Molecular characterisation of the effects of Poloxamer 188 on CHO cell viability

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Abstract:

The pressing demands for efficient biopharmaceutical production have necessitated a deeper understanding of the factors influencing cell culture viability, particularly within bioreactor environments. This thesis addresses these necessities by investigating the role of Poloxamer 188 (P188), a non-ionic triblock copolymer, in safeguarding Chinese Hamster Ovary (CHO) cells from mechanically induced stress, a common concern in stirred tank bioreactors.

Building on the existing knowledge that mechanical agitation can adversely affect cell survival, this study leverages a scaled-down model using baffled Erlenmeyer flasks to simulate the sparging and shearing forces prevalent in industrial bioreactors. It was observed that P188 significantly delays the onset of apoptosis in CHO cells, as indicated by the retarded appearance of cleaved caspase-3 (c-casp3). This effect is contrasted with other surfactants, which displayed variable impacts on apoptosis, dependent on their molecular weight and PEO/PPO content, underlining the specificity of P188's protective action. In order to understand how the P188 can interact with mammalian cells, a thorough assessment of its surfactant characteristics was carried out. The physicochemical characterization of P188 has proven useful because its protective efficacy is closely linked to its molecular structure and purity. If P188 is contaminated with impurities or if it interacts with other surfactants can significantly alter its surface-active properties. Enhanced surface activity of P188, due to these impurities or interactions, might adversely affect its ability to protect cells. Enhanced surface activity could lead to a quicker decline in cell viability, indicating the need for stringent quality control in surfactant manufacturing. In electroporation studies, the addition of P188 was seen to improve transfection efficiency in CHO-S cells, hinting at its role as a facilitative agent for genetic material uptake. Zeta potential measurements further substantiate the stabilising physical interactions between P188 and transfection reagents, offering a plausible explanation for the observed enhancement in transfection outcomes.

P188 emerges as a vital additive for the maintenance of cell viability in mechanically stressful environments, with its function extending beyond mere surfactant properties to include modulation of cell death and survival pathways. This study not only contributes to the fundamental understanding of surfactant behaviour in cell culture systems but also paves the way for optimising biotechnological processes in the pharmaceutical industry.

Table of contents:

List of figures:

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 8

List of tables:

List of Abbreviations:

- **P188** Poloxamer 188
- **CHO** Chinese Hamster Ovary
- **C-casp3** Cleaved Caspase 3
- **PEO** Polyethylene Oxide
- **PPO** Polypropylene Oxyde
- **ADCs Antibody**-drug conjugates
- **mAbs** Monoclonal Antibodies
- **GAPDH** Glyceraldehyde 3-phosphate dehydrogenase
- **LC3** Microtubule-associated proteins 1A/1B light chain 3B
- **HPLC** High performance liquid chromatography
- **GS** Glutamine synthetase
- **DHFR** Dihydrofolate reductase
- **AAV** Adeno-associated virus
- **CAR** Chimeric antigen receptor
- **PTMs** Post-translational modifications
- **ER** Endoplasmic reticulum
- **ERAD** ER-associated degradation
- **UPR** Unfolded protein response
- **ADC** Antibody-drug conjugate
- **DO** Dissolved oxygen.
- **CFD** Computational fluid dynamics

- **PE** Phosphatidylethanolamine
- **ROS** Reactive oxygen species
- **PVDF Polyvinylidene** difluoride
- **SDS** Sodium dodecyl sulphate
- **APS Ammonium** Persulfate
- **TBST** Tris-buffered saline
- **PBST** Phosphate-buffered saline
- **PM** Plasma membrane
- **MSC** Mesenchymal stem cells
- **YFP** Yellow Fluorescence Protein
- **XGene** Xtreme GENE

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Voglio infine dedicare il mio lavoro a due persone importanti,

ai miei Maurizi.

Author’s declaration

I declare that this thesis is a presentation of original work, and I am the sole author. This work has not previously been presented for a degree or other qualification at this University or elsewhere. All sources are acknowledged as references. The work presented in this thesis was performed between October 2018 and February 2023. Experiments were carried out in the Department of Biology, University of York, in the laboratory of Professor Nia Bryant, and in the Snaith branch of Croda, supervised by Dr James Humphrey.

Chapter 1: Introduction

1.1 Biologics in biotechnology

The advent of recombinant DNA technology has revolutionised the field of biopharmaceuticals, particularly with the rise of biologics and monoclonal antibodies (mAbs). These biotherapeutic proteins have become a big player in the treatment of various diseases, including cancers, autoimmune disorders, and infectious diseases. Biologics can be designed to mimic or modulate natural biological processes, potentially leading to fewer side effects and better patient outcomes. Central to the production of these biologics are Chinese Hamster Ovary (CHO) cells, which have emerged as the most widely used mammalian host for the industrial production of recombinant proteins (Wurm, 2004) (Huang et al., 2012).

Biologics are rather complex molecules which consist of proteins, carbohydrates, nucleic acids or a group of these substances. Examples of biologic therapeutics include antihistamines, hormones, vaccines, monoclonal antibodies, recombinant proteins, gene and cell therapies, cytokines and even insulin. Nowadays, biologics are already taking up a very important place in medicine and there is an expectation for them to become even more vital in targeting complex disorders in the future. Although today they would be routinely prescribed to patients with complex disorders and autoimmune diseases, they would have been classed as nothing less than a miracle from physicians entering the profession just a short half century ago. (Capasso, 2021)

The success in the administration of such drugs has mainly been extrapolated from the positive outcome of several diseases that were thought to be untreatable across different medical disciplines. Classic chemical pharmaceutical development involved empirical processes but nowadays there is a new forward approach which shifted towards the understanding of the disease pathology and genetic deregulations in order to make drugs which would target the molecular pathways that work abnormally in the disease (Cacabelos, 2022). Biologics generally target specific proteins or genes. The process

begins by isolating the relevant recombinant DNA from human genetic material. This DNA is then inserted into bacterial or mammalian cells, which are cultured to grow and express the target gene. After sufficient growth, the protein of interest is extracted from these cells. The extracted proteins are subsequently purified, mixed with physiological solutions, and prepared for individual dosing. (Viglio et al, 2020) (Figure 1).

A large portion of orally taken, small molecule pharmaceuticals are compounds which are organic, with a small molecular weight (0.1 - 1 kDa). They are normally produced by chemical synthesis, they have a well-defined and stable chemical structure and are thermostable, they are nonimmunogenic and can have a wide range of polarity. Biologics are immunogenic therapies as they are capable to stimulate the immune system to recognize and respond to them as foreign substances. This response can occur because biologics, often composed of proteins or other large molecules, may have structures that differ slightly from the body's own molecules, leading the immune system to produce antibodies against them. Chemically defined drugs can often be administered orally and are readily absorbed in the organism through the intestinal lining. Further permeability via the intestinal epithelium is mediated by a combination of passive diffusion and paracellular traffic (Jones and Dean, 2012) (Makurvet, 2021). Biologics tend to be much larger molecules, with a larger molecular weight (>1 kDa) than smaller drug molecules, they are polar, sensitive to changes in temperature and can easily degrade (Table 1). Biologics and larger molecular drugs have to normally be dispensed parenterally due to their instability and high molecular weight (Makurvet, 2021).

	Biologics	**Small molecule pharmaceuticals**
Synthesis	Chemical processes, relatively simple organic structures	Living organisms or cells that have been genetically engineered

Size of molecule	Relatively small (0.1 - 1 kDa)	Over 1 kDa
Selectivity	Usually bind to various off-target sites, rendering side-effects or toxicity	Highly specific to the targets
Physical factors sensitivity	Molecules are physically and thermally stable	Higher molecular instability due to size and composition
Contamination issues	Low susceptibility to contamination	Higher susceptibility to contamination
Structure of molecule	Simple structures which can be determined analytically	Complex molecular structures
Complexity	Pure chemicals	Mixture of complex molecules (excipients, impurities, by-products)
Immunogenicity	Non antigenic (mostly)	Antigenic
Absorption/ distribution	Faster metabolic absorption	Slower metabolic absorption
Business aspects	Comparatively lower price Higher competition from chemical equivalent generics Simpler research and	Higher prices for biologics therapies Less fierce competition More expensive and

	cheaper manufacturing	complex R&D

Table 1. Biologics and small molecule pharmaceutical differences. Differences Table illustrating the main differences in physical, chemical, biological and cost effectiveness between biologics and small molecules pharmaceuticals.

