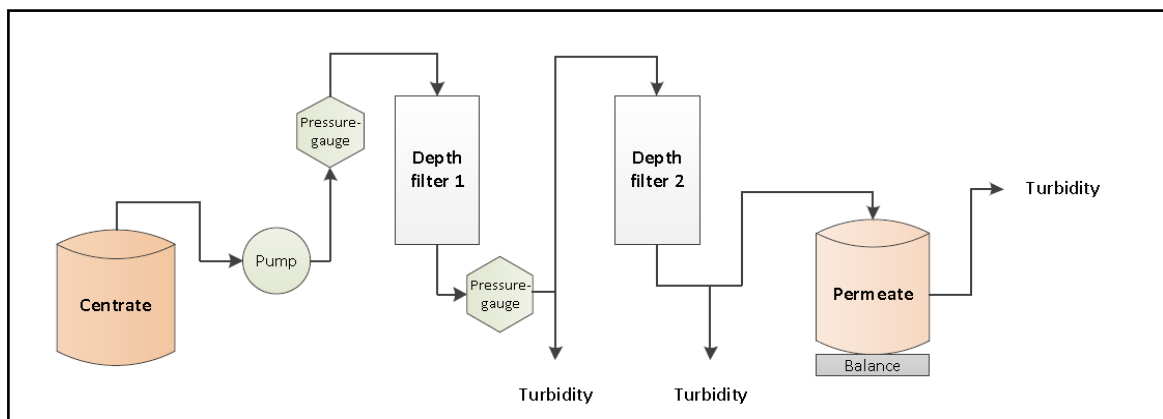


Figure 22. Depth filtration setup.

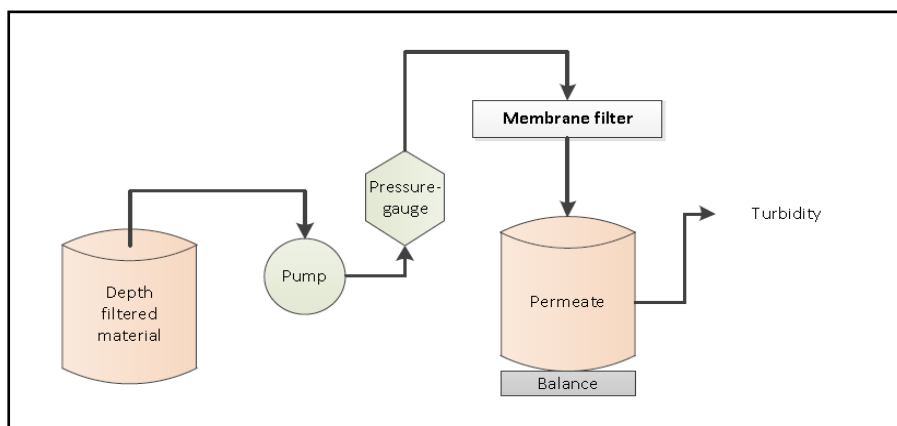


Figure 23. Membrane filtration setup.

5. Titer and product quality analysis

Titer was measured using the Octet® QK (Pall Fortébio) and/or protein A HPLC. Clarified cell culture fluid was purified using Protein A chromatography carried out on an ÄKTAXpress system (GE Healthcare). Product quality was assessed on the chromatography eluate with charge variant analysis (iCE3, ProteinSimple) and size exclusion chromatography (Acquity UPLC, Waters).

Isoelectric Capillary Electrophoresis (iCE) is a common analytical method used to characterise protein charge heterogeneity. This heterogeneity is directly related to some structural modifications such as deamidation, C-terminal lysines, sialylation, oxidation or glycation. The separation of each charge isoform is carried out through a pH gradient, based on its isoelectric point (pI).

Size exclusion chromatography (SEC) is an easy method for quantification of aggregated and dissociated antibodies. The components of a sample are separated according to differences in their molecular size thanks to porous beads. The dissociated proteins penetrate deep into the porous matrix on the contrary to large proteins (aggregates) which do not enter inside the beads. Each molecule group therefore has its own retention time.

Section 3. Results & discussion: case studies

This section comprises 4 case studies each displaying different aspects and applications of mammalian USP development for monoclonal antibodies or fragment antibodies. Data presented have been normalised and will display a reference with 3 standard deviations (SD).

A. Case study 1: Scale-down model, comparison 2000L vs 2L

1. Purpose

This case study focuses on 2L bioreactor experiments for scale-down model optimisation of a project currently in clinical trials. The drug substance is a full-length mAb produced in CHO cells, forming the basis of a therapy for auto-immune diseases. A scale-down model is a representation of a more complicated, expensive and/or physically larger system that enables prediction of quality, and can be used to support the extrapolation of operating conditions across multiple scales and equipment. Data should be provided demonstrating the model is suitable for the fine-tuning of the control strategy for commercial scale(39,40).

2. State of the art

Parameter	2L (n=8)	2000L (n=5)
IVCC (%)	94.8	100 +/- 16.3
Maximum VCC (%)	76.1	100 +/- 22.8
VCC at harvest (%)	89.7	100 +/- 30.3
Viability at harvest (%)	116.5	100 +/- 9.5
mAb titer at harvest (prot A HPLC) (%)	92.7	100 +/- 43.6
APG iCE (%)	104.3	100 +/- 4.7
BPG iCE (%)	120.2	100 +/- 91.7
HMWS (%)	144.8	100 +/- 110.3

Table 2. Case study 1. Parameter comparison between the reference 2000L scale and the scale-down model at 2L scale.

For development of a robust, comparable SDM it is preferable to find operating conditions achieving comparable VCC, titer, and PQA, such that results from optimisation studies at small scale can be transferred directly to manufacturing scale.

The parameters displayed in **Table 2** show remaining discrepancies which are the maximum VCC and the viability at the end of the process. Another discrepancy in the output parameters was also identified. **Figure 24** shows offsets between the reference i.e. the

2000L scale and the scale-down model for glutamate concentration from day 9 to day 13. This offset can be correlated with a difference in pH profiles: the pH is slightly lower for the 2L scale. However this pH difference is not significant i.e. it is included in the variability of the process.

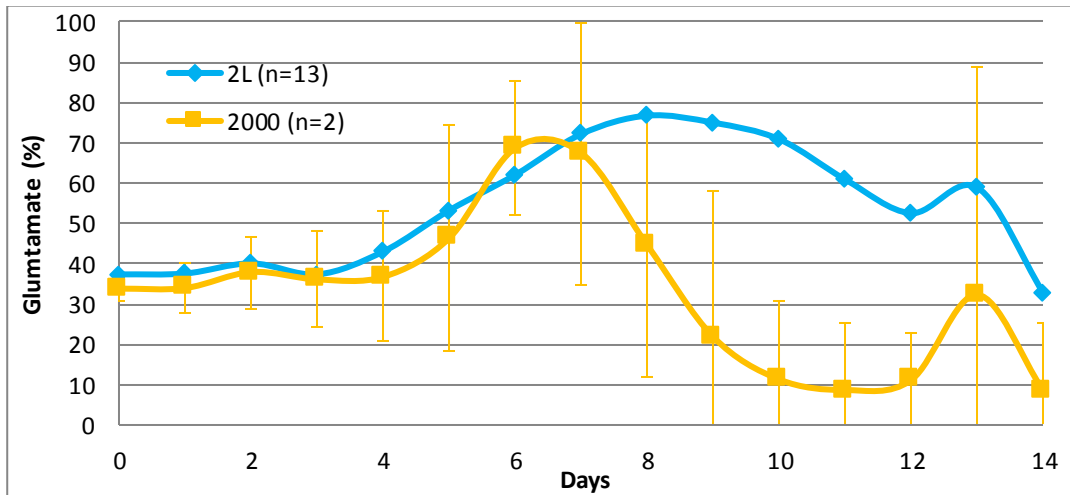


Figure 24. Case study 1. Glutamate concentration profiles 2L vs 2000L (3SD).

2L scale experiments were designed and have been performed in order to eliminate or reduce these offsets and further optimise the scale-down model.

3. Scale-down model strategy

Identified parameters differences	Status
Mode of Feed 2 addition	Tested
Feed / medium contact material	Not tested
Feed 2 precipitation (stalactites)	Not tested
N-1 type of vessel	Tested
Evaporation and sampling volume	Tested
Initial glutamine concentration	Not tested
Antifoam amount	Not tested
Impeller centrifugation	Tested
Stirring speed (tip speed) & power input	Not tested
Aeration cascade	Not tested
Overlay	Tested

Table 3. Case study1. Process differences: 2L vs 2000L.

Table 3 displays the identified parameter where differences were observed between the two scales. In terms of strategy, experiments were designed for testing identified parameters where differences were observed between the SDM and the 2000L scale such as the N-1 type of vessel. The latest endeavour of further optimisation was focused on the aeration and mixing parameters. The overlay was tested on two different

parameters: the overlay flow rate and the percentage of CO₂ in the overlay. None of the conditions tested could reduce the VCC and

viability discrepancies between the 2000L manufacturing scale and the 2L laboratory scale. However, some trends could be observed: when the air flow rate decreases, the VCC and pH increase and the glutamate concentration decreases whereas an increase in CO₂ percentage leads to the opposite effect. For this experiment, no impact on the PQA was observed.

The largest focus was put on the aeration cascade. The idea was to scale-down the 2000L aeration profile and test it at 2L to verify if it had an impact on VCC and viability. The obtained “optimised” cascade and the former one are presented in **Table 4**.

2L standard aeration cascade				2L optimised aeration cascade			
Output	N ₂	Air	O ₂	Output	N ₂	Air	O ₂
%	cm ³	cm ³	cm ³	%	cm ³	cm ³	cm ³
0	25	0	0	0	50	0	0
20	0	50	0	20	0	25	0
40	0	25	50	40	0	50	0
60	0	0	100	60	0	65	20
80	0	0	200	80	0	85	45
100	0	0	300	100	0	85	65

Table 4. Case study1. 2L standard and 2L optimised aeration cascade.

4. Design of Experiment

In a first experiment, the optimised cascade was coupled with 2 different impeller configurations (one standard with 3-blade impeller, and one equipped with a combination of a 3-blade and a Rushton at the bottom) and different starting speeds:

- Standard configuration + start at 400rpm: stirring speed higher than usual (280rpm) to compensate for the new cascade with lower O₂ input.
- Standard configuration + start at 225rpm: calculated so that the power input will be the same as the 2000L.
- Combination 3-blade & Rushton + start at 150rpm: same initial power input than at the 2000L scale

$$P/V = \frac{\rho * P_0 * N^3 * D^5}{V}$$

P/V = volumetric power input (W/m³)
 ρ = density (kg/m³)
 P₀ = power number
 N = stirring/60 (rpm)
 D = impeller diameter (m)
 V = working volume (m³)

P is the power required in an aerated bioreactor. The volumetric power input P/V was calculated as it is a tool commonly used and known to be efficient for scale-up or scale-down of mammalian cell culture processes.

A second experiment was designed to test the optimised cascade with the overlay parameter in order to confirm the positive trend previously observed with the new cascade, test the tip speed parameter and test once again the optimised cascade since the aim was to have repeatable results before changing a parameter in the SDM. The DoE (design of experiment) is described in **Table 5**:

Bioreactor tag	Cascade & agitation	Overlay flow rate & %CO ₂	Tested parameter
2L	Std + 280 rpm	No	Previous experiments' controls (n=11)
Bioreactor 1	Std + 280 rpm	No	None: control
Bioreactor 2	Std + 280 rpm	No	
Bioreactor 3	Std + 280 rpm	No	
Bioreactor 4	Opt + 510 rpm (tip speed)	No	Tip speed
Bioreactor 5	Opt + 510 rpm (tip speed)	No	
Bioreactor 6	Opt + 510 rpm (tip speed)	0.3 + 0%	Overlay
Bioreactor 7	Opt + 510 rpm (tip speed)	0.3 + 2%	
Bioreactor 8	Opt + 510 rpm (tip speed)	0.3 + 5%	
Bioreactor 9	Opt + 510 rpm (tip speed)	0.3 + 8%	

Table 5. Case study1. Experiment n2 design of experiment. Std: standard aeration cascade; opt: optimised aeration cascade.

The overlay is a layer of gas, usually air, on top of the cell culture fluid during cell culture. At 80L and 2000L scale, it is mainly used for protecting the exhaust filter. It needs protection because, at these scales, there is no condenser hence particles can reach the exhaust filter and clog it which leads to overpressure in the bioreactor and increases the risk of contamination. The overlay tackles both these issues: as there is a constant air circulation through the filter, it reduces humidity and clogging. The pressure the overlay applies to the liquid surface also helps reducing the foam level hence contamination risk during culture. Based on previously observed trends, a low flow rate was applied as it seems favorable for pH and VCC and a range of CO₂% in the overlay were tested. The 2000L tip speed was scaled down for 2L scale giving 510 rpm.

5. Results

5.1. Experiment n°1

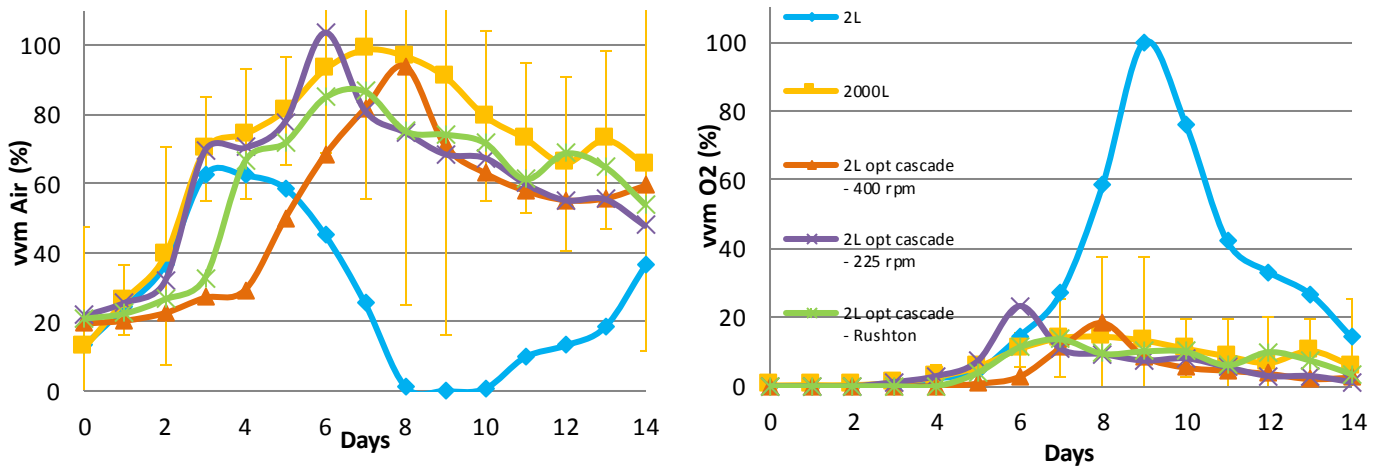


Figure 25. Case study1. Experiment n°1 O₂ and air aeration profiles in vvm i.e. L/L/m. 2000L curve is an average (n=5); 2L curve is an average (n=11); 2L opt cascade - 400 rpm curve is an average (n=4); 2L opt cascade - 225 rpm is an average curve (n=5); 2 opt cascade - Rushton is an average curve (n=3).opt means optimised.

In this experiment PQA were not impacted and significantly similar to the 2000L.

The aeration profiles in **Figure 25** were calculated in liter of air per liter of medium per minute in order to be able to compare both scales. All bioreactors with the optimised cascade condition displayed aeration profiles more similar to the 2000L scale compared to the results obtained with the standard cascade.

In terms of VCC (**Figure 26**), the condition which falls more into the range of the 3 standard deviation of the 2000L curve is the optimised cascade with 400 rpm condition which has

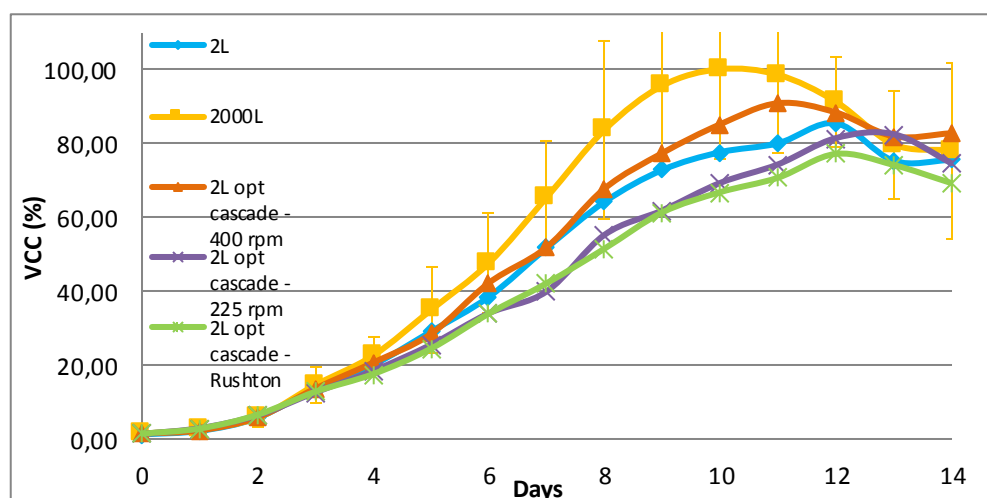


Figure 26. Case study1. Experiment n°1 viable cell concentration. 2000L curve is an average (n=5); 2L curve is an average (n=11); 2L opt cascade - 400 rpm curve is an average (n=4); 2L opt cascade - 225 rpm is an average curve (n=5); 2 opt cascade - Rushton is an average curve (n=3).

higher VCC than the control one from day 8 to 12.

These observations suggest that an optimised cascade, when coupled with a high agitation, is beneficial for the VCC. Furthermore, optimised aeration profiles clearly further align with the 2000L ones. However, the optimised cascade must be retested in order to confirm if this observation can be confirmed.

5.2. Experiment n°2

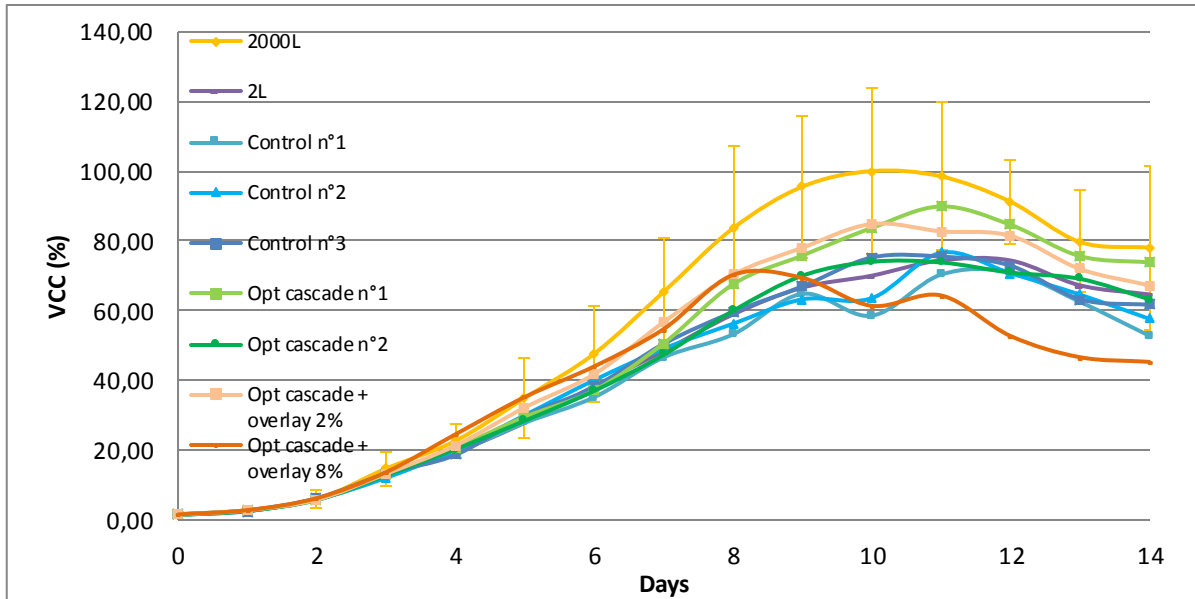


Figure 27. Case study1. Experiment n°2 viable cell concentration.

2000L curve is an average (n=5); 2L curve is an average (n=11); for all the other conditions n=1.

Due to early contaminations during the cell culture period, bioreactors 6 and 8 of experiment n°2 were lost hence did not provide any data.