Almost all biologics can induce an immune response and the production of antibodies, the small molecule therapeutics are generally too small to be considered immunogenic and are not readily recognisable by the immune system. In some cases, such as vaccines or immunotherapies for cancer, the ability of biologics to stimulate an immune response is a very important benefit, as it helps the body recognize and fight infections or malignancies. When biologics, especially the antibody therapeutics, are introduced in the organism, the immune system quickly recognises the molecule and a measured immune response is stimulated (Makurvet, 2021). Given their increasing importance in therapeutic interventions and the challenges associated with their production, focusing on biologics, especially on enhancing manufacturing techniques like improving cell viability in bioreactors, is essential for advancing healthcare and meeting the growing demand for these sophisticated therapies. The study will investigate how surfactants can be utilised to mitigate cell death during the cultivation of CHO cells, which are crucial for the industrial production of recombinant proteins.

1.1.2 Biologics manufacturing in industry:

Biologics are not a novelty, human growth hormone, insulin and red blood cell agents were created many decades ago, using live cells as vessels. Since then, the therapeutic targets have vastly increased thanks to a wider knowledge of the genome, disease aetiology and molecular pathways. Many biological and chemical fields are involved in the refining of biologics synthesis. Their development relies on expertise in genomics and

proteomics, cell culture, molecular and cell trafficking pathways, the physics and chemistry of upscaling cell cultures (Malaviya and Mehra, 2018).

There are multiple steps which are involved in the process of biologics manufacturing (Figure 1). As aforementioned, biologics therapeutics have larger, more complex and less stable structures compared to chemically defined drugs. Moreover, protein-based therapeutics are made relying on living systems which require extra care and precise conditions in order to produce the maximum amount of targeted therapy, which also needs to be bioactive in humans and non-toxic. This can make the process lengthy and costly (Jones and Dean, 2012).

The manufacturing of biologics consists in four main steps (Figure 1):

1. Identifying and engineering the cell line which contains the target gene that makes the desired biologics

2. Amplify the cell culture in order to maximise the production of the desired protein

3. The protein is then isolated and purified using many different purification methods

4. The biologic drug is prepared in doses ready to be administered to patients

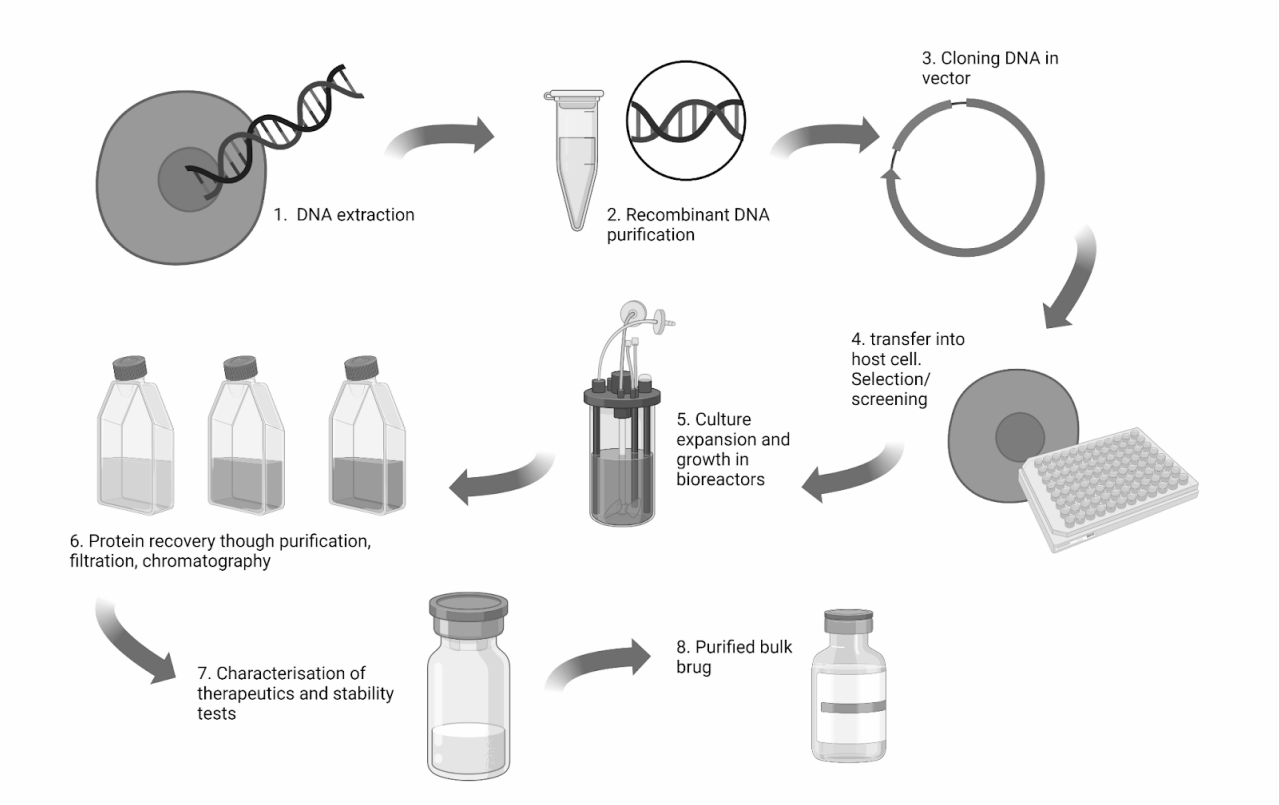


Figure 1: The steps of mAb manufacturing. The diagram illustrates the comprehensive process of biologics manufacturing, divided into eight critical steps. Step 1 involves the extraction of DNA, where the genetic material is isolated from the source organism. In Step 2, the recombinant DNA is purified to ensure it is free of contaminants. Step 3 focuses on cloning the DNA into vectors, facilitating the transfer of genetic material into host systems. During Step 4, the recombinant vectors are transferred into host cells, followed by selection and screening to identify successful transfections. Step 5 encompasses the culture expansion and growth of these genetically modified host cells in bioreactors to produce the desired protein at scale. In Step 6, the protein is recovered from the cell culture through various extraction and purification methods. Step 7 involves the characterization of the therapeutic proteins and conducting stability tests to ensure efficacy and safety. Finally, in Step 8, the purified bulk drug is prepared for formulation and eventual distribution.

The process of manufacturing the biologics starts with the identification of target DNA which will code for the protein of interest. All the new proteins that are identified have to undergo a cell-based process which can give us information on how the specific protein can change molecular mechanisms which are inhibited, over or under expressed in the phenotypes of multiple disorders (Maniadakis et al., 2018).

The protein production process continues with cloning of the relevant gene into a complementary DNA (cDNA) mammalian vector which is then transferred into a host cell (normally as E. coli). The DNA is then transfected into host mammalian cells, which are then checked and selected through expression screenings. Quality control can be achieved by gene sequencing, and the levels of expressions are also checked using techniques such as Elisa (Morrow, 2004).