5.2.1. Growth parameters

As can be seen in **Figure 27**, the optimised cascade + 8% of CO₂ in its overlay falls out of the

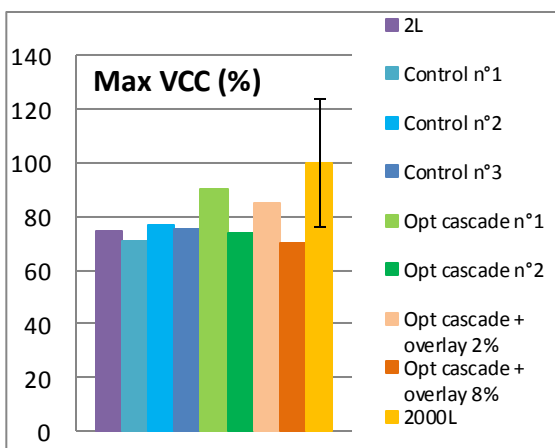


Figure 28. Case study1. Experiment n°2 maximum viable cell concentration.

2000L curve is an average (n=5); 2L curve is an average (n=11); for all the other conditions n=1.

variation of the 2000L process from day 9. The other conditions show no difference from the controls hence no improvement except for the optimised cascade n°1 and optimised cascade + 2% CO₂ in the overlay conditions. Both VCC profiles are higher and closer to the 2000L one and fall into the 3 standard deviations.

The same two conditions also stand out in terms of maximum VCC (see **Figure 28**): they are the only 2L conditions that have a higher maximum VCC and are comparable to the 2000L VCC.

The second condition with an aeration cascade (opt cascade n°2) does not align with the first one. This difference is observed in most of the graphs.

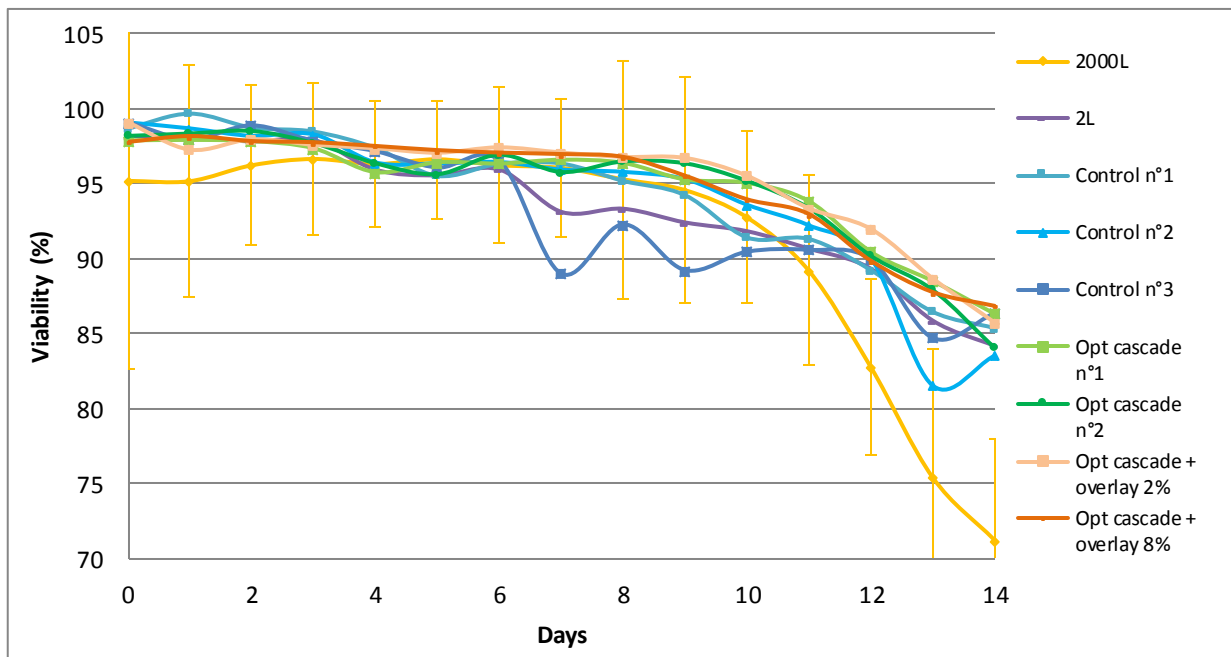


Figure 29. Case study1. Experiment n°2 cell viability.

2000L curve is an average (n=5); 2L curve is an average (n=11); for all the other conditions n=1.

Regarding the viability (**Figure 29**), no condition allows an improvement towards the 2000L profile. Variations of control n°3 from day 6 to 10 might be due to sample measurement errors.

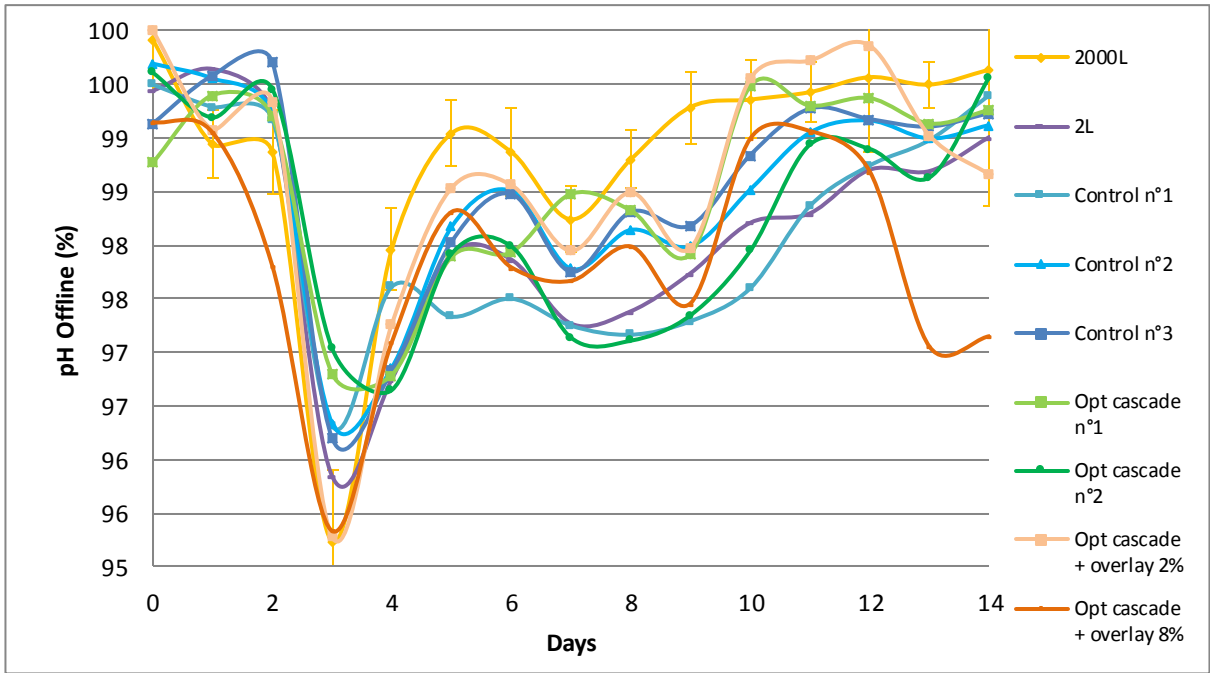


Figure 30. Case study1. Experiment n°2 offline pH profiles.
 2000L curve is an average (n=5); 2L curve is an average (n=11); for all the other conditions n=1.

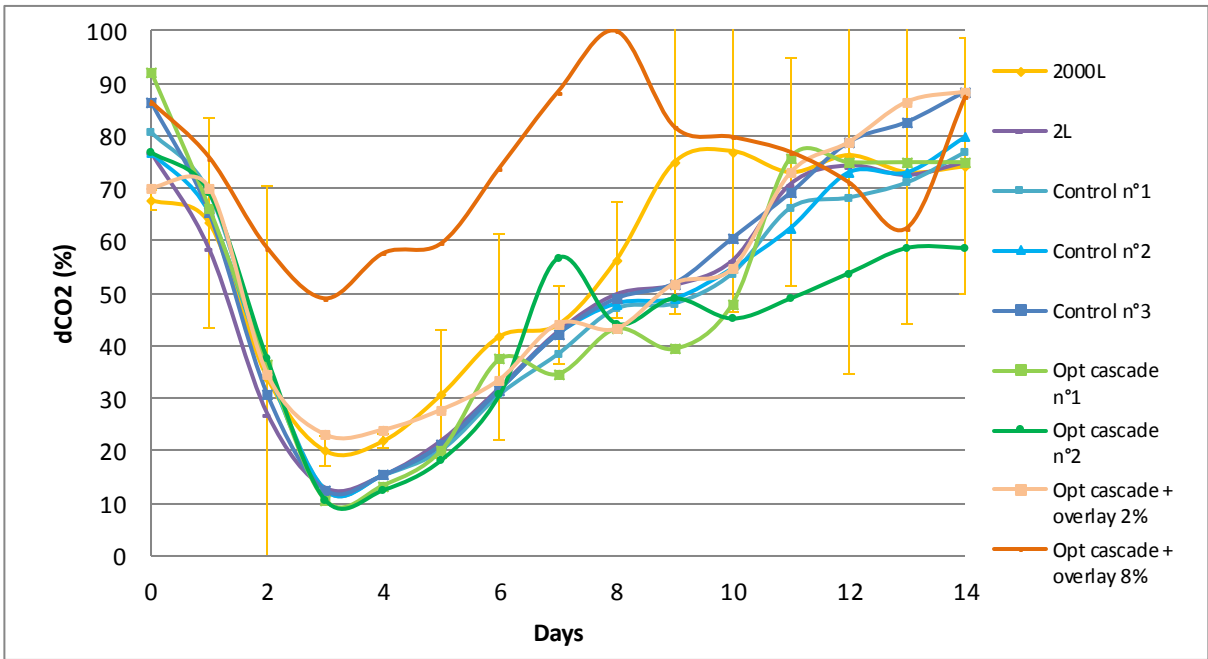


Figure 31. Case study1. Experiment n°2 dCO₂ profiles.
 2000L curve is an average (n=5); 2L curve is an average (n=11); for all the other conditions n=1.

pH and dCO₂ profiles (respectively **Figure 30** and **31**) do not allow to clearly conclude whether improvement is made for any of the conditions even though optimised cascade with 2% CO₂ in the overlay seems have a pH profile that is partly more similar to the 2000L one. Optimised cascade n°1 and n°2 conditions still fluctuate within the variability of the process.

Profiles of optimised cascade + 8% of CO₂ in the overlay condition significantly fall out of the 3 standard deviation range in terms of pH with a drastic drop from day 12 which is also correlated to the dCO₂ profile of the condition that is the highest throughout almost all the process period and present an offset with the 2000L profile. Especially, significantly higher levels of lactate and glutamate concentration from day 10 correlate with the drop of pH for this same condition (see **Figure 32** and **33**).

Regarding the glutamate concentration profiles, optimised cascade with 2% CO₂ in the overlay and optimised cascade n°1 conditions have a smaller offset with the 2000L profile. The same tendency can be observed for lactate concentration profiles but remains minimal.

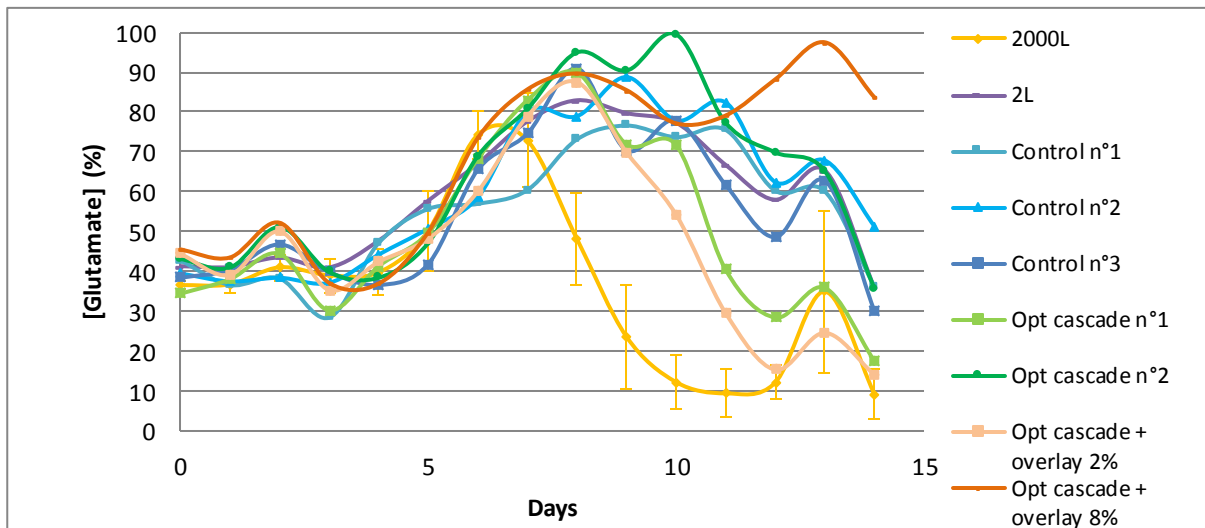


Figure 32. Case study1. Experiment n°2 Glutamate concentration profiles. 2000L curve is an average (n=5); 2L curve is an average (n=11); for all the other conditions n=1.

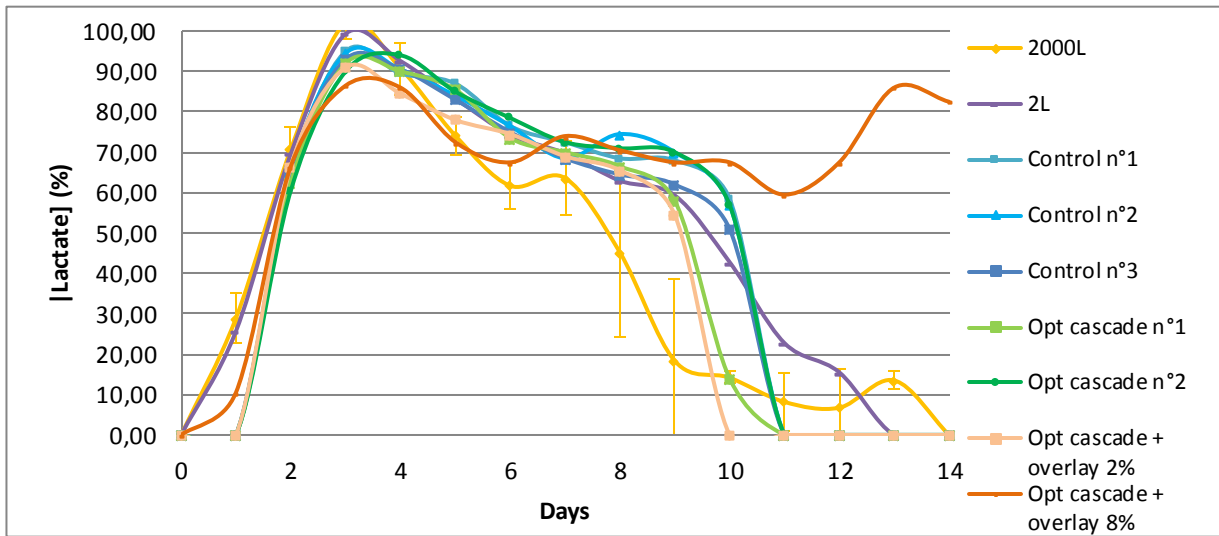


Figure 33. Case study1. Experiment n°2 lactate concentration profiles. 2000L curve is an average (n=5); 2L curve is an average (n=11); for all the other conditions n=1.

5.2.2. Titer and product quality attributes

Figure 34 summarises titer and PQA data from experiment n°2.

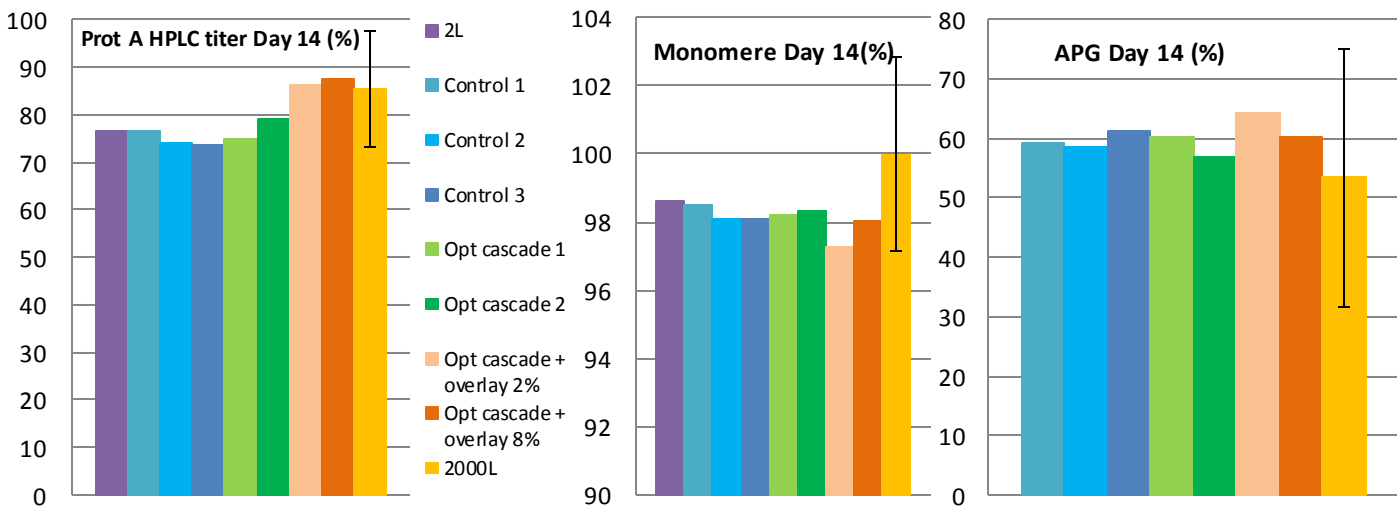


Figure 34. Case study1. Experiment n°2 titer and PQA. From left to right: titer, SEC and iCE results. APG = Acidic Peak Group. 2000L curve is an average (n=5); 2L curve is an average (n=11); for all the other conditions n=1.

All conditions are within the variability of the 2000L process.

6. Case study conclusion

The goal of this experiment was principally to reduce the VCC and glutamate discrepancies between the 2000L scale and the 2L scale. The SDM has already acceptable results: the PQA and mAb production are comparable between scales, i.e. the parameters are in the three

standard deviations of the 2000L batches. These values have to stay in this range during experiments.

With the optimised aeration cascade, aeration profiles were indubitably closer to the 2000L and there was no impact on the PQA. The second experiment allowed detecting a possible tendency of the optimised cascade to reduce the offset in VCC, maximum VCC and glutamate concentration but one must bear in mind that the results are not statistically significant as they were not repeated. In addition, the 2 bioreactor with the same condition were not aligned in most cases. This phenomenon has no clear root cause but one explanation could be that these differences are due to the variability of the process. Therefore the possible beneficial effect of the new cascade on these output parameters cannot be proven.

Regarding the overlay, the 8% CO₂ condition is clearly detrimental for almost all growth parameters and has the opposite effect of creating new offsets with the 2000L scale. This condition is not to be retained. Reversely, the overlay with 2% of CO₂ seems to be prone to reduce the VCC, max viability and glutamate offsets. Still, similarly to the aeration cascade conditions, results are not statistically significant as they have not been repeated and hence should be confirmed with additional studies.

Interestingly, all PQA data were not impacted i.e. comparable to the 2000L scale. This demonstrates the process robustness.

The optimised cascade can be implemented as it clearly does not affect PQA and improve the aeration profiles. Even so, this condition which is also coupled with 510 rpm (high agitation) is more difficult to regulate (see **Appendix 4** and **5**). The final decision must be made weighing all these elements along with the future SDM strategy.

B. Case study 2: 80L scale & scale-up consideration

1. Purpose

This case study is about the manufacturing process development of a new molecule which is a fragment antibody. Due to the restricted timelines available to develop a process prior GMP manufacturing, it was decided to skip the early process development & optimisation step in 2L scale and go straight into 80L bioreactors using a platform USP. In order to support tight drug substance development, some indispensable development were to be done before scale-up to 2000L manufacturing. Key points to be developed were DSP, analytical methods and formulation. For this purpose, three 80L plus two 10L cell culture runs were performed with the aim to produce representative material to be sent to the respective departments for development. These 5 batches represented several challenges, the first one being the feeding strategy which needed close monitoring and control in order to adapt it for the cell line. Another one was the primary clarification step as its yield had to be optimal in order to provide enough material to DSP, analytical and formulation departments. This critical step had to be scalable as well in order to implement it at 2000L scale. The first 80L batch was performed in parallel with the two 10L batches and the two other 80L runs followed subsequently. This case study will focus on the scale comparison between 10 and 80L regarding the growth data (in all graphs n=1 for each batch). This scale comparison between 10 and 80L is key as it enables to assess process reproducibility across scales which is necessary before scaling up the process at 2000L scale.

2. Results

2.1. Culture viability and VCC

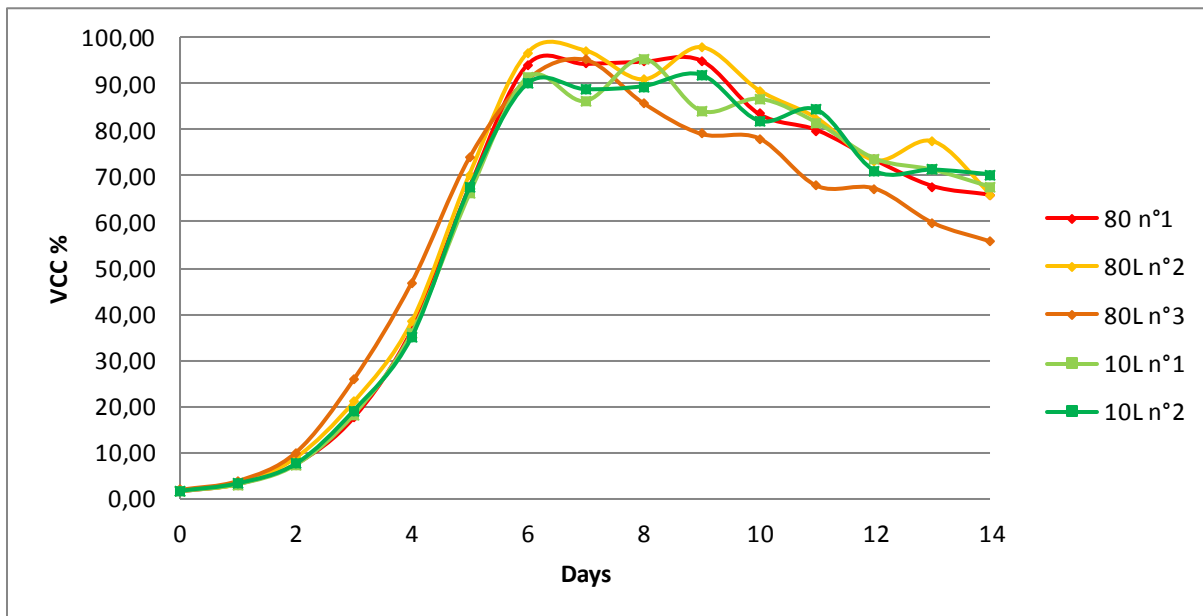


Figure 35. Case study 2. VCC profiles during the 14 days of culture. Samples were taken and analysed daily.

All VCC profiles are comparable except one: the 80L n°3 (see **Figure 35**). Indeed for this batch, the VCC has a more drastic increase than the others and also, after the maximum VCC is reached on day 7, a more pronounced drop in its VCC which results in a lower final value on day 14.

This is most likely due to an issue during the production run preparation which caused a 24-hour delay for the 80L inoculation. In other words, instead of inoculating the bioreactor with a 3 days old N-1, which is the standard procedure, it was inoculated with a 4 days old N-1. Because of this overgrowth in the N-1, a potential lag-phase was expected at the N-stage. In order to compensate, the TSD was increased by 14%. The objective was also to compensate the depletion of nutrients at day 4 of the N-1 stage that may have had caused cell stress which could have a negative impact on the performance of the bioreactor production.

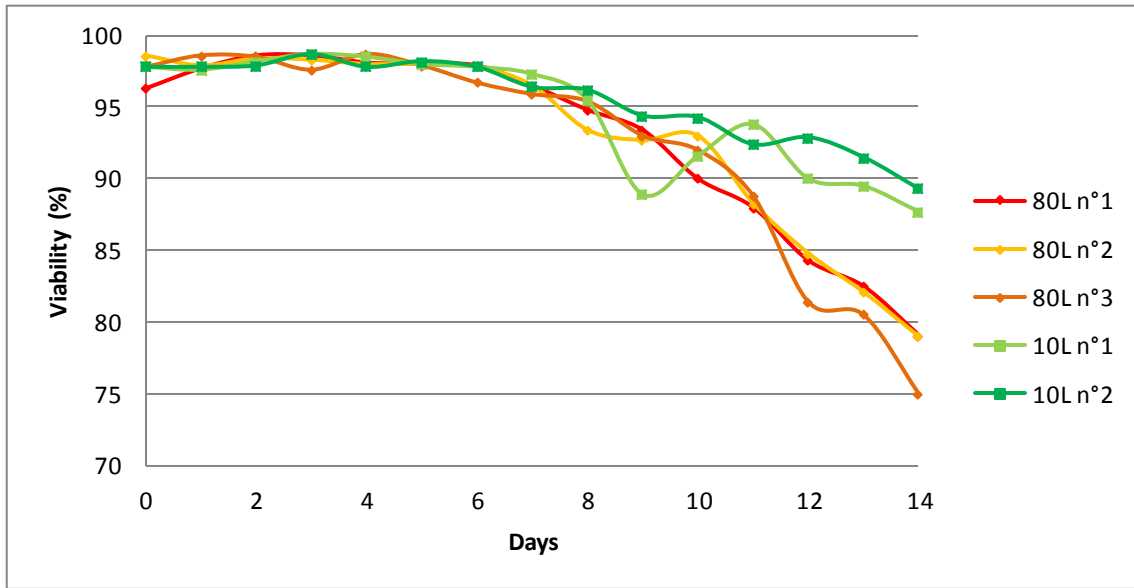


Figure 36. Case study 2. Viability profiles during the 14 days of culture. Samples were taken and analysed daily.

The same trend can be seen in **Figure 36** for the viability profiles: the profile of the 80L n°3 batch has a more drastic decrease in viability from day 11 and reaches a lower final viability compared to the other two 80L runs. Apart from this, the two 10L batches have similar profiles (day 9 of 10L n°1 seems to be a measurement error) and 80L n°1 & 2 also display similar profiles. Interestingly, the two 10L runs have a higher final viability than the 80L runs. This difference which seems to be a trend is most likely due to the scale difference.

2.2. Feeding strategy

The feeding strategy adopted for this new molecule was based on knowledge of previous

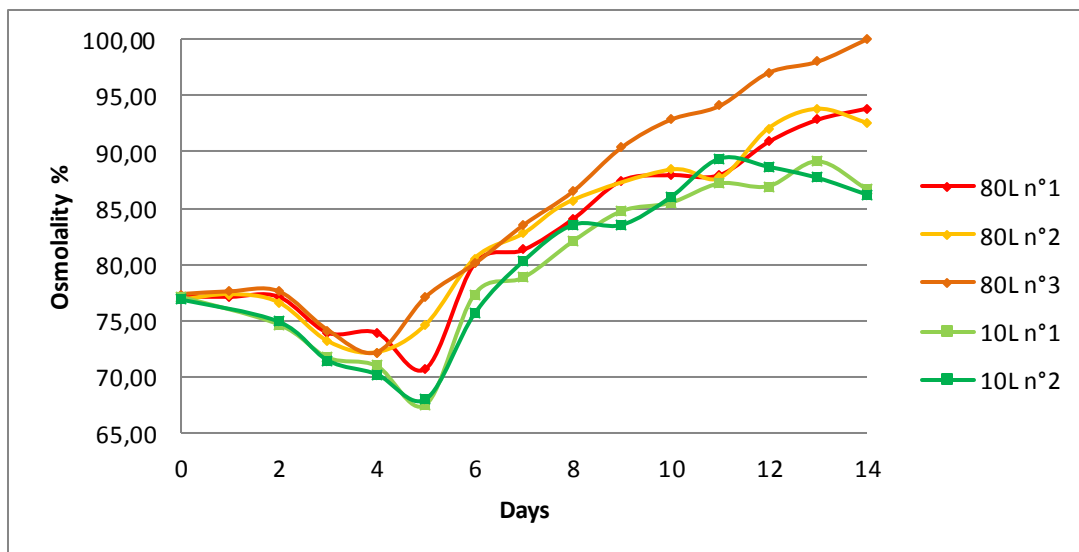


Figure 37. Case study 2. Osmolality profiles during the 14 days of culture. Samples were taken and analysed daily.

projects. It is was a critical point to master as a underfed cells would die or have a lower specific productivity of the therapeutic protein whereas overfeed cells leads to toxic accumulation of byproducts and high osmolality which is detrimental to cell viability. Parameters to verify for the feeding strategy are the osmolality, glucose, lactate and ammonium concentrations.

Figure 37 displays similar profiles of osmolality. In a first phase, the two 10L and 80L n°1 have similar profile with a trough in osmolality on day 5 that led to the decision of starting supplementing them with glucose on day 5 instead of day 6 usually. This trough on day 5 matches a trough in glucose concentration for the same bioreactors (see **Figure 38**). Following these runs, it was decided to further advance the glucose supplementation on day 4 in order to avoid the trough on day 5; hence smooth the osmolality profile. The outcome of this implementation can be observed for the two last 80L runs which have a gentler drop in osmolality until day 4 then an increase until the end of the culture. Glucose profiles also changed accordingly with a trough on day 4 instead of day 5 for 80L n°2 and 3. Due to the N-1 discrepancy and adjustment of the TSD, 80L n°3 batch has a higher osmolality from day 9 which match with the drops of VCC and viability: there are less cells hence less feed and glucose consumption which results in an overfeeding associated with a higher osmolality.

The final osmolality is higher for 80L than for the 10L. This is most likely due to a scale variability. The rest of the profiles are similar (day 10 for 10L n°1 and n°2 are measurement

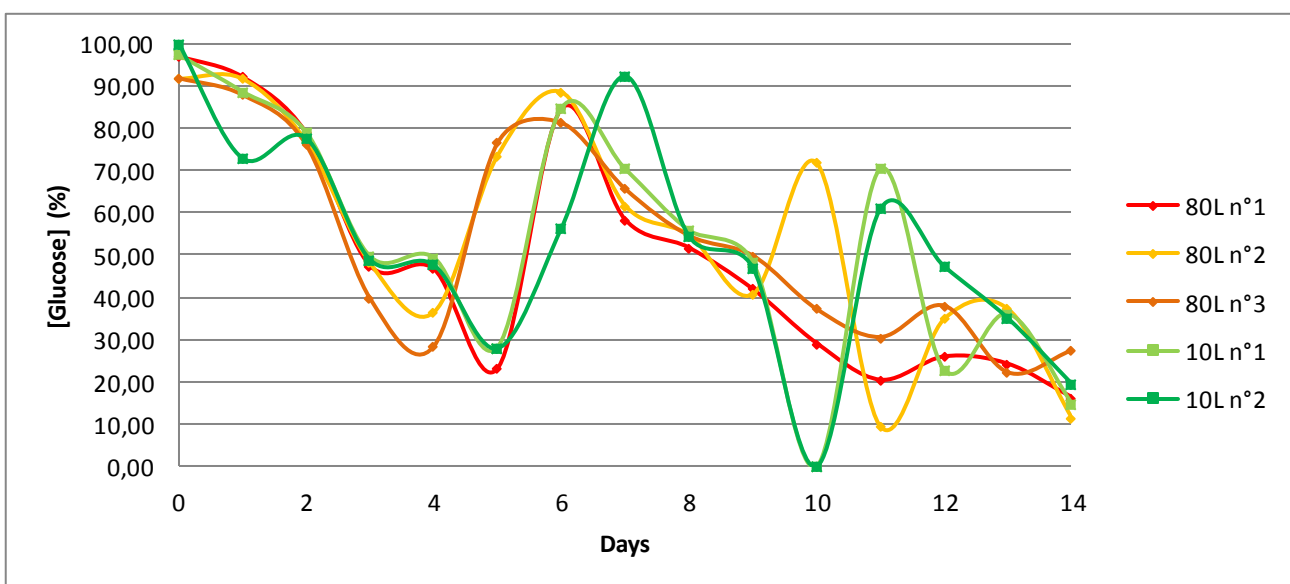


Figure 38. Case study 2. Glucose concentration profiles during the 14 days of culture. Samples were taken and analysed daily.

errors).

Figure 39 and **40** display similar glutamine and glutamate concentration profiles for both scales respectively. As expected, the third 80L batch drifts from the other from day 9

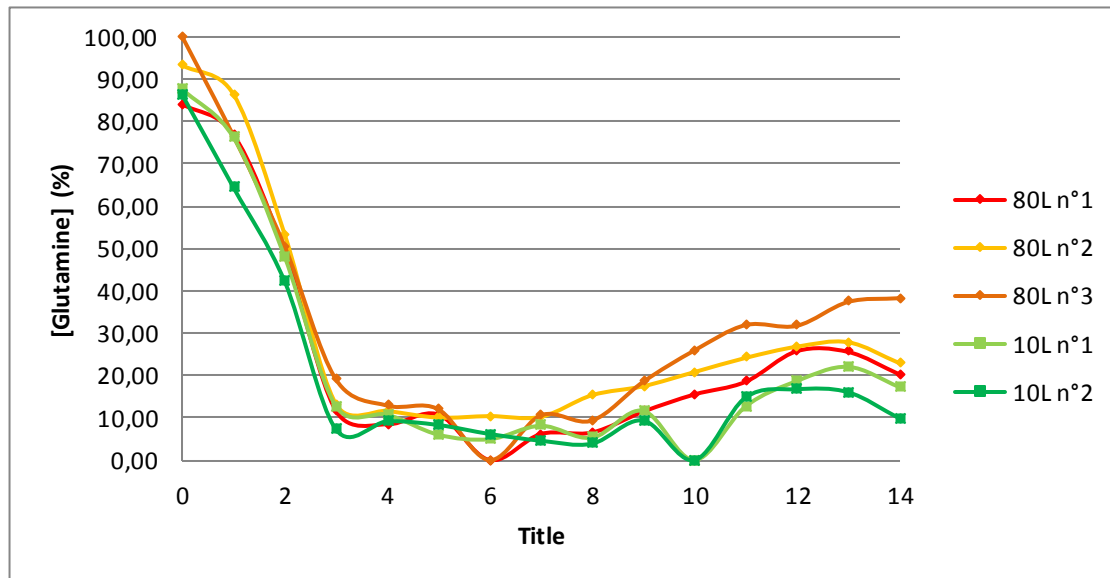


Figure 39. Case study 2. Glutamine concentration profiles during the 14 days of culture. Samples were taken and analysed daily.

onwards: the glutamine concentration is higher. This is a direct effect of its lower VCC and viability; there are less cells hence less glutamine and glutamate consumption. The other batches have similar profile regardless of the scale.

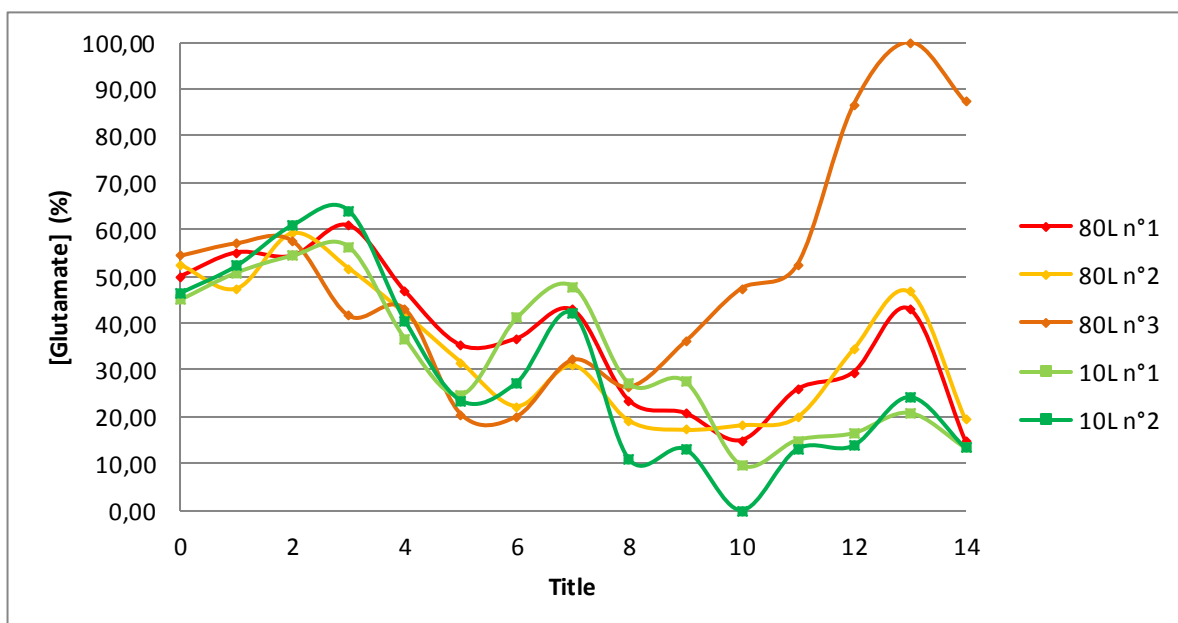


Figure 40. Case study 2. Glutamate concentration profiles during the 14 days of culture. Samples were taken and analysed daily.

The lactate profiles (**Figure 41**) show the process reproducibility as all batches of both scale are aligned. In addition ammonium concentration never reaches its toxic threshold (data not shown).

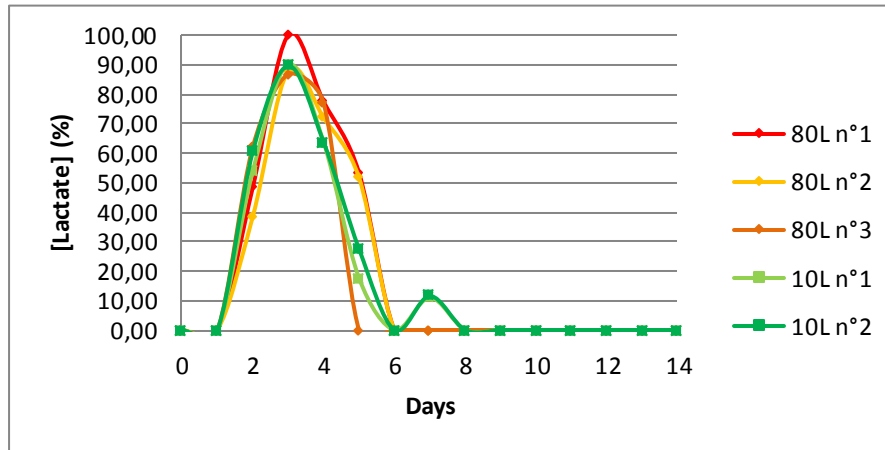


Figure 41. Case study 2. Lactate concentration profiles during the 14 days of culture. Samples were taken and analysed daily.

2.3. Scalability: pH and dCO₂

Figure 42 and **43** present, offline pH and dCO₂ profiles respectively. The trough in pH for all batches can be correlated with the peak of lactate on day 3. The trough in pH is less

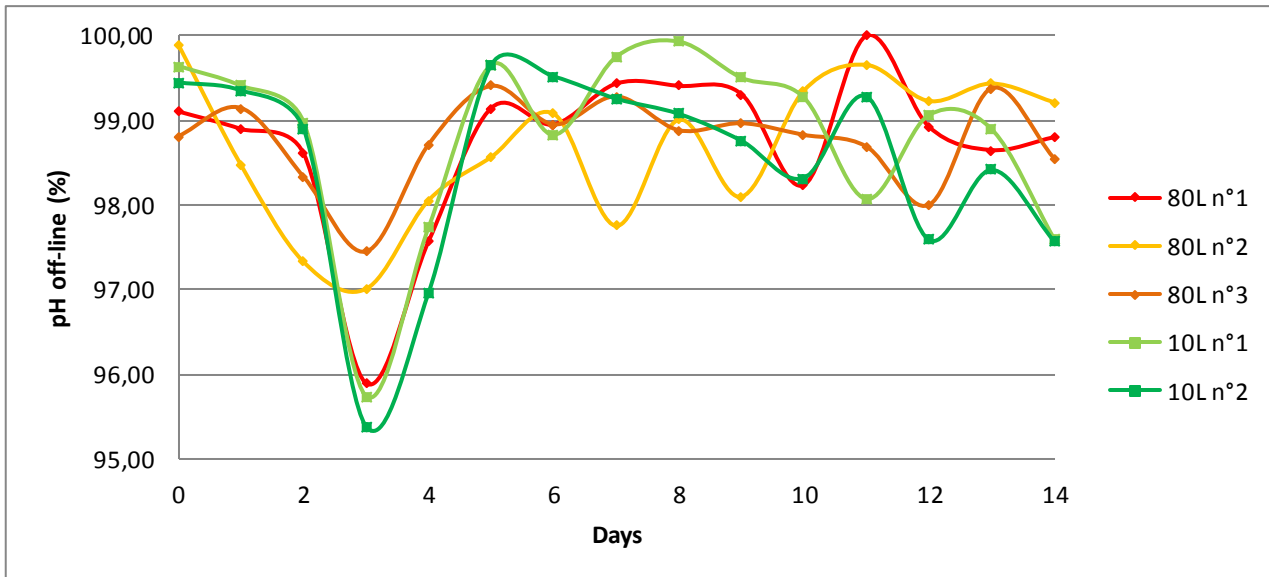


Figure 42. Case study 2. Offline pH profiles during the 14 days of culture. Samples were taken and analysed daily.

pronounced for 80L n°2 and n°3 batches but still fluctuates within the pH deadband. All profiles for both scales are similar (dCO₂ profile for 10L n°1 on day 4 corresponds to a measurement error). This shows the process is reproducible across scales.