Initially, multiple clones are generated by transfecting host cells with the gene of interest. These clones are screened for protein expression levels using techniques like ELISA or Western blotting. The top-producing clones are then subjected to functional assays to confirm that the protein exhibits the expected biological activity. Further assessments are conducted to evaluate the stability and quality of the expressed protein, including its post-translational modifications. The clone demonstrating the highest protein yield, correct functionality, and stability is then chosen for further development and production. Candidates of these selection are normally mammalian, yeast and bacterial cells. Mammalian cells are initially placed in plates, small flasks or petri dishes with the media and nutrients they need for growth. The scale-up process is then initiated, cells are transferred and grown in larger and larger vessels. The scale-up process starts in large volume flasks and the volume is further increased by the introduction of bioreactors. Bioreactors are large tanks of various materials and sizes (Yijuan et al., 2010) (from a minimum of 5L and can reach 20.000L vol). The conditions of growth of these cells are closely monitored at every stage. Bioreactors are environments where constant centrifuge forces and liquid turbulence is added to the cell culture in order to immit oxygen and nutrients as well as preventing cells from adhering to each other and clumping. Even small changes in this environment can cause a loss of cell viability and potentially changes in the protein that they produce (Dranitsaris, et al., 2011).

Scientists involved in the production of biologics closely monitor media environment variables such as temperature, pH, oxygen, sparging forces, sparging and nutrients concentration. It is worth remembering that biologic production is a very costly and lengthy process and in order to maximise production, it is important to prevent harmful alterations in the cell environment as well as bacterial or fungal contamination (Zhong, 2011). When cells have been successfully grown and have expressed the protein of choice, it is then isolated from the cells and the media. Different filtering methods are employed to purify the proteins. The purification revolves around discrimination by size, charge and molecular weight. Purification methods include HPLC with different bindings and elutes, ultrafiltration, bulk filtration. Once the protein has been readily purified, it is then added to sterile physiological solutions ready for injections and infusions. The final steps include packaging, labelling and distribution (Capasso, 2021).

1.2 CHO cells as a platform for biologics production

The first “biologics” to be introduced to the market were insulins and hormones to promote growth. Both were produced in cloned *E. coli* (Heinemann et al., 2022). while *E. coli* and yeast can achieve higher yields and lower costs, CHO cells are preferred for the production, CHO cells can perform complex post-translational modifications, such as glycosylation, which are crucial for the proper function and efficacy of many therapeutic proteins. E. coli cannot perform these modifications and may produce proteins that require additional processing. Yeast can perform some modifications, but they differ from those done by mammalian cells, potentially affecting the therapeutic quality and safety of the proteins produced. (Pham et al., 2019). Luckily, most eukaryotic cells are able to make these modifications, even the simplest. This is the reason why proteins of higher complexity are produced in yeast cells, which are easy to maintain and are low in cost. One of the main challenges in producing biologics in yeast is the difficulty in controlling protein expression levels, which can lead to inconsistent yields and product quality. Additionally, proteins expressed in yeast may not be fully bioactive in humans because

yeast-specific post-translational modification pathways, such as glycosylation, differ significantly from those in mammals. These differences can alter the protein's structure and function, potentially reducing its efficacy and increasing the risk of immunogenicity when used as a therapeutic. (Table 2) (Rathinam and Sani, 2019).

These are the main reasons why most of the current biologics are produced in mammalian cells, even though they are more expensive to maintain and more difficult to grow than bacterial and yeast cells. Another downside of mammalian cells is the fact that they have a relatively lower yield of recombinant proteins. Thanks to the high quality and effectiveness of biologics made in mammalian cells, the process and the prices are factored in and the business of biologic manufacturing is one of the most up and coming in the biopharmaceutical industry. The global biologics market size was USD 299.72 Billion in 2020 and is expected to reach USD 567.96 Billion in 2028. mAb such as Enbrel, Remicade, Herceptin and Humira yield a total annual revenue of over 5 billion dollars, making their production incredibly profitable and biologic research and development a very viable venture (Emergen Research, 2022).

1.2.1 History of CHO Cells

Due to a lack of mammalian cells that would survive and multiply, maintaining a stable and reliable cell line for studies of human and animal genetics, the compact genome of Chinese hamsters made them the perfect candidate in trying to produce a sturdy cell line for molecular and cell biology (Wuest et al, 2012). Initially, CHO cells were primarily used in cytogenetic studies and as a model system to study cellular physiology. Soon after, scientists recognised their potential in biomedical research: their diploid nature, ease of culture, fast growth, and large cell size, which facilitated microscopic analysis (F. M. Wurm, 2004), were all great characteristics for their future uses.

The most important role for CHO cells in biopharmaceutical production was released in the late 1970s and early 1980s. Dr. Kari Alitalo and his colleagues managed to

demonstrate the successful expression of recombinant proteins in CHO cells, taking a significant step for biomedical research. This breakthrough began a revolution for the production of therapeutic proteins with high yields and with an insured compatibility with human physiology.

The genetic stability and favourable post-translational modification of CHO cells further solidified their position as the cell line of choice for therapeutic protein production (Zhu et al 2017). The CHO cell genome was found to be stable and easy to genetically manipulate and ensuring consistent protein expression over extended culture periods. Since these early discoveries, CHO cells have played a critical role in the production of a wide range of therapeutic proteins, including monoclonal antibodies, recombinant enzymes, growth factors, hormones, and viral vectors for gene therapy.

1.2.2 Advantages of CHO Cells in Protein Production

Recombinant Chinese Hamster ovary cells (rCHO) have been a massive help to scale up production of therapeutic proteins, they can yield more than 10g/L thanks to recent advances and optimisation of cell culture technology (Meskova et al., 2023). CHO cells are by far the most commonly used mammalian cell vessel for the production of industrial-scale biologics (Kim et al., 2011). These progresses are linked to the advances in the culturing methods, environment and conditions as well as optimisation of media additives and ingredients ratios.

The vast popularity of CHO cells can be attributed to multiple factors: it is easy to obtain safety clearance from governing authorities, as these cells have been proven without reasonable doubt to be safe hosts for protein production. The low productivity of protein, typical of mammalian cells, can be overcome by gene amplification (dihydrofolate reductase (DHFR)-mediated or glutamine synthetase (GS)- mediated gene amplification). CHO cells are able to adapt to stable, serum free suspension conditions, which is vital for the upscale of industrial production. CHO cells are capable of post-translational modification and are able to produce proteins whose glycoforms are not only compatible in human organisms, but also bioactive (Kim et al., 2011). CHO cells possess similar protein synthesis mechanisms and perform essential post-translational modifications,

such as glycosylation, disulfide bond formation, and proteolytic processing, not dissimilar to human cells. This compatibility with human post-translational modifications is crucial for the efficacy, safety, and immunogenicity of therapeutic proteins produced by CHO cells, and, most importantly, their safety for human use (Wuest, 2012).

CHO cells are renowned for being able to produce high levels of recombinant proteins compared to other mammalian cells (Meskova et al., 2023). For example, in some cases, CHO cells can produce up to ten times more protein than HEK293 cells under similar conditions. The productivity advantage of CHO cells is largely due to their robustness, scalability in industrial bioreactors, and the industry's extensive experience in optimizing their use for large-scale production. They possess an efficient and robust protein expression mechanism, allowing to produce complex proteins at large commercial scales (Gray, 1997). Additionally, CHO cells offer a favourable environment for the correct folding, assembly, and secretion of recombinant proteins, contributing to their high expression yields qualities. Various strategies have been employed by researchers in this field to further enhance the productivity of CHO cells, including cell line engineering, optimization of culture conditions (like cell media studies), and development of novel expression vectors. These efforts have resulted in significant improvements in protein yields, making CHO cells a preferred choice for industrial-scale production.

CHO cells also offer exceptional scalability, allowing for the production of therapeutic proteins on a large scale. The ability to cultivate CHO cells in large multi-liter bioreactors and scale up the production process makes them highly suitable for meeting the demands of the biopharmaceutical industry, especially now that demand is higher than ever. Moreover, the use of CHO cells as a production host has gained regulatory acceptance, with many CHO cell-derived therapeutics receiving approval from regulatory agencies worldwide. This regulatory acceptance further solidifies the position of CHO cells as a reliable and well-established platform for therapeutic protein production. CHO cells maintain genetic stability and product consistency over prolonged culture periods and multiple passages, which is vital for commercial production where consistency and quality are paramount (Lee et al., 2009).