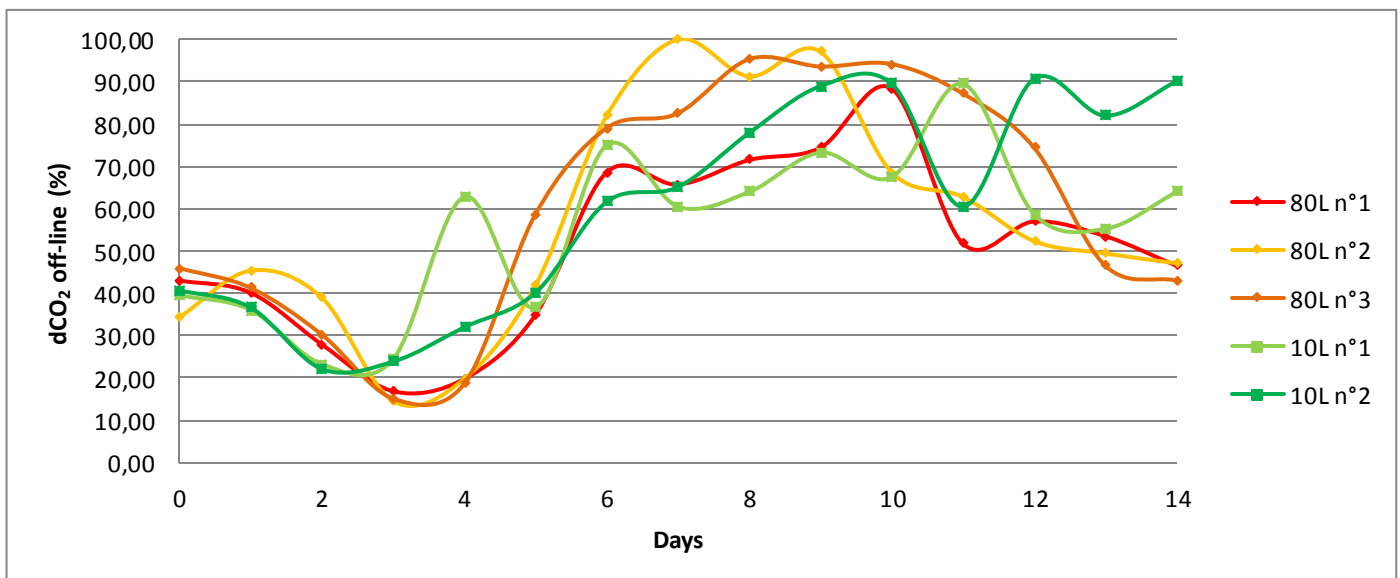


Figure 43. Case study 2. dCO₂ offline profiles during the 14 days of culture. Samples were taken and analysed daily.

2.4. Titer

Since analytical methods were being developed, no PQA result is available. Only titer measurements based on protein A HPLC were available and shown in **Figure 44**.

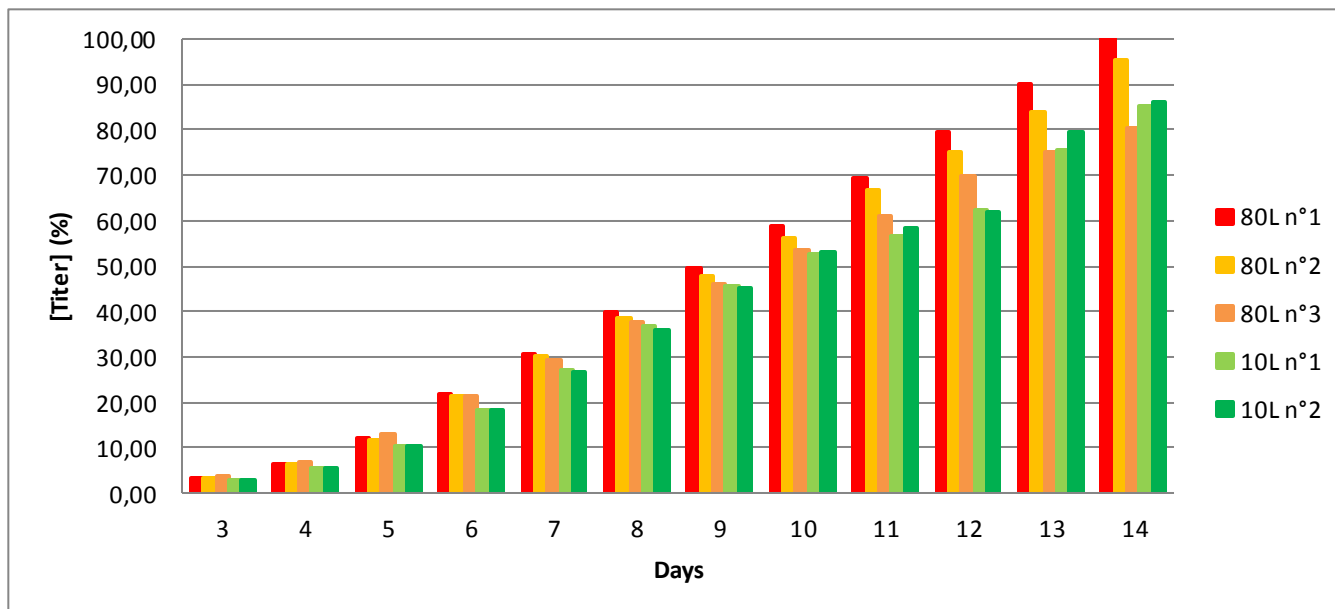


Figure 44. Case study 2. Protein A HPLC titers.

Overall, 80L batches provided fragment antibody titers superior to the 10L titers with one exception: 80L n°3 batch showing the lowest titer on day 14. This is related to the inoculation discrepancy of the batch which led to a lower viability and VCC. There is no difference intra-scale for the 10L batches nor for 80L n°1 and n°2 (the slight difference between 80L n°1 and n°2 batches is not significant and due to the variability of the process or of the analytical method).

3. Case study conclusion

Intense monitoring enabled to overcome all challenges and to conduct all batches until their term. The objective to provide enough material to the different departments i.e. DSP, analytical and formulation departments for development purposes was fulfilled. Despite the discrepancy at the N-1 step and the strategy to increase the TSD at production inoculation step for the third 80L batch, all cell growth parameters and indicators demonstrated that the process seems robust and comparable across scales. This discrepancy was actually beneficial for the scale up study of the process as it gives data on the impact of such discrepancy on process performance particularly in final therapeutic protein titer. It reveals

process robustness when performed as per procedure but also its limits in term of protein yield which is essential for the soon scale-up at 2000L.

Regarding the feeding strategy, all parameters i.e. osmolality, glucose, lactate and ammonium concentration were consistent and acceptable therefore the feeding strategy was endorsed and will be used for the transfer at GMP 2000L scale. As a continuous improvement, the feeding strategy will be further refined at a later point in the development of the process.

C. Harvest development

Cell culture engineering and process monitoring have led to the rapid development of high yielding and robust manufacturing processes for monoclonal and fragment antibodies. Biopharmaceuticals are now usually produced at large scale using platform approaches. This high performance processes come with other challenges, particularly developing scalable and reliable primary clarification process. Indeed this step which connects USP and DSP has a direct impact on yield and product quality. As a result, harvest is becoming a critical step which needs development and optimisation.

When it comes to harvest development, scalability is key. Depth filtration performance is highly dependent on the flow rate applied during filtration. Flow rate is usually expressed in L/m²/h (LMH) and is kept constant throughout scales. There is an optimal flow rate for each type of filter and each process. At a certain flow rate, filter performance is described as the amount of material that can be filtered by surface unit: volumetric throughput (L/m²).

The following case studies present different aspect and approach to be considered when developing harvest processes.

1. Case study 3: Back-up filter development

1.1. Purpose

The goal of this case study was to define a secondary filter supplier for the large-scale process. 2000L bioreactor runs were performed and harvested on day 14. The cell culture fluid was centrifuged and filtered at large-scale. A portion of the centrate was collected after the centrifugation step for a filterability study. Different series of two depth filters and different membrane filters were evaluated using filters designed for quick and efficient filter sizing studies.

1.2. State of the art: experiment n°1

A previous study (experiment n°1) screened filters from two suppliers based on their characteristics and compared them to the depth filtration train currently set up at large-scale. After depth filtration, permeates were filtered on the same membrane filter used at 2000L scale. **Table 6** presents the depth filter combinations tested with the associated results. The first combination with filters from supplier A is the one currently used in the large scale manufacturing process hence it is the control condition. The test performed on one depth filter only, B_{D3}, was performed on site by supplier B within same work conditions. For all the conditions tested, the turbidity of the centrate was 223.3 NTU.

For all depth filtration experiments,

- **Initial filtration flow rate:** 40 LMH.
- **Monitored parameters:** inlet pressure, permeate weight, flow rate and turbidity.
- **Threshold parameters:** 2 bar maximum increase, high final turbidity (> 20NTU) and flow rate decrease.
- **Target:** 100 L/m².

Depth filter	Maximum inlet pressure (bar)	Turbidity post 1 st depth filter at end of filtration (NTU)	Turbidity post 2 nd depth filter at end of filtration (NTU)	Final turbidity of total permeate (NTU)	Permeate flow (LMH)		Volumetric throughput (L/m ²)	
					Start	End	At end of filtration	Target
A _{D1} – A _{D2}	1.3	139.9	37.7	18	40.9	24.6	111.4	100
B _{D2} – B _{D3}	2.0	> 153.6	> 47.9	19	32.6	5.2	46.5	
B _{D1} – B _{D3}	0.4	195.3	81.8	41	42.4	42.4	93.9	
B _{D3}	1.6	47.3	N.A	47	40.2	43.35	75.1	
C _{D1} – C _{D2}	0.1	> 170.9	> 129.5	63	42.0	33.6	53	
C _{D3} – C _{D2}	0.4	203.5	143.1	90	43.2	33.6	34	
C _{D4} – C _{D2}	0.4	190.4	101.9	71	40.8	40.8	18.6	

Table 6. Case study 3. Depth filter results of screening experiment n°1. Pressure data was collected using a pressure gauge between the pump and the first filter; turbidity data was collected using a portable turbidimeter on samples, flow data was collected using a measuring tube to collect material after the 2 depth filters for one minute to obtain the volume per minute.

1.2.1. Depth filtration results

No filter combination outperformed the control combination, A_{D1} - A_{D2}, currently set up at 2000L scale. Filters from supplier B seemed to be more efficient for particle removal (size exclusion) than filters from supplier C but are still above the 20 NTU threshold. Indeed, the adsorption surface area of supplier C filters is quickly saturated and small particles seem to be going through as the pores are too big (no pressure increase with high turbidity).

Condition B_{D2} - B_{D3} seemed to be clogged at the end of the experiment: low permeate flow rate and inlet pressure reaching 2 bars.

B_{D3} alone displayed good results in terms of permeate flow which remained stable but, despite a relatively high volumetric throughput (75.1 L/m²), did not reach the 100 L/m² target. Condition B_{D1} - B_{D3} had also positive results regarding the flow rate which remains steady plus a volumetric throughput reaching the target. Yet, both conditions had a total permeate turbidity that is the double of the threshold. If we compare those two conditions, filter B_{D3} alone has a lower volumetric throughput and a higher final turbidity than B_{D1} – B_{D3} condition. It seems filter B_{D1} retains particles before B_{D3} but not efficiently enough to protect the second depth filter.

1.2.2. Impact on membrane filtration performance

Subsequent membrane filtration results were not acceptable for all condition except the control. The volumetric throughput target was not met by using B_{D3} or B_{D1} – B_{D3}. This may be related to the high turbidity (over 40 NTU) after depth filtration.

1.2.3. Experiment outcome

Product quality and titer were not impacted by the filters (data not shown). B_{D3} filter is present in condition with most potential (B_{D3} alone and B_{D1}-B_{D3}) but B_{D2} and B_{D3} seemed to not retain lots of particles. Filters from supplier C did not give satisfying results.

As a result, the filtration train could be made of only one depth filter: B_{D3}. The sizing study enable to estimate that at least 27 m² would be needed for B_{D3} at large scale instead of the 20 m² for the control condition. This sizing should be confirmed with a new filtration study.

1.3. Experiment n°2 & n°3: depth filtration performance

1.3.1. Design of experiment

Experiment n°2				
Depth filter 1	Depth filter 2	Ratio (%)	Initial flow rate (LMH)	Volumetric throughput target (L/m ²)
A _{D1}	A _{D2}	50/50	75 - 80	100
B _{D3}	N.A	100		
B _{D1}	B _{D3}	75/25		
B _{D1}	B _{D3}	25/75		
D _{D1}	D _{D2}	50/50		
D _{D1}	D _{D3}	50/50		

Table 7. Case study 3. Experiment n°2 DoE.

Table 7 presents the DoE of the second depth filter screening experiment. In this experiment, filters from a fourth supplier, supplier D, were tested. The promising depth filter, B_{D3}, was tested again alone and associated with B_{D1} however the combination B_{D1} – B_{D3} was tested using different ratios. The reasoning behind ratios was to see if more than one, i.e. three, B_{D1} filters would protect B_{D3} by stopping small particle more efficiently or if three B_{D3} filters instead of one would reach the target of depth filtration efficiency. Both ratios were tested: 75/25 i.e. three B_{D1} in parallel followed by one B_{D3} and 25/75 which means one B_{D1} followed by three B_{D3} in parallel.

Initial filtration rate was between 75 and 80 LMH. The turbidity of the portion of centrate collected after centrifugation at 2000L scale was 291.7 NTU. After depth filtration, permeates were filtered on the same membrane filter used at 2000L scale.

Experiment n°3				
Depth filter 1	Depth filter 2	Ratio (%)	Flow rate (LMH)	Volumetric throughput target (L/m ²)
A _{D1} (dead end centrifugation)	A _{D2} (dead end centrifugation)	50/50	80	100
A _{D1}	A _{D2}	50/50	80	
A _{D1}	A _{D2}	50/50	Low: 40	
A _{D1}	A _{D2}	75/25	Low: 40	
B _{D1}	B _{D3}	75/25	40	
B _{D1}	B _{D3}	75/25	Low: 20	

Table 8. Case study 3. Experiment n°3 DoE.

Experiment n°3 was performed in order to test most reliable conditions from experiment n°2 once again and also test them regarding the initial flow rate (see **Table 8**). A low flow rate was tested as it is more representative to the current large scale filtration process. This parameters was also investigated in order to see if it could impact the filtration performance.

Initial filtration rate was 80 LMH for standard flow rates and 40 LMH for low flow rate conditions. The turbidity of the portion of centrate collected after centrifugation at 2000L scale was 241.3 NTU.

Another control was performed using the two standard filters A_{D1} – A_{D2} at the standard flow rate of 80 LMH, but with the difference that the material used for this condition had been centrifuged with a lab scale centrifuge (dead-end centrifuge) which resulted in a post centrifugation turbidity of 120.95 NTU. This condition corresponds to the first row in **Table 8** and is labeled in the result section as A_{D1} – A_{D2} Lab ctrl.

After depth filtration, permeates were filtered on the same membrane filter used at 2000L scale.

1.3.2. Results

a. Depth filtration performance

The three following figures (**Figure 45, 46 & 47**) display the flow rate, the total permeate turbidity and the inlet pressure respectively.

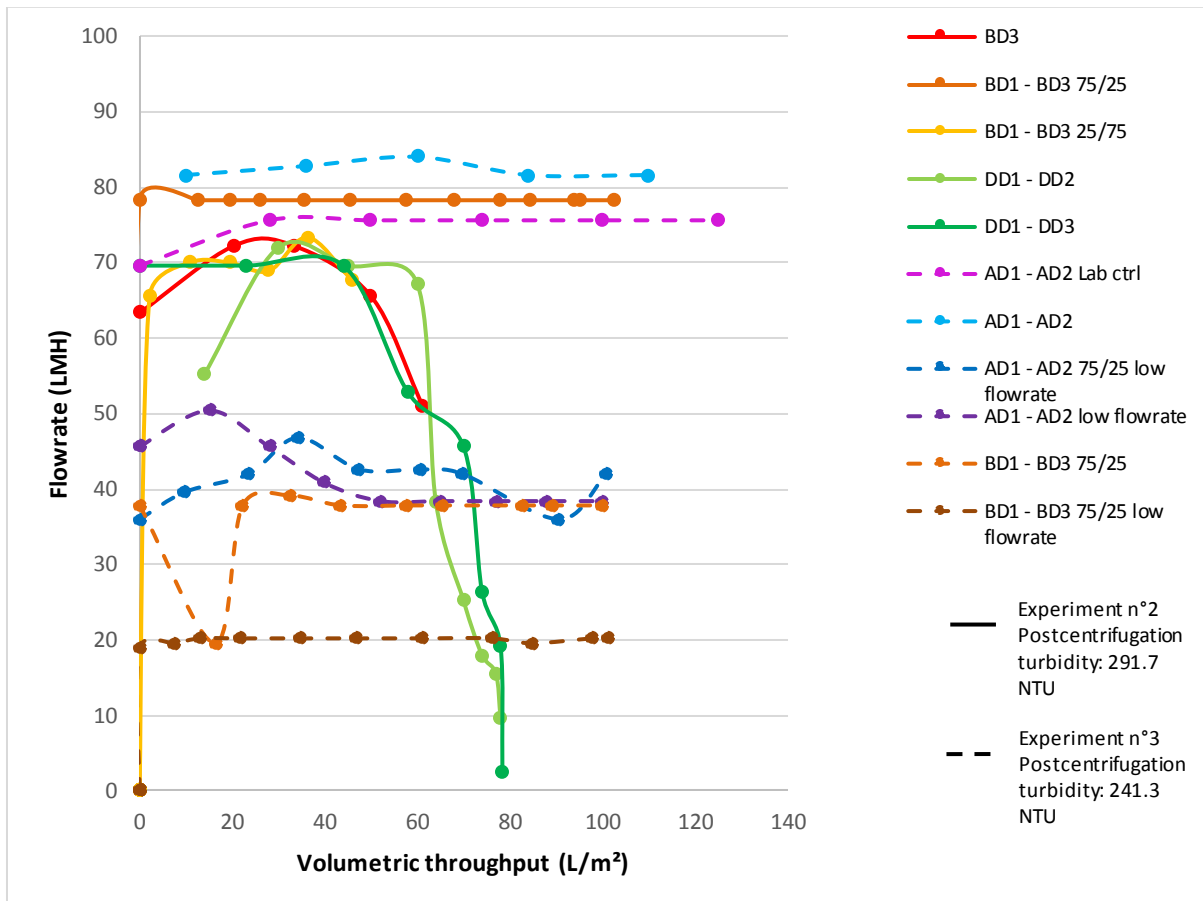


Figure 45. Case study 3. Experiment n°2 & 3 inlet flow rates (LMH) in fonction of the volumetric throughput (L/m²). Data was provided using a measuring tube to collect material after the 2 depth filters for one munute to obtain the volume per minute. Measures were performed every 10 minutes but was adjusted in case of variation of the parameter.

The conditions where the filter B_{D3} was predominant i.e. B_{D3} alone and $B_{D1} - B_{D3} 25/75$ conditions were quickly stopped as they seemed to be clogged: decrease in flow rate and increase in pressure exceeding 2 bars. These two conditions were therefore discarded from the list of potential back-up filters.

After 60 to 80 L/m², $D_{D1} - D_{D2}$ and $D_{D1} - D_{D3}$ conditions displayed a drastic drop in flow rate (which is supposed to remain constant during the filtration for good performance), high permeate turbidity and high pressure exceeding the corresponding thresholds i.e. 20 NTU and 2 bars respectively. The two conditions were also discarded.

All the other conditions displayed a relatively constant flow rate.