	Advantages	Yield	Disadvantages
Bacterial cells	Cost effective. High quantities of proteins Easy to culture	Tens to hundreds of gramps per litre. Very high yields; lacks post-translational modifications like glycosylation.	Unable to modify proteins post-translationally.
Yeast cells	Can express more complex proteins. Can form disulfide bridges. Low cost	Tens of grams per litre. High yields; can perform some modifications, but different from mammalian cells.	Hard to control protein expression. Different pathways compared to mammalian cells
Chinese Hamster Ovary cells	Can express most proteins. Can be made to increase their production when genetically engineered. Resilient cell line	Up to several grams per litre, Lower yields compared to microbial systems, but capable of complex modifications.	High maintenance costs Low protein yield Complex upkeep

Table 2. Live cells for production of recombinant therapies. This table illustrates the advantages and disadvantages of different types of live cells for the production of biologic therapies.

The useful characteristics of CHO cells as well as their high adaptability and resilience, make them likely to continue to be the top candidates for the industrial production of biologics. Fast progress in the optimisation of their production and conditions is and will continue to be a matter of great importance in the biopharmaceutical industry (Kim et al., 2011). Even with the current advances, the demand for these therapies is exponentially

increasing, defining a need for innovative ideas to increase cell density and sustain cell viability in bioreactors.

1.2.3 Applications of CHO cells in therapeutic production

CHO cells have become the primary platform for the production of monoclonal antibodies, which represent a significant portion of the current biopharmaceutical market. The ability of CHO cells to produce large quantities of properly folded and glycosylated mAbs has been instrumental in the development of numerous successful therapeutic antibodies (Nmagu et al., 2021). Several approved mAb therapeutics, such as Rituxan, Herceptin, and Avastin, are all produced by CHO cells.

In addition to mAbs, CHO cells have also been used for the production of a wide range of recombinant proteins and enzymes. Their ability to perform complex post-translational modifications, such as glycosylation and disulfide bond formation, makes them ideal for the expression of proteins that require these modifications for proper functionality. Examples of therapeutic proteins produced in CHO cells include cytokines, growth factors, hormones, and clotting factors. CHO cells have also found applications in the production of viral vectors for gene therapy, such as adeno-associated virus (AAV) vectors (Nagy et al, 2023). AAV-based gene therapies prospect an incredible potential for treating genetic diseases, and CHO cells provide a reliable platform for their large-scale production. Furthermore, CHO cells are being explored in the context of cell-based therapies, where they serve as a production host for therapies such as including chimeric antigen receptor (CAR) T-cell therapies (VWang and Rivière, 2016).

1.2.4 CHO cell engineering and cell line development:

In industry, mammalian cells are grown in very large mechanical vessels called bioreactors. In order to maximise productivity, cells are grown at very high density and in large quantities. Bioreactors facilitate the growth of cells as they are meticulously controlled environments. Cells are normally cultured at high densities, which, while economically advantageous, poses a great deal of challenges that can severely impact cell viability and longevity (Fang et al., 2022). The high-density cultures create a microenvironment where even minor conditions which do not meet the cell's requirements can have disproportionately adverse effects, leading to a cascade of cellular stress responses. Continuous sparging, the process of gas introduction in bioreactors to maintain dissolved oxygen levels, coupled with the resultant shear forces, can mechanically disrupt plasma membranes. Moreover, these high-density conditions can lead to rapid nutrient depletion, oxygen scarcity, accumulation of metabolic byproducts, and increased osmolarity, further exacerbating cellular stress. These stressor factors can push cells toward various fates, including necrosis, which is often considered an uncontrolled form of cell death characterised by the loss of membrane integrity and an unregulated release of cellular contents, potentially provoking an inflammatory response. On the other hand, cells may undergo apoptosis or autophagy, both of which are regulated pathways programmed for cell death or survival under duress. Apoptosis is a form of programmed cell death that systematically breaks down the cell in a controlled manner, preventing damage to neighbouring cells, while autophagy is a protective mechanism that allows cells to degrade and recycle cellular components, thus providing an internal source of nutrients during periods of stress (Elmore, 2007).

To counteract these challenges and improve recombinant protein yields, cell engineering strategies have been rigorously pursued by many scientists and a lot of investments have been made by industrial companies to maintain cell viability in bioreactors for as long as possible. These strategies aim to enhance the resilience of CHO cells within the bioreactor environment and increase their productive lifespan. Genetic and metabolic engineering techniques have been harnessed to bolster cellular defences against the

stresses of high-density growth (Liu *et al.*, 2023). By genetically modifying cell cycle regulators, scientists have been able to manipulate the proliferation rates of CHO cells, enabling sustained growth even under stressful conditions. These engineered modifications can range from the overexpression of genes that promote cell cycle progression, to the downregulation or knockout of genes that would otherwise trigger cell death pathways.

Furthermore, there has been an effort to fortify the intrinsic stress response mechanisms within the cells. This includes the enhancement of antioxidant defence mechanisms to prevent oxidative stress, and the refinement of the unfolded protein response to better manage the load of misfolded proteins, which can accumulate due to the increased production rates necessitated by high-density cultures (Chong, et al, 2017). The engineering of apoptotic pathways, through the modulation of pro-apoptotic and anti-apoptotic proteins, has also been a critical area of focus to improve cell viability in bioreactors.

Beyond genetic modifications, metabolic engineering plays another important role in optimising the media composition and culture conditions which can support cell growth and industrial productivity. Metabolic engineering can involve the supplementation of media with specific nutrients or growth enhancers as well as the adjustment of osmolarity to prevent cellular dehydration, and the close monitoring of pH and gas levels to maintain a healthy homeostasis. Moreover, the development of fed-batch and perfusion bioreactor systems is a technological advance which allows for the continuous feeding and sparging of fresh medium and the removal of waste products. Bioreactor systems have greatly improved the sustainability of high-density cultures (Ullah et al., 2021)

1.2.5 post-translational modifications (PTMs) and protein folding in CHO cells.

Post-translational modifications (PTMs) and correct protein folding monitoring are extremely important for the functionality and stability of recombinant proteins produced in CHO cells in industry. PTMs are chemical modifications that occur after protein biosynthesis, they involve a broad spectrum of changes including phosphorylation, glycosylation, ubiquitination, methylation, acetylation, lipidation, and proteolytic processing (Figure 2). Glycosylation, which is the attachment of sugar moieties to specific amino acid residues (Hollman et al., 1999) Glycosylation is one of the most critical PTMs in therapeutic protein production, as it influences a protein's efficacy, immunogenicity, and plasma half-life. CHO cells are particularly favoured in the biopharmaceutical industry due to their glycosylation patterns which are similar to the ones of the human cells, this characteristic is essential for the clinical effectiveness of glycoprotein-based therapeutics.

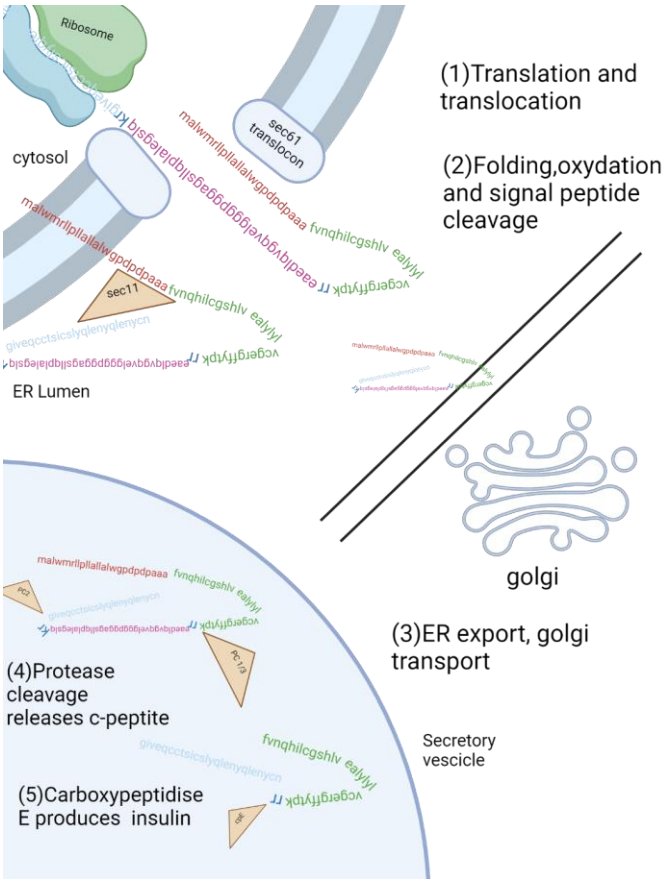


Figure 2: Post-translational modification of insulin. The diagram shows the post-translational modification of insulin: Initially, a ribosome decodes a mRNA sequence to synthesise the insulin protein (1). This protein is then channelled through the endoplasmic reticulum, undergoing processes such as slicing, folding, and stabilisation through disulfide (-S-S-) bonds (2). Subsequently, the protein moves to the Golgi apparatus (3), where it is enclosed in a vesicle. Within this vesicle, further segments of the protein are trimmed away, resulting in the formation of mature insulin (4-5).

Protein folding, a process intricately linked with PTMs, is just as important. As proteins are synthesised, they fold into specific three-dimensional structures to achieve functional conformations (Díaz-Villanueva et al., 2015). Misfolded proteins can lead to loss of

function and can aggregate, leading to cytotoxicity and reduced product yields. In CHO cells, the endoplasmic reticulum (ER) is the site where newly synthesised polypeptides are folded and undergo quality control mechanisms. The ER possesses an elaborate network of chaperones and folding enzymes that assist in protein folding and PTM attachment. Proteins that fail to fold correctly are typically retained within the ER and eventually targeted for degradation by the proteasome, a process known as ER-associated degradation (ERAD) (Halperin, et al, 2014).

The capacity of CHO cells to properly fold proteins and execute PTMs is a key attribute that explains their success in the production of therapeutic proteins. The cell line's robust folding machinery and its ability to carry out complex PTMs make it a versatile and dependable system for producing a wide array of biologically active proteins. Furthermore, the PTMs in CHO cells can be fine-tuned to enhance the therapeutic properties of produced proteins, such as increasing their stability or reducing their immunogenicity. Manipulating the glycosylation patterns in CHO cells, for instance, has become a central focus in cell line engineering to produce glycoproteins with desired efficacy and pharmacokinetics (Zhu et al, 2017).

However, the efficiency of protein folding and PTM processes in CHO cells can be compromised under sub-optimal culture conditions, such as those caused by the mechanical stresses encountered in bioreactors. Shear stress and other physical forces can disrupt the delicate intracellular environment, leading to ER stress and the unfolded protein response (UPR), which can hinder the cell's ability to properly fold and modify proteins. Understanding and mitigating the effects of such stresses are critical for maintaining the fidelity of protein production in CHO cells (Li, et al, 2022).

1.3 Process Optimization and Bioprocessing

Process optimization and bioprocessing are the basis of industrial biotechnology, particularly in the context of maximising the output and quality of products derived from CHO cells. The development and refinement of culture media is a critical aspect of this, as the composition of the media directly impacts cell metabolism, growth, viability, and the production of recombinant proteins (Mandenius et al., 2008). Researchers in the field have looked into the development of tailored media formulations, devoid of animal-derived components, to not only enhance the biosynthesis of proteins but also to align with regulatory guidelines and consumer demand for safety and consistency in biopharmaceuticals. These advanced media are designed to address metabolic bottlenecks and support the high-density growth of CHO cells by providing an optimal balance of nutrients, such as amino acids, sugars, vitamins, and trace elements, which are essential for cellular functions and energy generation (Coulet et al, 2022).

Advanced control systems are employed to monitor these parameters in real-time, making adjustments as needed to maintain homeostasis within the bioreactor. This tight regulation is critical as even minor deviations can lead to stress responses that may hinder cell performance and protein production. For instance, shifts in osmolality can impact cellular water balance, enzyme activities, and the proper folding of proteins, while fluctuations in pH can affect protein structure and stability, as well as cell metabolism and viability (Hasan et al, 2019).

Beyond the physical and chemical aspects of the culture environment, addressing mechanical stress is also of paramount importance. Mechanical agitation and aeration, necessary for mixing and oxygen transfer in bioreactors, can subject cells to shear stress, which may result in membrane damage and cellular disruption (Chisti, 2001). To mitigate this, researchers have innovated by engineering media components that increase the viscosity of the solution to cushion cells, or by designing bioreactors that minimise turbulence. The incorporation of shear protectants like Poloxamer 188, a surface-active agent, has been shown to form a protective layer around cells, shielding them from the

mechanical damage that occurs upon collision with air bubbles or reactor surfaces (Percival et al, 2019).

Downstream from the cultivation and harvest, the purification of recombinant proteins is a process of equal importance and complexity. The downstream processing is meticulously designed to separate the desired proteins from a complex mixture of cells, cell debris, and media components. This typically involves a series of chromatography steps, each exploiting different properties of proteins, such as charge, size, or affinity, to achieve high purity levels (Prapulla and Karanth, 2014). Advanced chromatographic materials and methods have been developed to increase separation efficiency and capacity, reducing processing time and loss of product. Moreover, filtration techniques such as microfiltration and ultrafiltration are employed to concentrate the protein solutions and remove impurities, further refining the product.

The integration of single-use technologies and automation has helped revolutionise CHO cell-based protein production systems. Single-use bioreactors, disposable bioprocess containers, and flexible tubing systems have simplified process scalability, reduced contamination risks, and enabled faster turnaround times. Automation, including robotic systems and high-throughput screening platforms, has facilitated process monitoring, optimization, and quality control, streamlining the production workflow in the biotechnology and pharmaceutical industry.

1.4 Future Perspectives for CHO cells in biotechnology

Emerging technologies in biotechnology such as next-generation sequencing and omics-based approaches hold great potential for advancing CHO cell-based production systems. These technologies can provide deeper insights into cell metabolism, protein folding, and glycosylation patterns, facilitating the development of tailored strategies for enhancing protein production and its quality. Additionally, the application of artificial intelligence and machine learning in process optimization and cell line engineering holds promise for further improving the production of biologics and other therapeutic proteins (Hong *et al.*, 2018). As the field of therapeutics continues to evolve, CHO cells are

expected to play a vital role in the production of novel therapeutic modalities. This includes the production of bispecific antibodies and antibody-drug conjugates (ADCs). Moreover, the rise of personalised medicine and cell-based therapies opens new avenues for CHO cell utilisation, as they can serve as a platform for producing patient-specific cell therapies and engineered cell products.

Despite the remarkable success of CHO cells in therapeutic protein production, challenges remain. Heterogeneity in glycosylation patterns, the need for precise control over protein quality attributes, and the potential impact of alternative expression systems are areas that require further attention (Edwards et al, 2022). Bringing down costs and optimising the production and manufacturing processes is also of the utmost importance when it comes to the development of new strategies of use for CHO cells, and mammalian cells in general. A great deal of attention is being directed to the maintenance of high viability of the cells during the manufacturing processes as loss of viable cell density and cytotoxicity can be problematic towards a successful protein production yield (Verma et al, 2020). Addressing these challenges will facilitate the development of robust and efficient CHO cell-based production processes, ensuring the availability of safe and effective therapeutics.