In these experiments, the control condition i.e. $A_{D1} - A_{D2}$ underperformed compared to historic data of sizing and large scale (GMP manufacturing). Indeed, the filtered material reached a too high turbidity before reaching the volumetric throughput target of 100 L/m^2 .

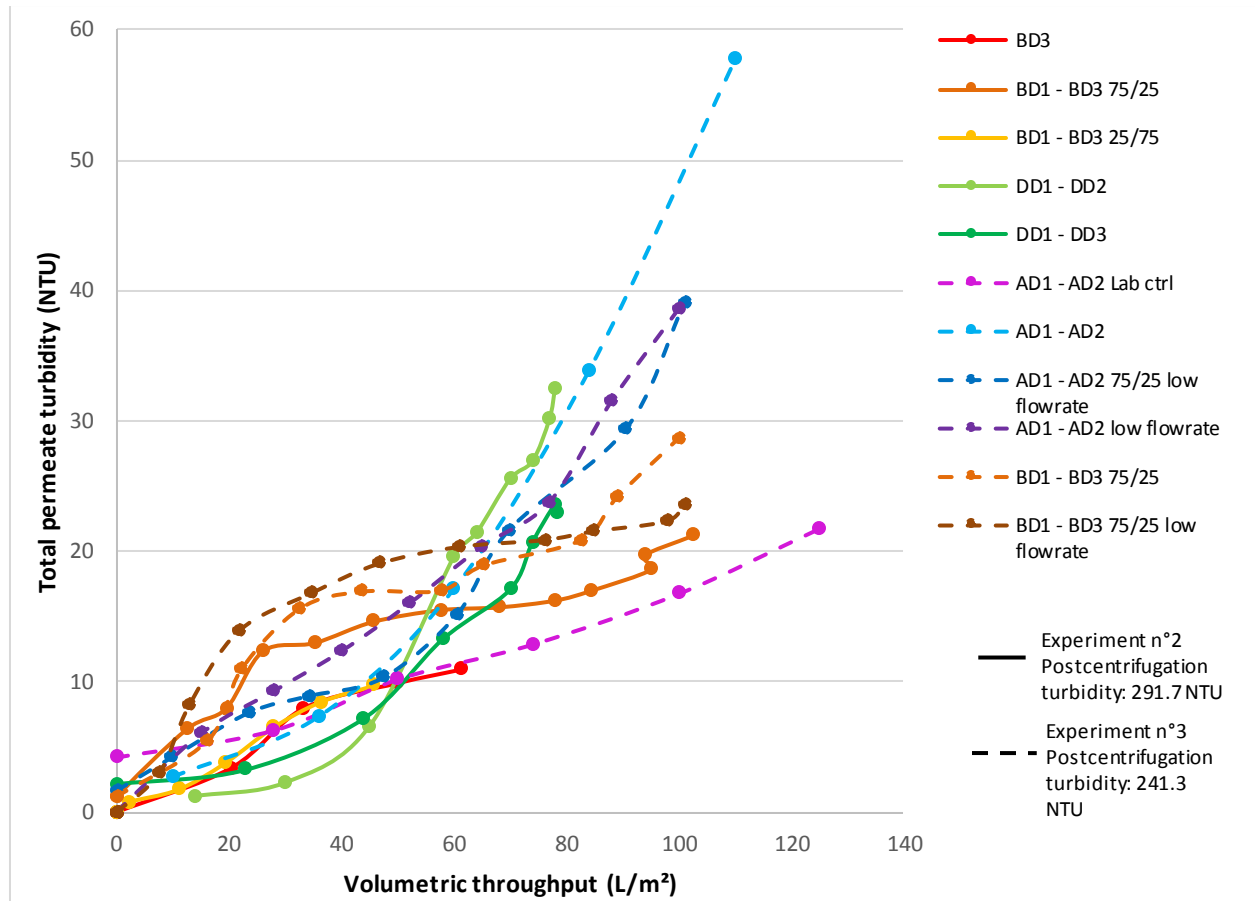


Figure 46. Case study 3. Experiment n°2 & 3 total permeate turbidity (NTU) in function of the volumetric throughput (L/m^2). Data was collected using a portable turbidimeter on the bulk filtered material. Measures were performed every 10 minutes but was adjusted in case of variation of the parameter.

It was due to a default of the filters used in the study. The same default had been identified in some other experiments. After an investigation in collaboration with the supplier, it was attributed to a wrong batch of filters which all presented the same defect. In addition, the fact that the material used for experiment n°2 was more turbid (than experiment n°3) might have led to a negative impact on depth filtration performance of the control. The link between the centrifugation performance and the subsequent turbidity of the permeate is obvious as the dead end centrifuged control ($A_{D1} - A_{D2}$ Lab ctrl) performed according to historical data with no turbidity breakthrough before reaching the 100 L/m^2 target.

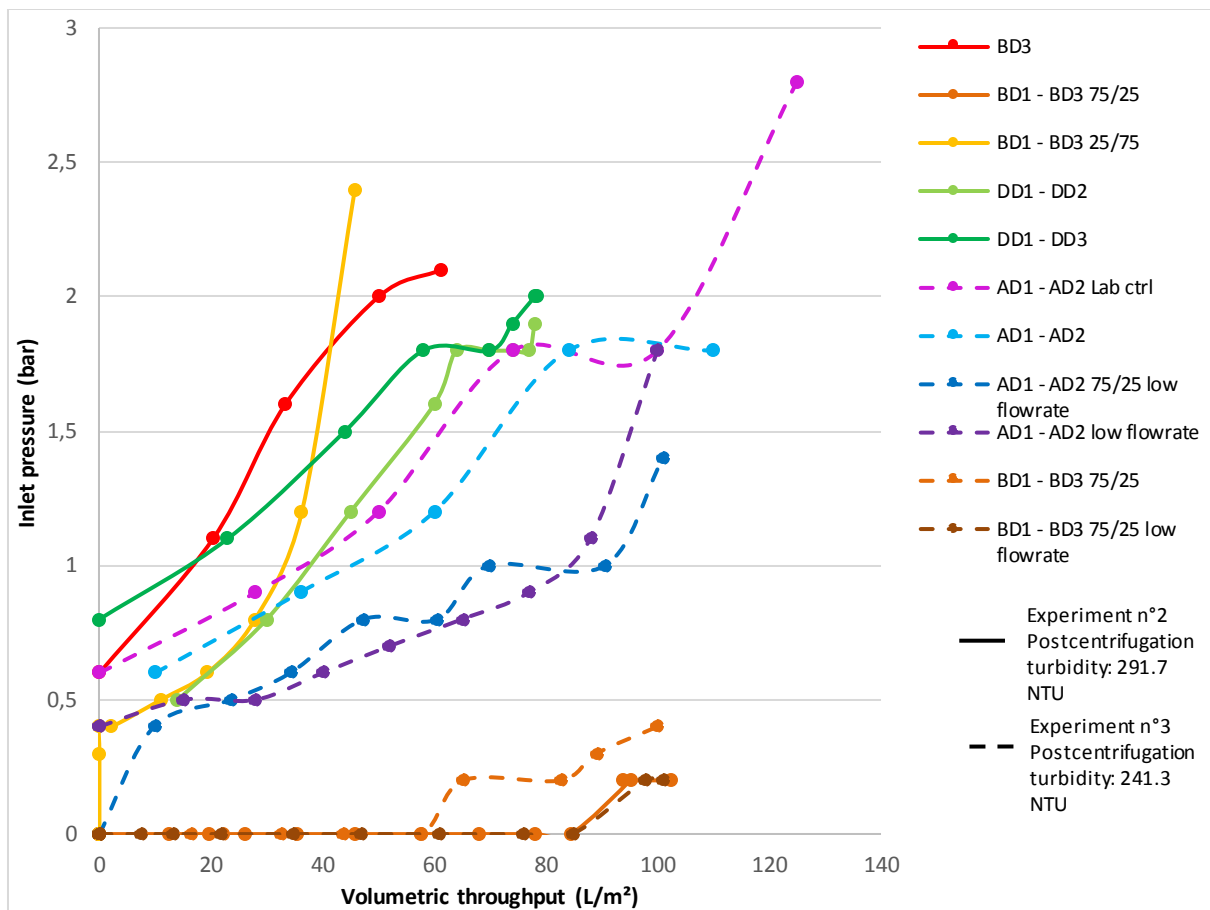


Figure 47. Case study 3. Experiments n°2 & 3 inlet pressure profiles (bar) in function of the volumetric throughput (L/m²). Data was collected using a pressure gauge connected after the pump and prior the two filters. Pressure was monitored throughout the filtration experiment and values were taken every 10 minutes but was adjusted in case of variation of the parameter.

Condition BD₁ – BD₃ 75/25 gave satisfying results for all three parameters. Indeed, this condition in experiment n°2 and experiment n°3 as well as BD₁ – BD₃ 75/25 with a slow flow rate in experiment n°3 showed, firstly, a steady flow rate throughout the filtration; secondly, they bested by far any other condition regarding the inlet pressure which was non-existent or at most under 0.5 bars; thirdly, these conditions reached the 100 L/m² target with a post depth filtration turbidity just equal or slightly higher than 20 NTU which is also an outperformance of the AD₁ – AD₂ (control) conditions.

BD₁ – BD₃ 75/25 slow flow rate condition did not give any positive nor negative impact on depth filtration performance as the turbidity and inlet pressure profiles are globally similar to the ones from the same condition with a “standard” flow rate from experience n°2 and n°3. The observation is strengthened with AD₁ – AD₂ slow flow rate and AD₁ – AD₂ 75/25 slow flow rate conditions whose profiles did not show drastic differences with AD₁ – AD₂ control

condition. This, overall, demonstrates that the flow rate applied to the depth filtration setup does not impact the depth filtration performance and is solely an indicator of this performance when measured after the filters.

$B_{D1} - B_{D3}$ 75/25 is clearly the best candidate to be an alternative depth filter combination for the clarification process of the molecule. Yet, the choice of this conditions depends on its impact on PQA.

b. Subsequent membrane filtration

Regarding experiment n°2, as it is the only filter combination that gave encouraging results, only $B_{D1} - B_{D3}$ 75/25 condition went through the rest of the clarification process. It consists in performing a last step which is a subsequent membrane filtration of the depth filtration permeate on the same filter that is used at 2000L GMP scale. After successful membrane filtration, samples were retained for PQA analysis and will be compared to the PQA of the GMP batch.

For experiment n°3, on the contrary, all conditions were successfully filtered after depth filtration using the same membrane filter used at 2000L GMP scale. Post-membrane samples were also retained in order to perform PQA analysis.

c. Titer and PQA

Figure 48 displays the titer results post membrane filtration i.e. post clarification. Focusing on the best back up-filter candidate condition, i.e. $B_{D1} - B_{D3}$ 75/25 condition. Data provided show that these filters have no negative impact on post-clarification titers. On the contrary, when compared with the GMP and all control conditions ($A_{D1} - A_{D2}$ and related conditions), $B_{D1} - B_{D3}$ 75/25 condition in both experiments as well as $B_{D1} - B_{D3}$ 75/25 low flow rate condition provided a slightly higher titer than any other conditions. This very positive results for $B_{D1} - B_{D3}$ 75/25 condition is another argument in favor of choosing this combination as back-up filters for this process.

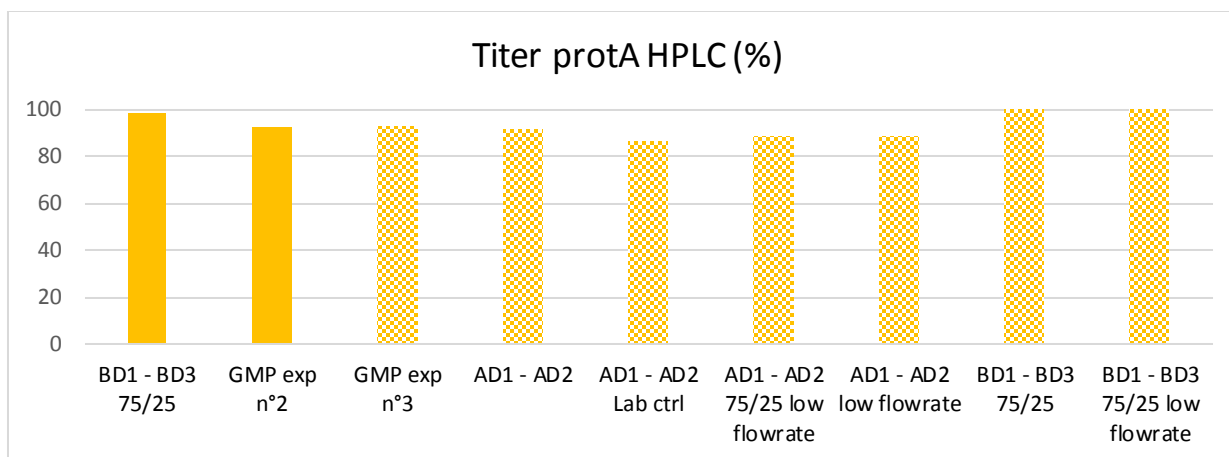


Figure 48. Case study 3. Experiments n°2 & 3 post clarification titer results. Titers were obtained using protein A HPLC analysis.

Figures 49 and **50** show SEC and iCE results respectively. The first analysis allows the investigation of potential aggregation or dissociation of the therapeutic molecule. The last one is also known as the charge heterogeneity analysis. It is focused on the characterisation of mAbs in terms of molecular isoforms. Heterogeneity can be caused by such molecular adaptations as C-terminal lysine modifications, deamidations or post translational modifications. These changes can affect biological activity, drug stability and patient safety.

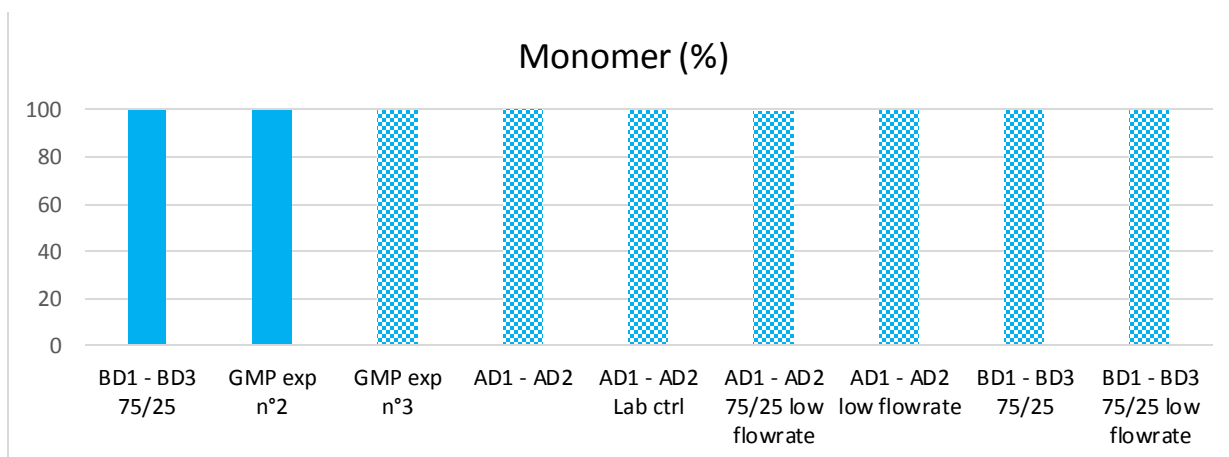


Figure 49. Case study 3. Experiments n°2 & 3 SEC results. The SEC analysis gives the proportion of monomer, aggregates (HMWS data not shown) and fragments (LMWS data not shown) of the therapeutic molecule.

Very small percentage of low molecular weight species (LMWS) and high molecular weight species (HMWS) were present compared to the main molecule (i.e. the main species) and no conditions gave an abnormal accumulation of HMWS or LMWS (data not shown). The therapeutic molecule was largely composed of acidic peak groups (APG) and main peak groups. The basic peak groups (BPG) were a minority and constituted less than 5% (data not shown).

shown). The differences between the conditions in APG were amplified with the data normalisation but were less than 1%.

Therefore, there are no significant difference regarding both SEC and iCE for B_{D1} – B_{D3} 75/25 condition and its related condition (B_{D1} – B_{D3} 75/25 low flow rate). As a matter of fact, none of the conditions demonstrates any positive or negative difference when compared with the GMP condition and control conditions (A_{D1} – A_{D2} and related conditions).

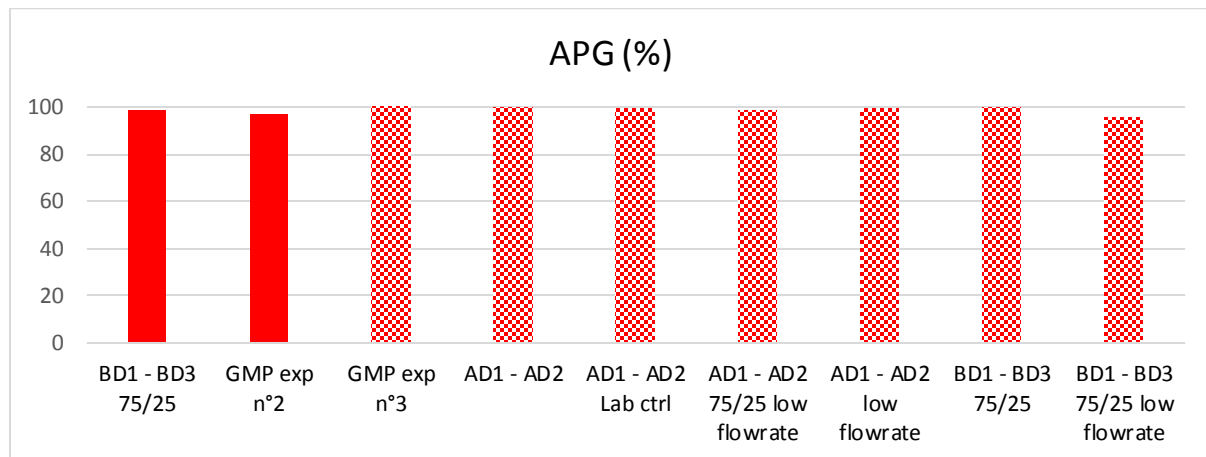


Figure 50. Case study 3. Experiments n°2 & 3 iCE results. iCE analysis gives the proportion of main (neutral) group (data not shown), acidic peak group which is the main impurity for this molecule and basic peak group which is insignificant for this molecule (data not shown).

Overall, PQA data were not impacted when using the new filter setup B_{D1} – B_{D3} 75/25. As per QbD, these filters could be validated to be the back-up filter train for this process.

1.4. Experiment n°4: membrane filtration performance

1.4.1. Design of Experiment

Initially, the centrate collected after the disc stack centrifugation step, was filtered on the current depth filter train (A_{D1} – A_{D2}) at bench scale. The depth filters had for filtering area 250 cm² which was chosen to be more extensive in order to provide enough material for the membrane filter assessment. The obtained permeate post depth filtration, was divided for membrane filter sizing afterwards. Three membrane filters were tested (A_{M1}, A_{M2} and B_{M1}) and compared to the membrane filter used at 2000L scale: C_{M1}.

For this membrane filtration experiment,

- **Initial filtration flow rate:** 2875 to 16428 LMH.
- **Monitored parameters:** inlet pressure, permeate weight.

- **Threshold parameters:** 2.5 bar maximum increase and flow rate decrease.
- **Target:** 200 L/m².

Turbidity after depth filtration was at 21 NTU.

After membrane filtration, samples were retained in order to perform titer and PQA analysis: SEC and iCE.

1.4.2. Results

a. Membrane filtration performance

The bulk permeate turbidity (post membrane filtration) and inlet pressure were monitored. Results are presented in **figure 51** & **Table 9**.