Their historical significance of CHO cells, together with their numerous advantages as a production host, has established CHO cells as the industry most used vessel for therapeutic protein production. With continuous advancements in the technology of cell line engineering, process optimization, and the integration of cutting-edge technologies, CHO cells are bound to remain at the forefront of biopharmaceutical manufacturing, facilitating the development of innovative and life-saving therapeutics.

1.5 The bioreactor environment for CHO cell cultivation

CHO cells are naturally adherent. This means they typically grow onto a surface, such as the bottom of a culture dish or flask. However, they can be adapted to grow in suspension culture, which is common in large-scale industrial applications for producing therapeutic proteins. Suspension culture adaptation allows for easier scaling up of production processes, as it facilitates the use of bioreactors and increases the efficiency of nutrient and gas exchange. Bioreactors provide an environment for the cultivation of CHO cells, as well as other types of mammalian cells, stem cells and insect cells, and they ensure the optimal conditions for growth and protein production. In these systems, parameters such as pH, temperature, dissolved oxygen, and nutrient supply are meticulously regulated to maximise cell growth and productivity. This controlled environment has become an essential part of biomanufacturing for maintaining the health and viability of CHO cells, directly impacting the yield and quality of the produced biologics (Fang0 et al, 2022).

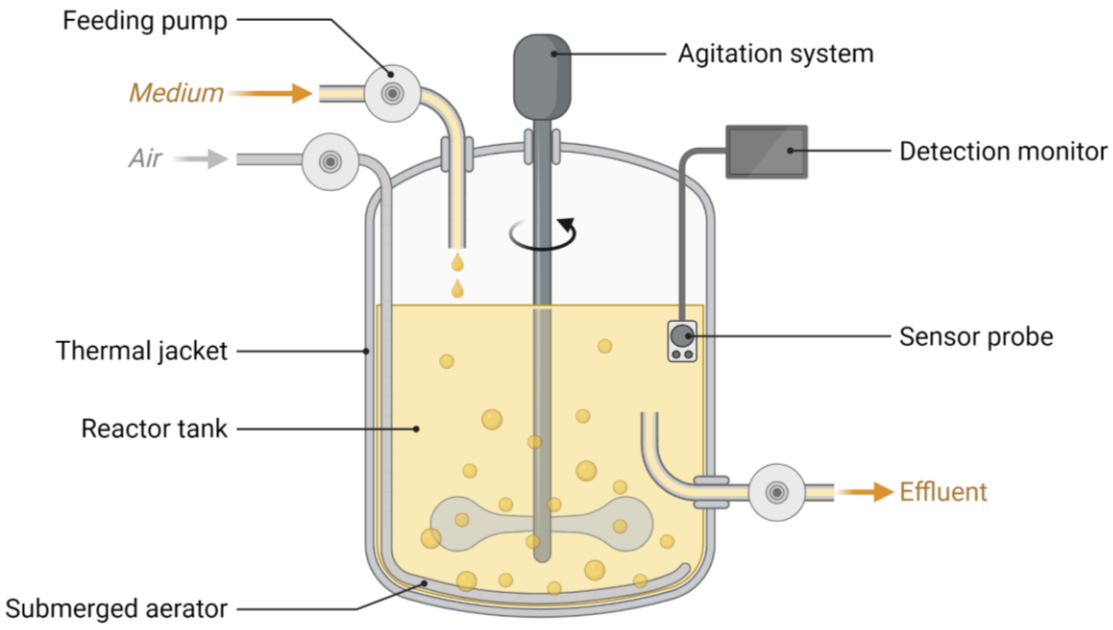


Figure 3: Diagram of a bioreactor. This diagram illustrates a bioreactor system, it its main components and operational mechanisms, such as the culture vessel, agitation system, oxygen supply, temperature control, and monitoring sensors (made with Biorender).

The most common types of bioreactors are multiple use ones. They are normally made of stainless steel and demand a considerable capital investment. Single use bioreactors are also a popular choice, they are more affordable but often with a much-reduced operational volume. While bioreactors offer advantages such as controlled culture conditions and scalability, limitations to productivity can easily arise, particularly linked to cell death and loss of cell viability over time. These issues are mostly due to the nature of long-term cell culture as well as the mechanical forces relative to the functionality of the bioreactors themselves (Jacquemart *et al.* 2016).

pH is typically tightly controlled using acid or base addition, while temperature is maintained using jacketing or internal metal coils. DO (dissolving oxygen) levels are regulated through aeration, agitation and sparging. These forces make sure that oxygen supply is constant and available, oxygen is normally introduced through capillary tubes. Nutrients, such as glucose and amino acids, are supplied through medium replenishment or fed-batch strategies (Jossen et al., 2018).

1.5.1 The bioreactor environment: challenges and strategies in bioreactor cultivation of CHO cells for recombinant protein production

CHO cells require a balanced supply of nutrients and media components for growth and biologics production. This includes the constant addition of essential amino acids, vitamins, glucose, and other energy sources, as well as trace elements and growth factors. The optimization of nutrient composition and media formulation has proven to be an essential step to support robust cell growth and high protein yields. Proper oxygenation of CHO cells in bioreactors is critical for their metabolic activity and productivity (Kim et al, 2020). Adequate oxygen supply is achieved through the sparging of air or oxygen into the culture medium, the oxygenation itself creates bubbles that rise and come into contact with the cells (Figure 3). Efficient bubble-to-cell attachment ensures effective oxygen transfer and avoids oxygen limitation. The presence of bubbles and foam in the media can however negatively impact as they can cause different damages to cells directly (mechanical damage) or indirectly (bubbles trapped in foam and unable to reach nutrients in media) (Riley et. al, 1997). Mechanical and hydrodynamic forces, such as shear stress and turbulence, are routinely generated within the bioreactor environment. These forces can affect cell viability and productivity. High shear stress levels can induce physical cell damage and trigger apoptotic pathways, leading to decreased cell viability and lower production. Optimization of agitation speed, impeller design, and other parameters can help minimise the detrimental effects of these forces. Careful control of gas flow rates, sparging methods, and bubble size distribution is necessary to optimise oxygenation while minimising the negative impact on cells.

Another significant challenge in CHO cell cultures within a bioreactor's environment is the management of the delicate relationship between nutrient supply and waste product removal. As cells metabolise substrates and proliferate, they deplete the medium of essential nutrients, such as glucose and amino acids, which are essential to keep up cellular energy levels and synthesise proteins. The accumulation of metabolic byproducts, such as lactate and ammonia, can create a toxic environment, inhibiting cell growth and

leading to a decline in protein quality and yield (Frahm et al, 2019). Controlling the m11etabolic parameters requires precise feeding strategies, such as fed-batch or perfusion culture systems, to provide a continuous supply of fresh nutrients and removal of waste (Li et al., 2013). The development of these feeding strategies, however, is non-trivial and must be tailored to the specific metabolic profile of the cell line being cultured. As with any other parameter, it plays on a fine balance between excessive sparging of nutrients and successful batch feed.

Furthermore, the high cell densities typically required for industrial-scale production can lead to increased viscosity and oxygen limitations, presenting additional challenges for mixing and mass transfer. Achieving homogeneous conditions throughout the bioreactor is essential to avoid gradients of nutrients and waste products, which could lead to cellular heterogeneity and affect product consistency (Li et al., 2013). The design of the bioreactor, including the impeller and sparger configuration, plays a very important role in optimising mass transfer while minimising shear stress, which can cause physical damage to the cells and impact their viability. Shear stress, in particular, is the cause of big challenges in bioreactor cultures. While agitation is necessary for mixing nutrients and oxygenation, excessive shear forces can disrupt the plasma membrane integrity, leading to cell lysis or apoptosis (Brindley et al., 2011). The incorporation of shear protectants and the optimization of bioreactor design to minimise high-shear zones are strategies employed to mitigate this issue. Nonetheless, these interventions must be carefully calibrated, as they can also influence mass transfer and mixing efficiency.

Another area of concern is the potential for genetic instability in CHO cells cultured over extended periods of time. Prolonged cultivation can lead to changes in the genome, which in turn can lead to clonal variation, with some clones possessing advantageous growth or productivity characteristics being selected over time. While this can sometimes be beneficial, it can potentially lead to the loss of high-producing cell lines and heterogeneity in the product profile. This necessitates the implementation of robust clonal selection and cell line stabilisation strategies to ensure consistent product quality (Torres et al, 2023).

The control of infections caused by bacteria, viruses, or mycoplasma is another critical challenge. Contaminations can lead to complete loss of cultures and are particularly problematic in large-scale operations. Rigorous aseptic techniques, stringent screening protocols, and the use of closed systems are essential to minimise the risk of contamination. The post-culture processing of bioreactor cultures, including the harvesting and purification of recombinant proteins, presents its own set of challenges. The downstream processing must be capable of efficiently purifying the desired product from the complex mixture of cells, debris, and media components while maintaining the integrity and functionality of the proteins. This often involves multiple stages of filtration, centrifugation, and chromatography, each of which must be optimised to maximise yield and purity (Tripathi and Shrivastava, 2019).

1.5.2 Surface tension, interface dynamic and cell death in bioreactors

Shear stress and surface tension are fundamental physical phenomena that have profound implications for the cultivation of mammalian cells in bioreactors. The interplay between these forces and the cellular environment can lead to many different outcomes, from cell viability to the quality of the bioproduct (Tanzeglock *et al.* 2009).

Shear stress in bioreactors is induced by mechanical agitation and aeration, which are necessary for maintaining homogeneity and adequate oxygen transfer in the culture medium. However, the cells in suspension are exposed to frictional forces as a result of the fluid's velocity gradient, which can be detrimental to cellular structures, particularly the plasma membrane. This loss of plasma membrane stability normally leads to apoptosis. The degree of shear stress encountered by the cells is a function of the bioreactor's design, including impeller speed, sparger design, and the viscosity of the medium. When shear forces exceed a cell's structural integrity, it can result in membrane rupture, detachment of cell surface receptors, or disruption of cell-to-cell communication, leading to apoptosis or necrosis (Chalmers and Ma, 2014). This is particularly critical for shear-sensitive mammalian cells, which lack a rigid cell wall for organelles and cytoplasm

protection. Understanding and mitigating the effects of shear stress require an in-depth analysis of fluid dynamics within the bioreactor. Computational fluid dynamics (CFD) models are extensively used to simulate and predict the patterns of fluid flow and shear distribution. These models help in designing bioreactors that can minimise areas of high shear while optimising mass transfer. In addition to bioreactor design, the use of microcarriers or encapsulation techniques can provide a physical barrier to shear forces, thereby protecting the cells by making the plasma membrane more robust. Surfactants are compounds that reduce surface tension between liquids or between a liquid and a solid. In bioreactor environments, they are important because they help prevent the formation of foam, improve the solubility of hydrophobic substances, and can enhance the mixing and mass transfer of gases and nutrients. This leads to more stable and efficient cell cultures, ultimately improving the yield and quality of the bioproducts being produced. The medium's viscosity is also manipulated by the addition of polymers like P188, which can create a more viscous layer around the cells, reducing the impact of shear (Chang *et al.*, 2017).

Surface tension is a property of the liquid-air interface and is critical in the formation of gas bubbles during aeration and it can be critically influenced by the addition of surfactants. These bubbles are necessary for oxygen supply but can become sites of cell damage upon their collapse or coalescence, a process which is called “cell-bubble interaction”. High surface tension can exacerbate this damage as cells are more likely to be dragged by the departing bubbles, potentially causing cell rupture or detachment from the growth surface (Chang *et al.*, 2017). To counteract this, surfactants are introduced into the culture medium to reduce surface tension, thereby mitigating the potentially harmful interactions between cells and bubbles. The surfactants act by adsorbing at the gas-liquid interface, reducing the cohesive forces between liquid molecules and thus lowering the energy required to increase the surface area.

The dynamics of the liquid interface, where gas and cells interact, is an area of bioprocessing that requires a much deeper understanding and control. The stability of this interface is influenced by both the physical properties of the medium, such as viscosity and surface tension and the biological characteristics of the cells. The interface is a region

of high-energy exchange, where cells can experience stress due to fluctuating pressures and the forces exerted by bubble formation and collapse. This interfacial dynamic can affect not only cell viability but also influence cellular responses such as the expression of stress-related genes and proteins that may alter the cell's metabolism and productivity (Chang *et al.*, 2017).

Moreover, the surface-active agents, while reducing surface tension, can also interact with cellular membranes due to their amphiphilic nature. They can alter membrane fluidity and permeability by embedding themselves in the plasma membrane. This can have downstream effects on membrane-associated processes such as signal transduction, nutrient uptake, and product secretion. The choice of surfactant, therefore, is not a step in this process to take lightly; it must be biocompatible and effective at the concentrations used, without interfering with cellular functions or product purity (Witika *et al.*, 2020).

The dynamic nature of the interface can also impact the trafficking of nutrients and waste products between the cells and the bulk medium. At low surface tension, bubbles tend to be smaller and more stable, which can enhance mass transfer due to a larger interfacial area. However, if the bubbles are too stable, they may not coalesce and release their oxygen content effectively, resulting in localised oxygen depletion. Conversely larger and more structurally stable bubbles and foams are created in environments with high surface tension, which can lead to increased cell damage upon collapse but may improve oxygen release. Optimising these outcomes is a delicate balance that requires fine-tuning of the surfactant concentration and aeration strategies (Chang *et al.*, 2017).

The interfacial dynamics in bioreactors are also influenced by the presence of proteins and other macromolecules secreted by the cells, which can adsorb at the interface and alter its physical properties. These biomolecules can compete with the added surfactants for space, potentially reducing the efficacy of the surfactants and changing the response of the cells to the interfacial stress. The complexity of this system necessitates continuous monitoring and control to maintain an environment that supports cell health and productivity.

1.5.3 Foam formation in bioreactors

Foam formation in bioreactors is a phenomenon which occurs as a result of gas being sparged into the culture medium to supply oxygen necessary for cell metabolism. This sparging, while essential, also introduces significant mechanical forces that can lead to the entrainment of air or gas bubbles, which unify to form foam. Foam is constituted of a complex matrix of gas-filled pockets stabilised by liquid films and is particularly problematic in bioprocessing due to its propensity to entrap cells, proteins, and other macromolecules (Chang *et al.*, 2017).

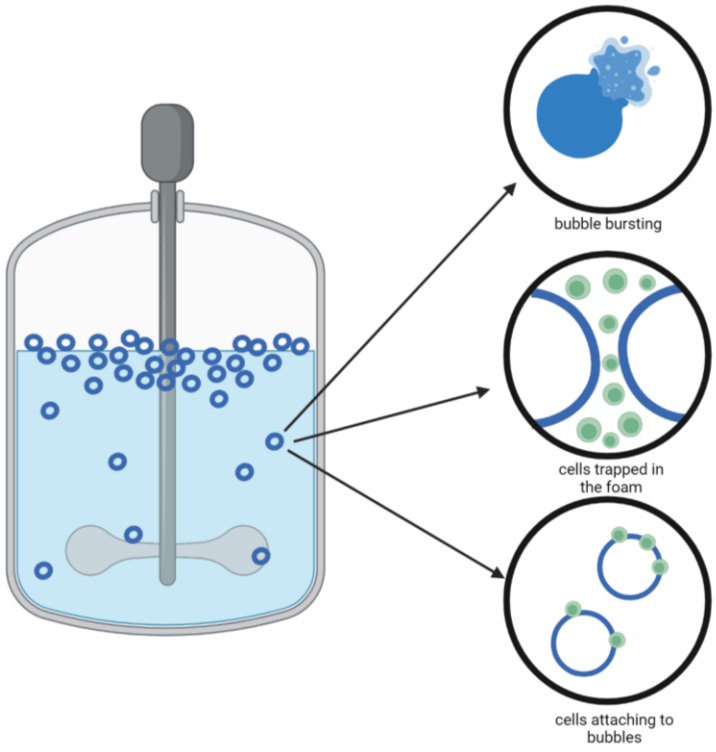


Figure 4: Cell attachment to oxygen bubbles in bioreactors. Diagram of a mixed and aerated bioreactor, it illustrates the surface interactions which may lead to cell death. In bioreactors, air bubble interfaces refer to the surfaces where gas bubbles meet the liquid culture medium. These interfaces are very important for oxygen transfer, as they allow oxygen from the air to dissolve into the liquid, which provides oxygen to the cells. Efficient gas exchange at these interfaces helps maintaining cell respiration and metabolism. However, excessive bubbles can cause cell damage or create foam, which can disrupt the culture process and reduce yield, making proper management of air bubble interfaces essential. Suspended cells adhere to ascending air bubbles, accumulating in the foam at

the top of the liquid interface. Here, they risk entrapment within the foam's structure or may sustain damage from rupturing bubbles. (Image made with Biorender).