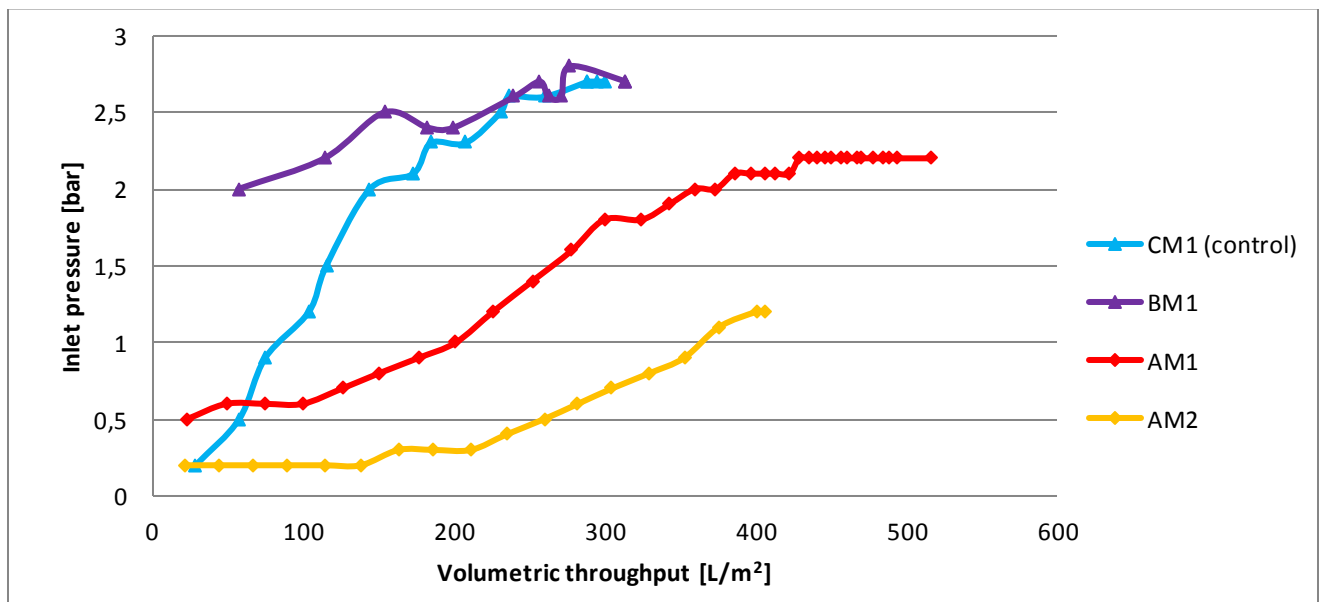


Figure 51. Case study 3. Experiment n°4. Inlet pressure (bar) in function of volumetric throughput (L/m²). Data was collected using a pressure gauge connected after the pump and prior the filter.

No filters were blocked too early and the feed rates were acceptable at the end of the filtration (over 2000 LMH). In addition, all filters gave a similar turbidity at the end of the filtration (data not shown). AM₁ and AM₂ had the better filterability performance with a volumetric throughput beyond the 200 L/m² target: 517 and 407 L/m² respectively. The inlet pressure at the end of the experiment for both filters did not exceed 2.2 bars which is tolerable for the device put in place. Filters from suppliers A and B are both good candidates for the membrane filtration step as they reached the minimum target for scale-up (200 L/m²). However, filters from supplier A seem best as they outperformed the control (C_{M1}) by

far. Filtration capacity aside, the CCCF quality must be assessed before determining if the membrane filters are acceptable as a back-up for the process.

Membrane filter	Volumetric Throughput L/m ²	Feed rate at the end (LMH)	Inlet pressure at the end (bar)
C _{M1} (control)	301	2254	2.7*
A _{M1}	517	2045	2.2
A _{M2}	407	2791	1.2
B _{M1}	314	3328	2.7

Table 9. Case study 3. Experiment n°4. Performance of the membrane filters AM1, AM2 and BM1 in comparison with the current membrane filter. * Pressure beyond the 200L/m² target.

b. Titer and PQA

Titer, monomer (SEC analysis) and APG (iCE) results are shown in **Figures 52, 53** and **54** respectively.

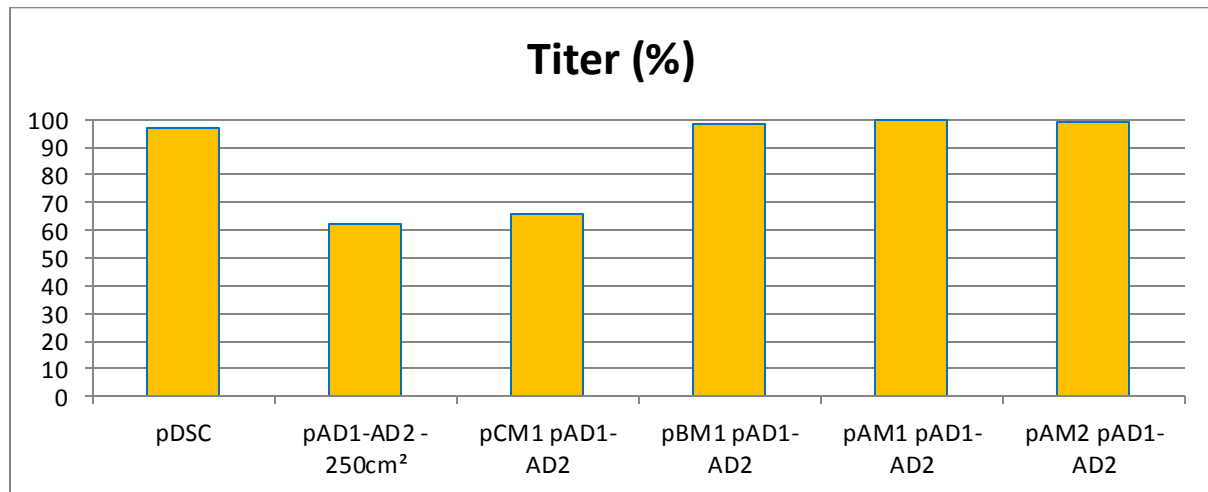


Figure 52. Case study 3. Experiment n°4. Titer post centrifugation, post depth filtration and post membrane filtration for the different membrane filters tested.

In order to assess the influence of the filters on the product yield, titers after all filtration conditions were compared to the titers after centrifugation. The normalised titer values are shown in **Figure 52**. It has to be noted that titer measurements come with a 15% margin of error. All titer values ranged from 96.6 to 100 % except for two conditions: after the depth filtration train A_{D1} - A_{D2} used to filter the material for the membrane filters assessment and after C_{M1} used as control. These low titer values were certainly due to measurement errors because the filtration train A_{D1} - A_{D2} is currently used at large-scale and has already been evaluated for this process. All membrane filters tested in this experiment have no concrete influence on product titer.

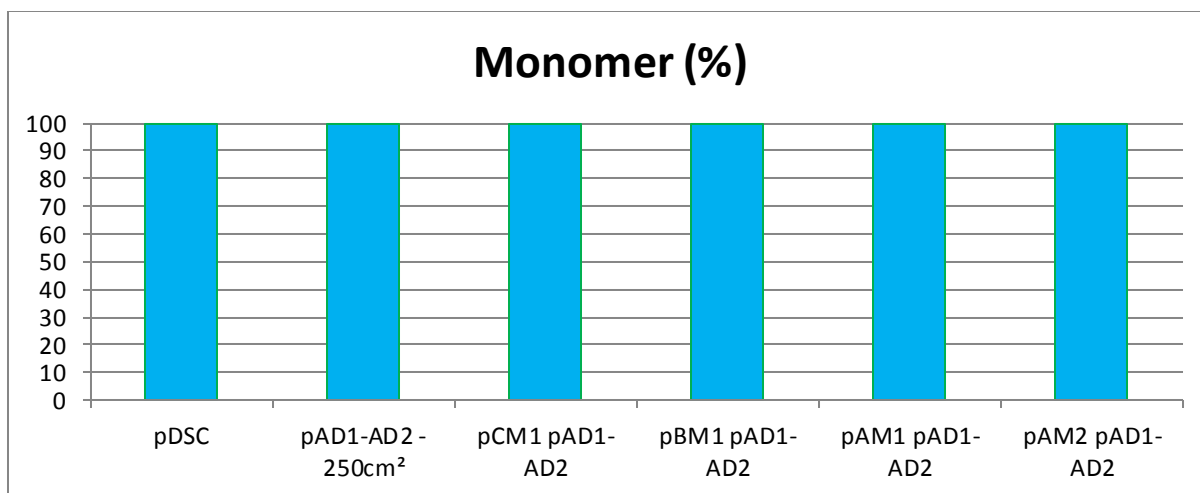


Figure 53. Case study 3. Experiment n°4. SEC results for post centrifugation, post depth filtration and post membrane filtration for the different membrane filters tested. The SEC analysis gives the proportion of monomer, aggregates (HMWS, data not shown) and fragments (LMWS, data not shown) of the therapeutic molecule.

Very small percentage of LMWS and HMWS were present compared to the main molecule (main species) and no conditions gave any higher accumulation of HMWS or LMWS (data not shown). The various filters tested have therefore no concrete influence on the amount of aggregated or dissociated product.

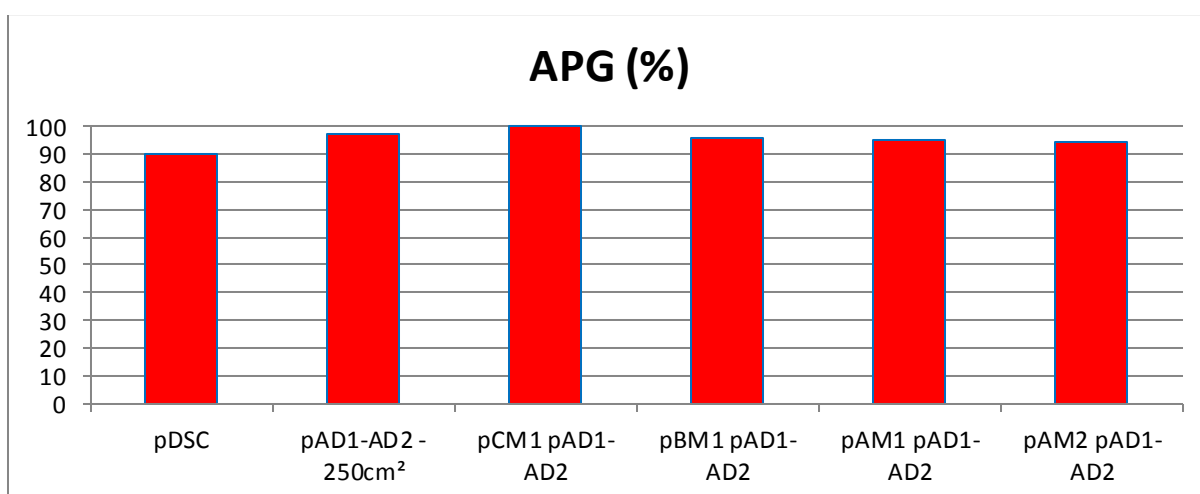


Figure 54. Case study 3. Experiment n°4 ICE results for post centrifugation, post depth filtration and post membrane filtration for the different membrane filters tested. ICE analysis gives the proportion of main (neutral) group (data not shown), acidic peak group which is the main impurity for this molecule and basic peak group which is insignificant for this molecule (data not shown).

The therapeutic molecule was largely composed of APG and main peak groups. The BPG were a minority and constituted less than 5%. The differences between the conditions in APG are amplified with the data normalisation but were less than 3%. The membrane filters tested did not influence the proportion of APG post membrane filtration.

Overall, PQA data were not impacted when using the alternative membrane filters. As per QbD, these filters could be validated to be the back-up filter for this process. Furthermore, A_{M1} and A_{M2} filters are the best candidates.

1.5. Case study conclusion

To summarise, the filtration capacity of all membrane filters and depth filters $B_{D1} - B_{D3}$ 75/25 were of appropriate for scale-up but membrane filters from supplier A were the best candidates in term of performance.

The results provided by these three experiments (n°2, 3 and 4) showed that the CCCF quality is acceptable. The cost of the filters should be taken into account for selecting the final second membrane filter supplier.

However, except for the depth filter condition $B_{D1} - B_{D3}$ 75/25 which was tested twice (in experiment n°2 and 3), these filtration performance, titer and PQA results must be taken with caution because only one experiment was done using the membrane filters. As a consequence, additional tests should be performed in order to confirm filtration performance titer and PQA results and further select the membrane filter to be chosen among the two tested from supplier A (most likely) or B.

Another necessary step is to retest the depth filter setup $B_{D1} - B_{D3}$ 75/25 and use the provided post depth filtration material to subsequently filter it using the membrane filters from supplier B and A. The main purpose of this experiment will be to assess the impact of $B_{D1} - B_{D3}$ 75/25 depth filters on the subsequent candidate membrane filters to confirm filtration performance titer and PQA results.

2. Case study 4: Cell culture fluid pre-clarification treatment comparison

2.1. Purpose

This case study focuses on the cell culture fluid treatment prior to the harvest with the objective of enhancing harvest performance. The harvest process in place was already consisting of a harvest pre-treatment step with a flocculating agent: pDADMAC polymer. Previous centrifugation and harvest development and optimisation had been performed in order to implement such treatment. However, the use of any additional treatment is a challenge as it is another impurity that needs to be purified hence it makes DSP more complex. In addition, DSP process validation must answer high standards in order to insure patient safety. It was decided to investigate other treatments particularly acid precipitation with the objective of enhancing the clarification performance and simplify the DSP and its validation.

2.2. Design of Experiment

An 80L run was dedicated to this study. It was divided into four batches corresponding to four conditions:

- 20L not treated, control
- 20L acid precipitated
- 40L flocculated (divided in two)

The flocculated batch was then subdivided into two 20L batches, a best case and a worst case depending on centrifugation conditions: the best case was centrifuged as per standard parameters described in the material and methods section whereas the worst case flocculated CCF was centrifuged at a higher bowl speed. The aim of the worst case condition was to evaluate the impact of poor centrifugation conditions on clarification performance. A higher bowl speed exposes the cells to higher forces of shear which generate small particles that cannot be removed from liquid using centrifugation. Therefore, it has a negative effect on yield, separation and clarification efficiency. Flocculation and acid precipitation were performed as per the procedure described in the material and method section.

After centrifugation, all batches undergone a conventional harvest i.e. depth filtration with two depth filters: DF₁ and DF₂ followed by membrane filtration with MF₂ for the control (0.2

µm pore diameter) and MF₁ for treated conditions (0.1 µm pore diameter). In case of filter blockage with MF₁, MF₂ filters were used.

2.3. Results

2.3.1. Impact of centrifugation

Figures 55 and **56** show the online turbidity of the different conditions during disc stack centrifugation and the off-line turbidities of the bulk centrate post-centrifugation.

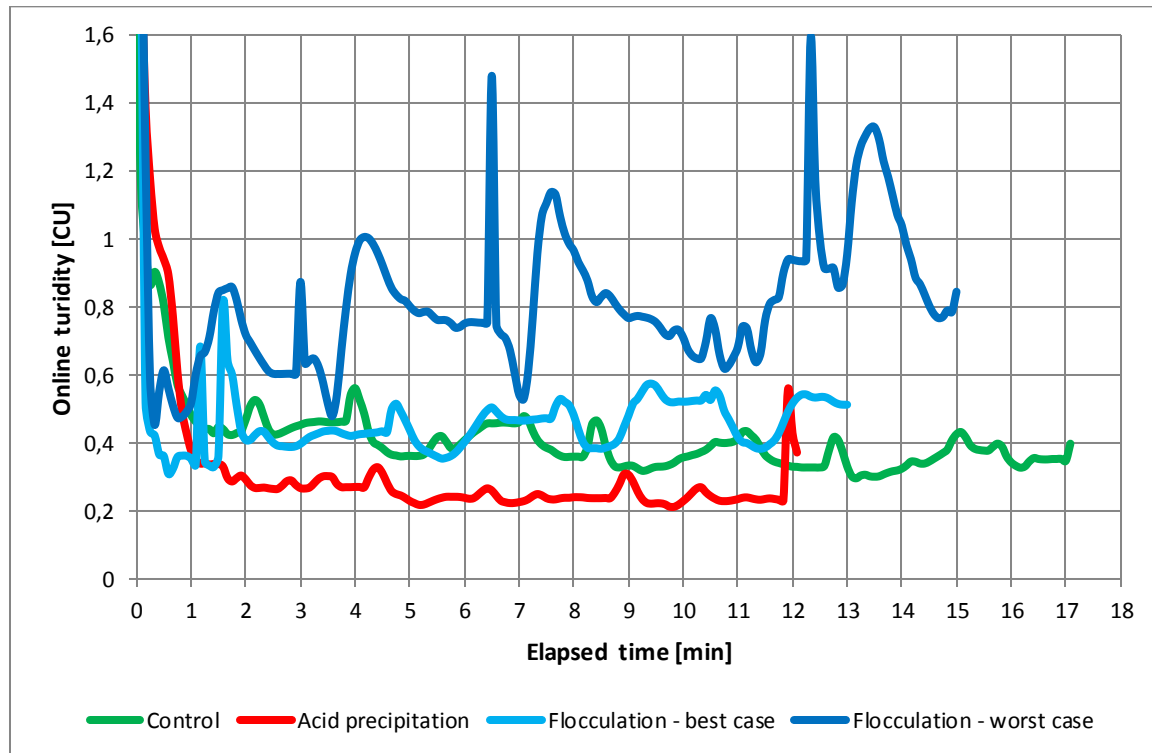


Figure 55. Case study 4. Online turbidity data obtained from centrifuge. Turbidity is in CU units.

The centrate turbidity for the acid precipitation treatment condition is lower than for the untreated material during the centrifugation step as well as regarding the post centrifugation bulk turbidity. It seems that the flocculation treatment does not improve post

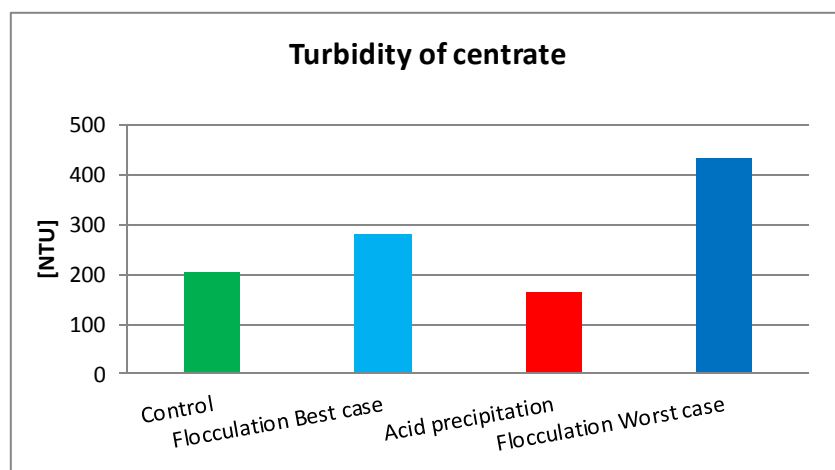


Figure 56. Case study 4. Turbidity values after disc stack centrifugation (in CU units).

centrifugation turbidity compared to the control condition. As anticipated, the worst case centrifugation for the flocculation treatment resulted in the highest centrifugation turbidity profile as well as post centrifugation turbidity.

2.3.2. Filter performance

Figure 57 displays the results of depth and membrane filtration for all conditions of the experiment. Acid precipitation treatment doubled the depth filtration volumetric

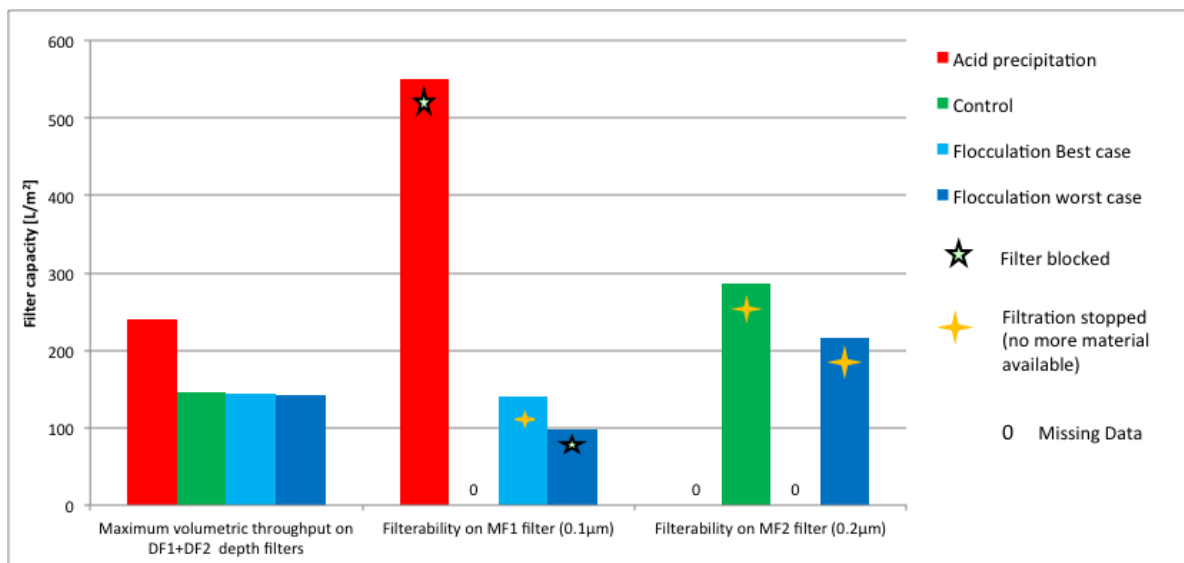


Figure 57. Case study 4. Filter capacity results for depth and membrane filtration.

throughput whereas the two flocculation conditions did not impact the volumetric throughput compared to the control. Concerning the membrane filtration, the acid precipitation treatment enabled filtration with smaller pore size than with the untreated material (0.1 µm instead of 0.2 µm with the control). In addition, the volumetric throughput for membrane filtration was 5 fold higher for the acid precipitated material than it was for the worst case flocculated one before blocking. On the one hand, best case flocculated material was membrane filtered with MF₁ however there was not enough material to say if or when the membrane filtration would have blocked. On the other hand, the worst case flocculated material blocked MF₁ filter and had to be filtered with MF₂.

Figure 58 shows that the post depth filtration turbidity was higher for the worst case flocculation condition than it was for the best case. This resulted in a lower membrane filtration capacity in MF₁ which suggest that high speed bowl during centrifugation can impair clarification efficiency. The lowest turbidity value after depth filtration was obtained

for best case flocculation treatment however, compared to the control, filter capacity was not improved.

Acid precipitation condition resulted in a slightly higher turbidity post depth filtration than the best case flocculation but its value was still half of the control turbidity. Finally, the acid precipitation treatment enabled to outperform by far all conditions in terms of filter capacity for both depth and membrane filtrations.

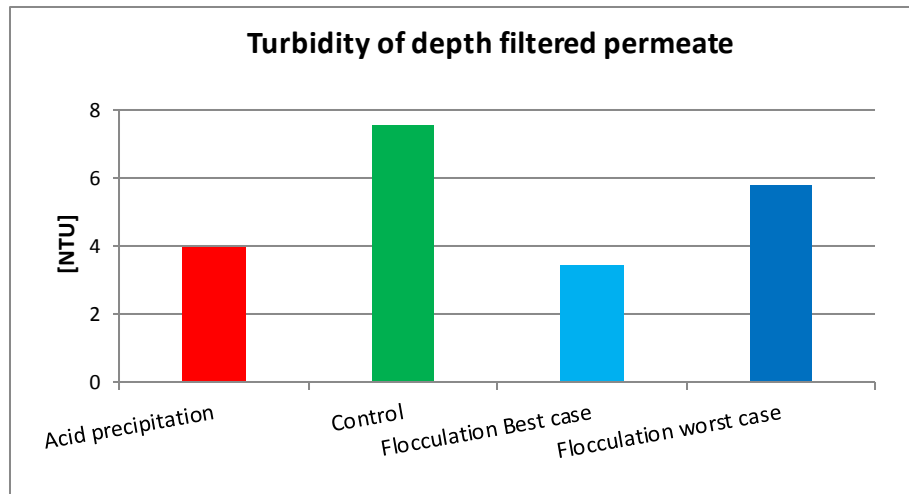


Figure 58. Case study 4. Turbidity results post depth filtration (in NTU).

2.3.3. Product recovery and PQA

After membrane filtration, samples were retained for analysis. Titer results displayed in

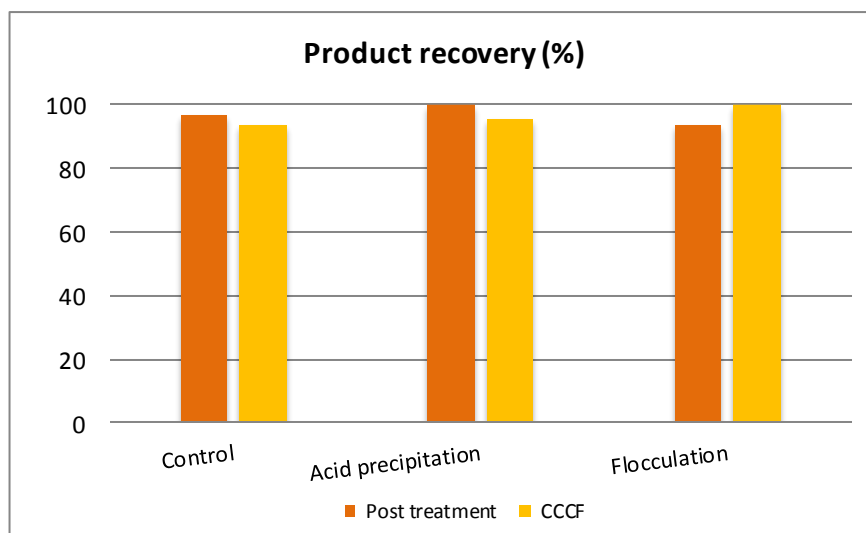


Figure 59. Case study 4. Product recovery results based on protein A HPLC analysis.

Figure 59 show that no product was lost after clarification neither for the acid precipitated

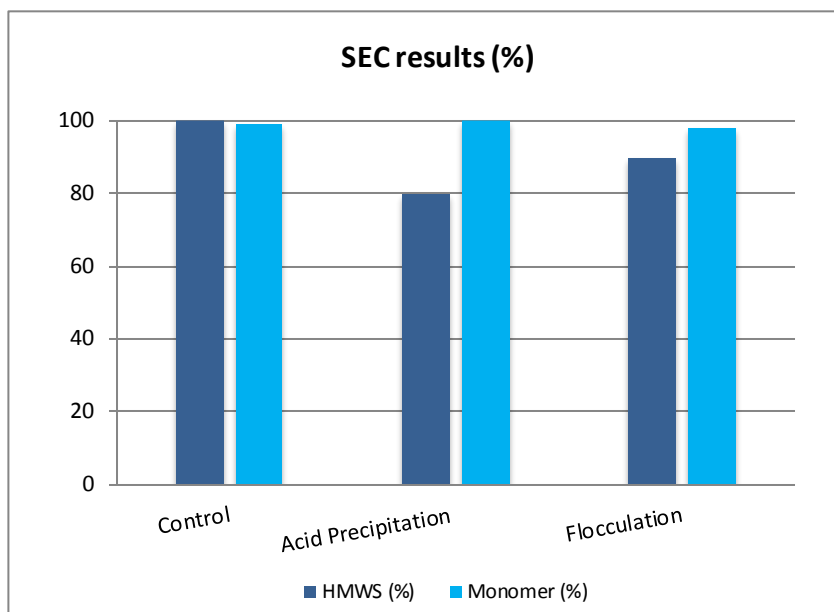


Figure 60. Case study 4. SEC analysis results showing distribution of the main molecule and HMWS.

material nor for the flocculated one. Thus, none of the pre clarification treatments tested

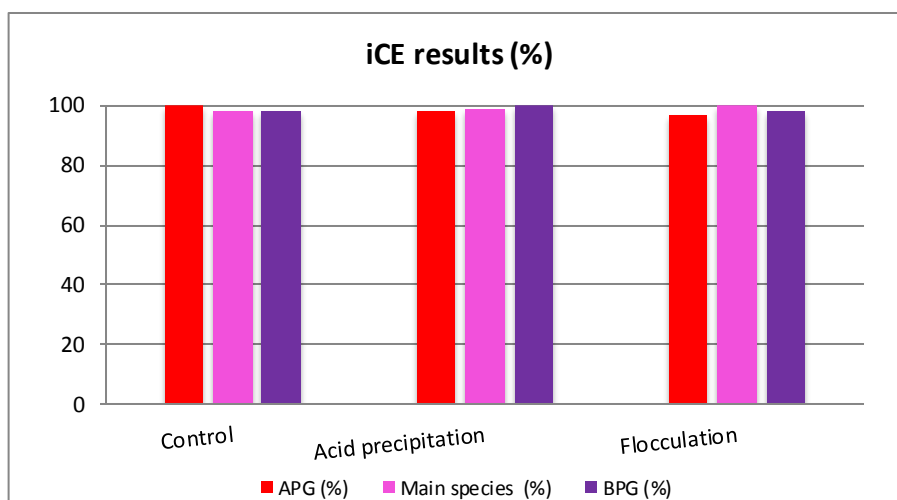


Figure 61. Case study 4. iCE analysis results showing the distribution between the main species i.e. the therapeutic molecule, APG and BPG.

have an impact on product titer.

Figures 60 and **61** show SEC and iCE results respectively. The first analysis allows the investigation of potential aggregation or dissociation of the therapeutic molecule. The second one is also known as the charge heterogeneity analysis. For both acid precipitation and flocculation conditions monomer and main species are similar to the control. Moreover,

HMWS, APG and BPG level did not increase. Therefore, both CCF treatments have no impact on product titer and PQA.

2.4. Case study conclusion

Titer and PQA analysis not only confirmed that the previously optimised and endorsed flocculation treatment does not affect PQA and product titer but also demonstrated that the alternative treatment tested i.e. acid precipitation does not have an impact either. As a result, the choice of the treatment depends on the filtration performance. In this regard, acid precipitation treated fluid gave, by far, the best results in term of depth and membrane filtration capacity. Furthermore, this treatment reduces by half the turbidity post depth filtration compared to the control condition (untreated material).

With these elements, the acid precipitation pre clarification treatment could be chosen. Yet, other parameters such as the subsequent purification process have to be taken into consideration.

Conclusion

In the last twenty years, there has been a significant expansion in the number of biotechnology-based drugs development and commercialised. In particular, monoclonal antibodies and fragment antibodies produced by CHO cell cultures have become a leading therapeutic class enabling treating rare and severe diseases.

Developing a new drug is a high-risk investment for a company as only one lead out of 10,000 molecules is marketed and its development is long and expensive. In addition, the increasing requirements from authorities during trials and prior giving market authorisation, the time limited-patent and the emergence of biosimilar are all challenges to be addressed by companies developing new biological entities.

The platform process approach has enabled to face such competitive requirements. Nowadays, mAbs production platform are mostly based on fed-batch mode and have led to high cell density cultures with titer achievement as high as 13 g/L(20). Such efficient cell culture processes require more optimisation to be scalable and ensure patient's safety.

Drug development combines preclinical and clinical trials with process development. Process development follows cell line development and engineering. Process is optimised and fine-tuned, scaled-up, characterised and validated for manufacturing. The four case studies were selected to present different aspects of both cell culture step and clarification step of process development in the context of a company developing NBEs.

Case studies 1 and 2 focused on cell culture process development but at two different stages. The first one was about the fine-tuning of a cell culture process by testing a new aeration cascade which enabled to have a more representative 2L scale-down model of the process. The second case study was an example of the start of a new molecule process development facing a short timeline for the first GMP batch production. The feeding strategy was optimised at 80L scale directly and a scale comparison (10L vs 80L) was performed to demonstrate the scalability of the process for its implementation at GMP scale.

Developing and optimising the primary recovery process has become critical as high cell density and high producing cultures are more challenging to clarify and harvest directly

impacts both product yield and quality. Case studies 3 and 4 both succeeded in selecting back-up filters for a process and enhancing centrifugation & filtration performance of another process respectively.

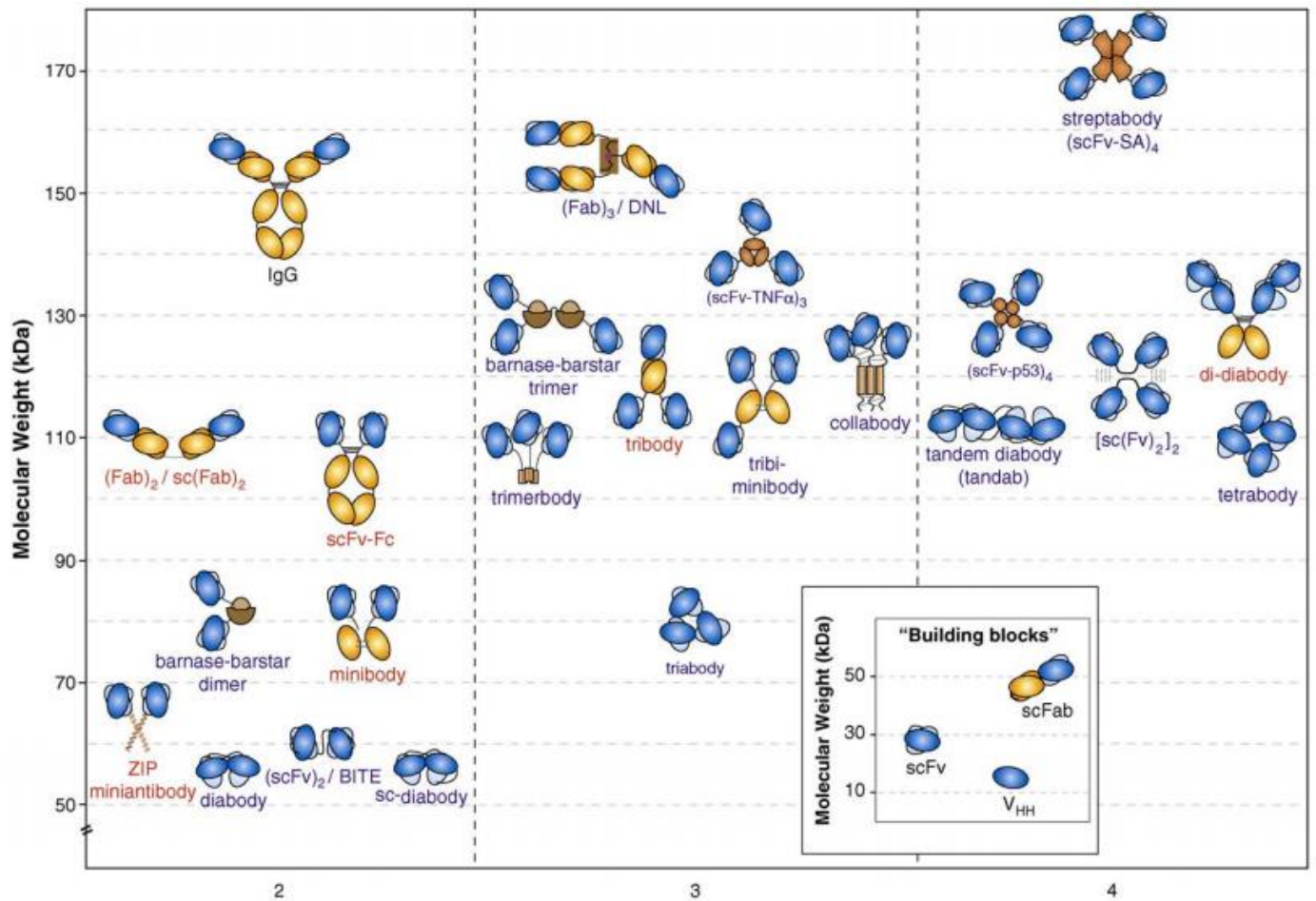
All experiments presented were designed based on the concept of quality by design which places the quality attributes of the therapeutic molecule at the center of the development. All decisions are made based on the impact on product quality. Holistic and systematic approaches such as QbD and production platforms enabled achieving positive results and improvements of the processes respecting the competitive timelines and requirements.

Other trends which have potential to simplify and accelerate process development are being investigated. The rise of disposable systems represents an alternative which offers several advantages such as reducing costs, shortening development times and process preparation time (assembly, dismantlement, cleaning), reducing risk of cross contamination, reducing the impact on environment and giving more flexibility (plug and play). Continuous processes could also give such benefits but the improvements would be the greatest if implemented for harvest and purification processes which represent the highest operational costs.

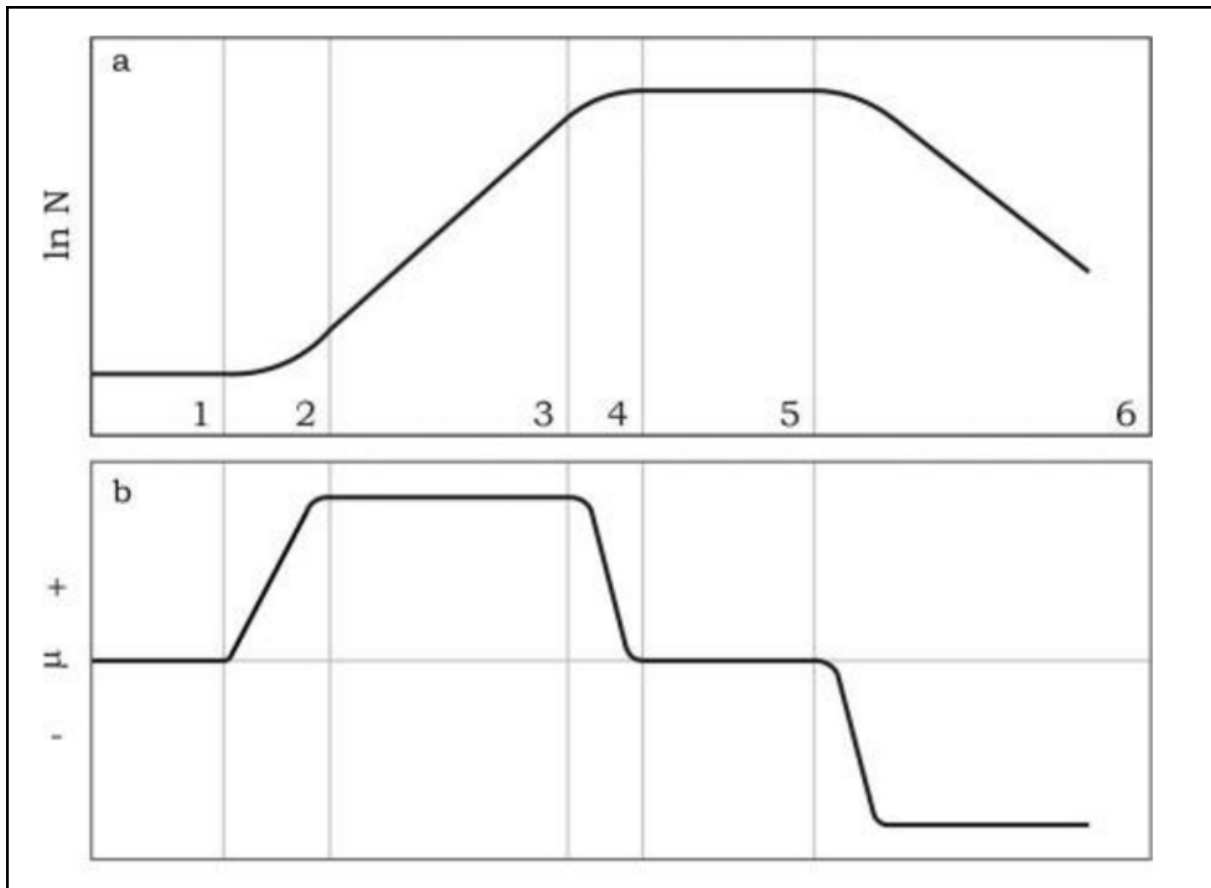
Appendix

Product	Company	Therapeutic group	Nature	Market share (%)
HUMIRA	ABBVIE	Antirheumatic	mAb	1.3
LANTUS	SANOFI	Antidiabetic	Recombinant protein	1.1
SOVALDI	GILEAD SCIENCES	Antihepatitis C	Chemical	1.0
ABILIFY	OTSUKA	Neuroleptic	Chemical	1.0
ENBREL	PFIZER	Antirheumatic	Recombinant protein (IgG1 fused with TNF receptor at constant end.	0.9
SERETIDE	GSK	Antiasthmatic	Chemical	0.9
CRESTOR	ASTRAZENECA	Anticholesterolemic	Chemical	0.9
REMICADE	MSD	Antirheumatic	mAb	0.9
NEXIUM/IN EXIUM	ASTRAZENECA	Antiulcer	Chemical	0.8
MABTHERA	ROCHE	Antineoplastic	mAb	0.7
Total				9.5

Appendix 1. 10 drugs most sold in 2014(5).



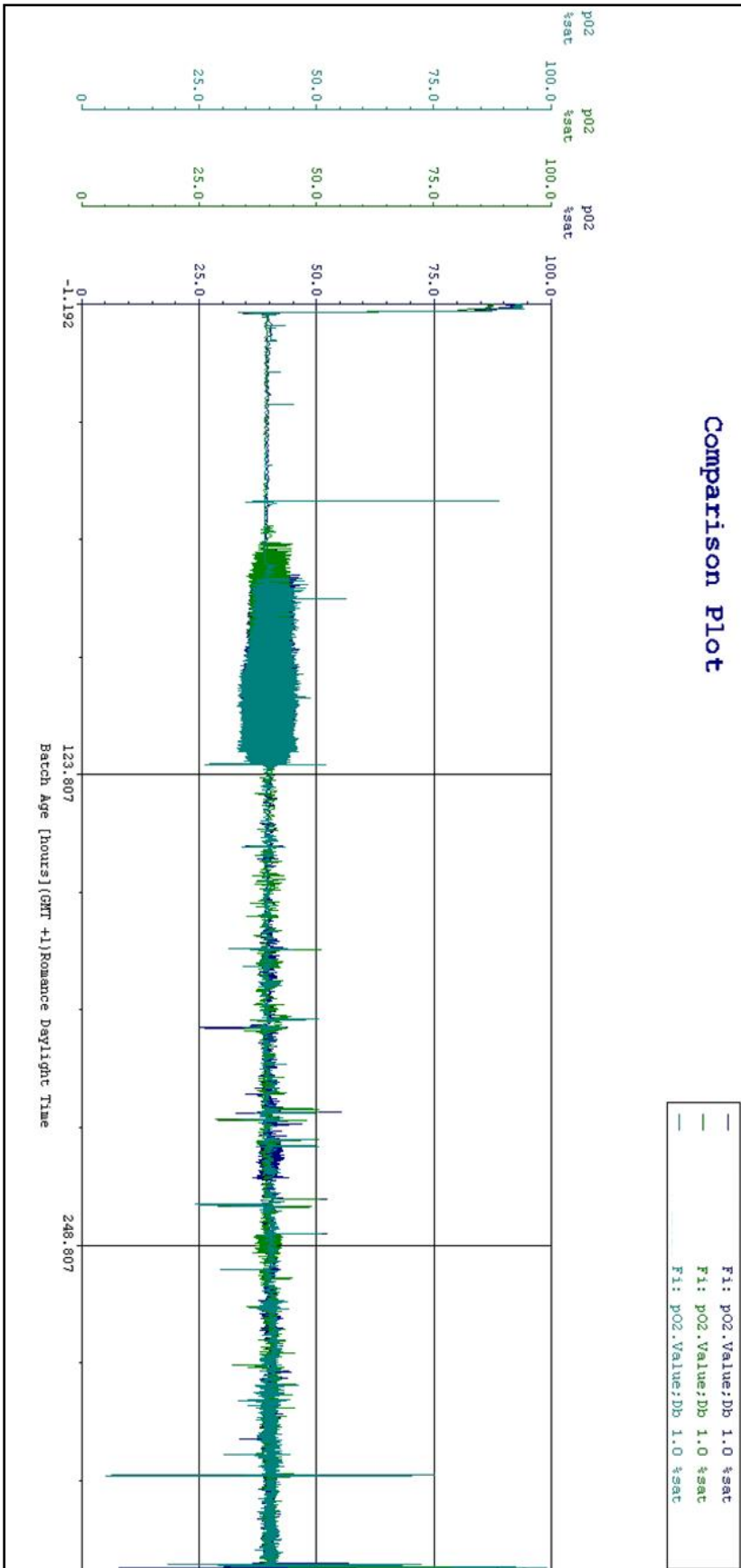
Appendix 2. Recombinant multimeric antibodies classified as either 'orthodox' (IgG-like) or 'heterodox' (non-IgG-like) formats according to their similarity to the native IgG.



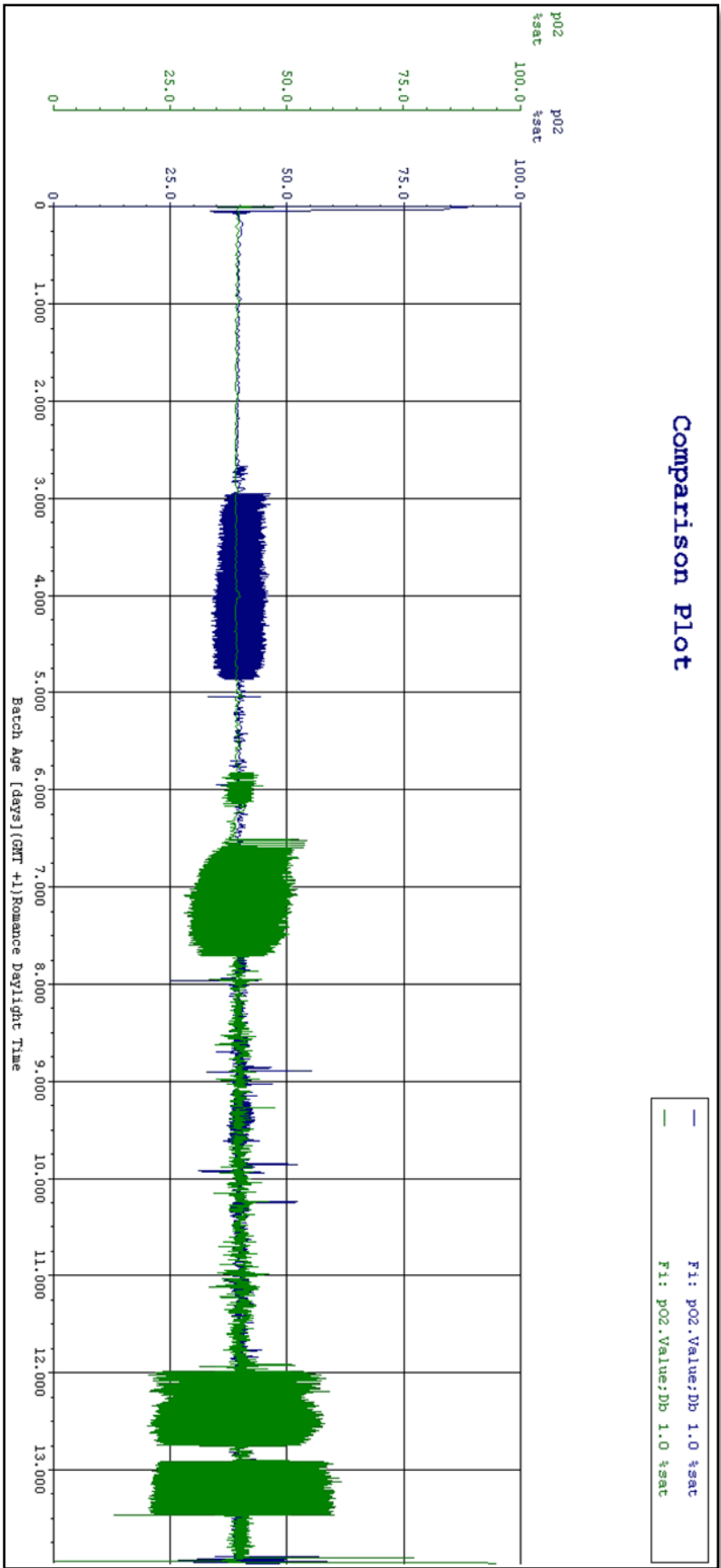
Appendix 3. Growth phases in batch mode.

(http://www.eplantscience.com/index/algae/algal_culturing/batch_cultures.php)

1. Lag phase: cells are adapting to their new environment/medium e.g. synthesising enzymes for the new substrate and replicating DNA preparing cell division; their growth rate μ is almost equal to zero.
2. Acceleration phase: Cells have adapted and start replicating. μ is increasing.
3. Exponential phase: growth rate is maximum and constant whereas death rate is equal to zero.
4. Retardation phase: Nutrients are being exhausted and wastes accumulating; μ is decreasing.
5. Stationary phase: $\mu=0$; cells replicated replace dead cells.
6. Decline phase: Cell death prevails because of a lack of substrate and an accumulation of toxic metabolites: μ is negative.



Appendix 4. Dioxygen regulation for bioreactor 1, 2 and 3 (controls) of SDM experiment n°2.



Appendix 5. Dioxygen regulation for bioreactor 1 (control) in blue vs bioreactor 4 (high agitation + optimised cascade) of SDM experiment n°2.

Bibliography

1. Calo-Fernández B, Martínez-Hurtado JL. Biosimilars: Company Strategies to Capture Value from the Biologics Market. *Pharmaceuticals*. 2012; 5(12):1393–1408.
2. Mahmoudian M. Biocatalysis: The Road Ahead. *Org Process Res Dev*. 2011; 15(1):173–174.
3. Sylvie Latieule. La 13e fusion. 2011; Available from: <http://www.industrie.com/pharma/la-13e-fusion,38665>
4. Aurélie Dureuil. Marché biotech : « Tous les indicateurs sont au vert ! ». 2015; Available from: <http://www.industrie.com/pharma/marche-biotech-tous-les-indicateurs-sont-au-vert,66034>
5. Leem. Bilan économique des Entreprises du Médicament - édition 2015 [Internet]. 2015. Available from: <http://www.leem.org/bilan-economique-des-entreprises-du-medicament-edition-2015>
6. Wurm FM. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat Biotechnol*. 2004; 22(11):1393–1398.
7. Selections From FDLI Update Series on FDA History - Celebrating a Milestone: FDA's Approval of First Genetically-Engineered Product [Internet]. Available from: <http://www.fda.gov/AboutFDA/WhatWeDo/History/ProductRegulation/SelectionsFromFDLIUpdateSeriesonFDAHistory/ucm081964.htm>
8. Walsh G. Biopharmaceutical benchmarks 2014. *Nat Biotechnol*. 2014; 32(10):992–1000.
9. Ghaderi D, Zhang M, Hurtado-Ziola N, Varki A. Production platforms for biotherapeutic glycoproteins. Occurrence, impact, and challenges of non-human sialylation. *Biotechnol Genet Eng Rev*. 2012; 28:147–175.
10. Loureiro LR, Carrascal MA, Barbas A, Ramalho JS, Novo C, Delannoy P, et al. Challenges in Antibody Development against Tn and Sialyl-Tn Antigens. *Biomolecules*. 2015; 5(3):1783–1809.
11. Cogné M, Duchez S, Pascal V. [Transgenesis and humanization of murine antibodies]. *Médecine Sci MS*. 2009; 25(12):1149–1154.
12. Bellet D, Dangles-Marie V. Anticorps humanisés en thérapeutique. *MS Médecine Sci*. 2005; 21(12):1054–1062.
13. Marasco WA, Sui J. The growth and potential of human antiviral monoclonal antibody therapeutics. *Nat Biotechnol*. 2007; 25(12):1421–1434.
14. Olafsen T, Wu AM. Antibody vectors for imaging. *Semin Nucl Med*. 2010; 40(3):167–181.

15. Cuesta AM, Sainz-Pastor N, Bonet J, Oliva B, Alvarez-Vallina L. Multivalent antibodies: when design surpasses evolution. *Trends Biotechnol.* 2010; 28(7):355–362.
16. Kelley B. Industrialization of mAb production technology The bioprocessing industry at a crossroads. *mAbs.* 2009;1(5):443–452.
17. BROU Paul R. Jr. CULTURE DE CELLULES ANIMALES DANS L'INDUSTRIE PHARMACEUTIQUE: PRODUCTION DE PROTEINES THERAPEUTIQUES D'UNE PETITE ECHELLE A UNE ECHELLE INDUSTRIELLE [Internet]. 2014 p. 43–45. Available from: http://hola.univ-lyon1.fr/ipac20/ipac.jsp?session=1Y5N987335Q75.354&profile=scd&source=~!bulyoni&view=subscriptionssummary&uri=full=3100001~!363462~!0&ri=2&aspect=basic_search&menu=search&ipp=20&spp=20&staffonly=&term=CULTURE+DE+CELLULES+ANIMALES&index=.GK&uindex=&aspect=basic_search&menu=search&ri=2
18. Wurm FM. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat Biotechnol.* 2004; 22(11):1393–1398.
19. Zhou Jiang KD, Zhaohui Geng. Fed-Batch Cell Culture Process Optimization A Rationally Integrated Approach. *BioProcess International* [Internet]. 2012; Available from: http://www.bioprocessintl.com/wp-content/uploads/bpi-content/BPI_A_121003AR09_O_176240a.pdf
20. Gronemeyer P, Ditz R, Strube J. Trends in Upstream and Downstream Process Development for Antibody Manufacturing. *Bioengineering.* 2014; 1(4):188–212.
21. Li F, Vijayasankaran N, Shen A (Yijuan), Kiss R, Amanullah A. Cell culture processes for monoclonal antibody production. *mAbs.* 2010; 2(5):466–479.
22. Lu F, Toh PC, Burnett I, Li F, Hudson T, Amanullah A, et al. Automated dynamic fed-batch process and media optimization for high productivity cell culture process development. *Biotechnol Bioeng.* 2013; 110(1):191–205.
23. Talley K, Alexov E. On the pH-optimum of activity and stability of proteins. *Proteins.* 2010; 78(12):2699–2706.
24. Castilho RL, Moraes AM, Augusto EFP, Butler M. *Animal Cell Technology: From Biopharmaceuticals to Gene Therapy.* Taylor & Francis; 2008.
25. Gigout A, Buschmann MD, Jolicoeur M. The fate of Pluronic F-68 in chondrocytes and CHO cells. *Biotechnol Bioeng.* 2008; 100(5):975–987.
26. Zhu MM, Goyal A, Rank DL, Gupta SK, Vanden Boom T, Lee SS. Effects of elevated pCO₂ and osmolality on growth of CHO cells and production of antibody-fusion protein B1: a case study. *Biotechnol Prog.* 2005; 21(1):70–77.
27. Kurano N, Leist C, Messi F, Kurano S, Fiechter A. Growth behavior of Chinese hamster ovary cells in a compact loop bioreactor. 2. Effects of medium components and waste products. *J Biotechnol.* 1990; 15(1-2):113–128.

28. Kandula AASJR. Harvest and Recovery of Monoclonal Antibodies from Large-Scale Mammalian Cell Culture [Internet]. Available from: <http://www.biopharminternational.com/harvest-and-recovery-monoclonal-antibodies-large-scale-mammalian-cell-culture>
29. Concerning Mechanisms of Particle Removal by Filters. In: Filtration and Purification in the Biopharmaceutical Industry, Second Edition [Internet]. CRC Press; 2007 p. 81–149. Available from: <http://dx.doi.org/10.3109/9780849379543-6>
30. GeorgeT Quigley. Prefiltration in Biopharmaceutical Processes. In: Filtration and Purification in the Biopharmaceutical Industry, Second Edition [Internet]. CRC Press; 2007 p. 1–21. Available from: <http://dx.doi.org/10.3109/9780849379543-2>
31. Protein Adsorption by Polymeric Filters. In: Filtration and Purification in the Biopharmaceutical Industry, Second Edition [Internet]. CRC Press; 2007 p. 257–95. Available from: <http://dx.doi.org/10.3109/9780849379543-11>
32. Roush DJ, Lu Y. Advances in primary recovery: centrifugation and membrane technology. *Biotechnol Prog.* 2008; 24(3):488–495.
33. Du Y, Walsh A, Ehrick R, Xu W, May K, Liu H. Chromatographic analysis of the acidic and basic species of recombinant monoclonal antibodies. *mAbs.* 2012; 4(5):578–585.
34. Leem. Bilan économique des Entreprises du Médicament - édition 2015 [Internet]. 2015. Available from: <http://www.calameo.com/read/002049284682054ac2c34>
35. Lonza, Protein & Vaccine Development Services. Design, Assess and Optimize for Clinical Success [Internet]. Available from: <http://www.lonza.com/custom-manufacturing/development-technologies/protein-and-vaccine-development-services.aspx>
36. Rathore AS, Winkle H. Quality by design for biopharmaceuticals. *Nat Biotechnol.* 2009; 27(1):26–34.
37. Li F, Hashimura Y, Pendleton R, Harms J, Collins E, Lee B. A systematic approach for scale-down model development and characterization of commercial cell culture processes. *Biotechnol Prog.* 2006; 22(3):696–703.
38. Singh N, Arunkumar A, Chollangi S, Tan ZG, Borys M, Li ZJ. Clarification technologies for monoclonal antibody manufacturing processes: Current state and future perspectives. *Biotechnol Bioeng;* 2015.
39. ICH. ICH HARMONISED TRIPARTITE GUIDELINE DEVELOPMENT AND MANUFACTURE OF DRUG SUBSTANCES (CHEMICAL ENTITIES AND BIOTECHNOLOGICAL/BIOLOGICAL ENTITIES) Q11 Current Step 4 [Internet]. 2012. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q11/Q11_Step_4.pdf

40. Christian Hakemeyer. Scale down models for cell culture [Internet]. 2013. Available from:
http://www.ema.europa.eu/docs/en_GB/document_library/Presentation/2013/04/WC500142339.pdf

Résumé

Développer un nouveau médicament est un investissement à haut risque pour les entreprises car, sur les 10 000 molécules criblées, une seule sera peut-être autorisée sur le marché. Ce développement est long et coûteux : environ 12 ans et 1 milliard d'euro. En outre, les exigences accrues des autorités de santé pour accorder l'autorisation de mise sur le marché, le brevet qui est limité dans le temps ainsi que l'émergence des biosimilaires sont autant de défis auxquels une entreprise développant ses produits doit faire face.

Le développement d'un médicament combine à la fois les développements précliniques, cliniques ainsi que le développement de procédé. Ce dernier succède au développement de la lignée cellulaire.

Cette thèse porte sur le développement de procédé : l'optimisation d'un procédé transférable à grande échelle qui précède sa caractérisation et validation en vue d'une production à échelle commerciale. Quatre études de cas ont été sélectionnées pour présenter des aspects différents de développement de la culture cellulaire et de l'étape de clarification ceci dans un contexte industriel d'une entreprise développant ses propres bio-médicaments, en particulier des anticorps monoclonaux.

Deux exemples présentés portent sur l'optimisation du procédé de culture cellulaire mais à différentes étapes du processus de développement. Cela permet de démontrer l'importance de l'optimisation d'un procédé afin d'obtenir un « scale down model » 2L approprié pour la stratégie de contrôle du procédé BPF à grande échelle. Le développement du procédé d'une molécule peut également débuter avec des contraintes de temps qui amènent à la mise en place du dit procédé directement à échelle 80L en utilisant une plateforme de production. Les éléments clés à optimiser sont alors la « feeding strategy » ainsi que la robustesse du procédé (étude comparative des échelles 10L et 80L) qui sont nécessaires pour démontrer la capacité de procédé à monter en échelle en vue d'une production BPF.

Le procédé de clarification est devenu crucial en raison de cultures à haute productivité et densité cellulaires plus difficiles à clarifier. Or cette étape a un impact direct sur les rendements et la qualité du produit. Développer et optimiser ces opérations peut inclure la sélection de filtres alternatifs pour une molécule ou encore l'amélioration du rendement de centrifugation et filtration d'une autre molécule thérapeutique par addition de traitements au milieu de culture avant la récolte.

Toutes ces expériences ont été régies par le principe du « quality by design » qui place la qualité du produit thérapeutique au centre des décisions en terme de développement de procédé. Le QbD et l'utilisation de plateformes production sont des approches holistiques et systématiques qui ont permis d'atteindre rapidement les objectifs visés dans un contexte compétitif où la pression sur les coûts et les délais est de plus en plus importante.

Mots clés: Développement de procédé, Bioréacteur, culture cellulaire, CHO, Anticorps monoclonaux, Clarification, Filtration, Quality by design, Performance, Transfert d'échelle, Robustesse.

Abstract

Developing a new drug is a high-risk investment for a company as only one lead out of 10,000 molecules is marketed and its development is long and expensive: around 12 years and 1 billion Euro. In addition, the increasing requirements from authorities during trials prior giving market authorisation, the time limited-patent and the emergence of biosimilar are all challenges to be addressed by companies developing new biological entities.

Drug development combines preclinical and clinical trials with process development. Process development is performed after the cell line development and engineering step.

This thesis focuses on subsequent process development: particularly on process optimisation, fine-tuning and scale-up, which are followed by characterisation and validation for commercial manufacturing. The four case studies were selected to present different aspects of both cell culture and clarification development in the context of a company developing new biological entities, particularly monoclonal antibodies.

Examples of cell culture process development are broached but at different stages. It shows the importance of fine-tuning a cell culture process to reach a 2L scale-down model which is suitable for the fine-tuning of the control strategy of a large scale GMP process. A molecule process development can also start facing short deadlines: the strategy will be to optimise the process directly at 80L scale using a platform process. Critical optimisation checkpoints are the feeding strategy and the process scalability assessment (scale comparison between 10L and 80L) which are needed for implementing the process at GMP scale.

The primary recovery process has become a critical step as high cell density and high producing cultures are more challenging to clarify and harvest directly impacts both product yield and quality. Developing and optimising these operations can include selecting alternative filters for a process but also enhancing centrifugation & filtration performance of another process by adding a treatment to the final culture broth prior to harvesting it.

Quality by design places the quality attributes of the therapeutic molecule at the center of the development. All design of experiment and decisions have been made in regards to the impact on product quality. Platform production and QbD are both holistic and systematic approaches which enabled achieving in a short time robust results and improvements of the processes in a competitive environment where pressure on cost and timelines is always greater.

Key-words: Process development, Bioreactor, Cell culture, CHO, Monoclonal antibodies, Clarification, Filtration, Quality by Design, Performance, Scalability, Robustness.