The mechanisms leading to foam formation are linked to the properties of the culture medium, including surface tension and viscosity, as well as the bioreactor's operational parameters such as the rate and method of gas introduction and sparging forces. High sparging rates, aimed at increasing oxygen transfer to meet the demands of densely cultured cells, can inadvertently elevate the rate of foam generation. The turbulent conditions allow the easy formation of bubbles, which upon rising to the surface, are not readily dissipated due to the presence of surface-active substances in the medium, including proteins secreted by the cells (Emery et al, 1995) (Figure 4).

The accumulation of foam at the air-liquid interface is a dangerous environment for cells due to several factors; cells entrapped within foam are increasingly exposed to air, which can lead to dehydration and osmotic stress, compromising cell integrity and function. Secondly, the physical forces applied on the cells during foam formation and collapse can induce shear stress, which, as previously discussed, can cause membrane rupture or initiate apoptosis. Furthermore, the cells caught in the foam layer are subject to nutrient depletion and waste accumulation due to their separation from the bulk medium. This isolation disrupts the exchange of metabolites, gases, and regulatory signals, which can rapidly lead to cell starvation and a build-up of toxic byproducts, both of which are detrimental to cell viability (Onal et al, 2022).

To mitigate foam formation and its negative impact on cell cultures, antifoaming agents or mechanical foam breakers are commonly employed. The mechanical methods can be more biocompatible, as they do not introduce additional chemicals into the culture medium. However, they also require careful implementation to prevent additional shear stress on the cells. Antifoaming agents function by reducing the surface elasticity and increasing the surface tension gradient, which will destabilise the foam bubbles leading to foam collapse. While effective, these agents must be used cautiously as they can also interact with plasma membranes and proteins, potentially affecting cell viability and product integrity. It is also crucial to recognize that foam carries with it not only cells but

also the product of interest, which in the case of CHO cells, is often a therapeutic protein (Chang *et al.*, 2017). The proteins trapped within the foam are at risk of denaturation due to the air-liquid interface's propensity to unfold proteins. Thus, foam control is not merely a matter of maintaining cell viability but also of protecting the product's quality and yield.

1.5.4 Strategies to mitigate hydrodynamic-induced cell death.

Mitigating hydrodynamic-induced cell death in bioreactor cultures, especially in the cultivation of mammalian cells such as CHO cells, is an area of crucial importance in the research into bettering bioprocessing and bioengineering. The physical forces generated in bioreactors, primarily due to agitation and sparging for mixing and oxygenation, can induce a range of cellular stresses leading to cell death. Addressing these challenges requires a multifaceted approach combining bioreactor design, optimization of culture conditions, and cellular engineering (Chevallier et al, 2020).

Bioreactor design is one of the main contributors in controlling the hydrodynamic environment. Traditional stirred-tank reactors, while effective in mixing and oxygen transfer, can generate regions of high shear stress, particularly near impellers and sparging devices. The development of novel bioreactor designs aims to reduce these high-shear zones. Air-lift bioreactors rely on the circulation of medium via a central draft tube, which provides a gentler method of mixing and oxygenating cell cultures. This design minimises the mechanical forces exerted on the cells, thus reducing the risk of hydrodynamic stress (Merchuk, 2003)**.** Similarly, wave-mixed bioreactors use a rocking motion to more gently agitate the culture medium, creating a low-shear environment suitable for sensitive mammalian cells. These alternative designs, while less conventional than stirred-tank reactors, offer promising avenues to mitigate hydrodynamic stress although they present issues with yield as nutrients are not homogeneously sparged (Zhan, *et al.*, 2019).

Optimization of culture conditions is another strategy to protect cells from hydrodynamic stress. This involves the careful control of operational parameters such as agitation speed, aeration rate, and culture viscosity. Reducing the speed of agitation and the rate of aeration can diminish the intensity of shear forces. This means that these adjustments must be balanced against the metabolic needs of the cells. Increasing the viscosity of the culture medium is another approach; higher viscosity can dampen the turbulent forces, providing a more protective environment for the cells (Hu, et al, 2011). This is often achieved by adding polymers such as hydroxyethyl cellulose or surfactants such as P188 to the medium. P188 is part of a class of polymers called poloxamers. Poloxamers, also known as Pluronics, are a class of non-ionic surfactants commonly used in bioreactors to enhance the stability and viability of cell cultures. They consist of block copolymers formed by a central hydrophobic block of polypropylene oxide (PPO) flanked by two hydrophilic blocks of polyethylene oxide (PEO). These polymers can form a protective layer around the cells, reducing the impact of shear forces. Surfactants like poloxamers can mitigate the formation of foam and improve the solubility of hydrophobic compounds, thereby enhancing nutrient and gas exchange in the culture medium. The balance between the hydrophobic PPO and hydrophilic PEO segments can be adjusted to tailor the surfactant properties for specific biotechnological applications, making poloxamers versatile agents in optimizing bioreactor conditions.

In parallel with these strategies, monitoring and controlling the metabolic health of the cell culture is vital. Cells under hydrodynamic stress can exhibit altered metabolic profiles, leading to the accumulation of toxic metabolites or depletion of critical nutrients. Real-time monitoring technologies, such as dielectric spectroscopy for cell density measurement and Raman spectroscopy for metabolite analysis, provide valuable insights into the culture's health and allow for timely interventions. Adjusting the feeding strategy in response to these metabolic changes, such as implementing nutrient-rich feeds or dialysis-like perfusion systems to remove waste, can help maintain a conducive environment for cell growth and productivity (Reyes, *et al.* ,2022). The development of computational models to predict and simulate the hydrodynamic conditions within bioreactors is an area of growing interest. These models, based on computational fluid

dynamics (CFD), can predict the distribution of shear forces and identify potential problem areas in bioreactor design. By simulating various operational scenarios, these models can guide the optimization of bioreactor operations, reducing the reliance on trial-and-error methods (Hutmacher and Singh, 2008).

Types of Bioreactors	Advantages	Disadvantages
Stirred tank bioreactor	Easy to scale up. Good mixing and oxygen transfer	Shear stress can damage cells. Harder to operate and maintain
Wave Bioreactor	Gentle mixing, low shear stress. Simple operation	Limited scalability. Lower oxygen transfer efficiency
Perfusion Bioreactor	Continuous product harvest. High cell density	More complex setup. Higher operational cost
Fixed-bed Bioreactor	High cell density. Low shear environment	Difficult to scale up. Limited nutrient distribution
Single-use Bioreactor	Reduced risk of contamination. Lower cleaning costs	Higher cost of disposables. Limited mechanical strength

Table 3: Overview of various bioreactor types used for therapeutic production with CHO cells, highlighting their advantages and disadvantages. The table includes stirred tank, wave, perfusion, fixed-bed, and single-use bioreactors.

1.6 Surface-active agents (Surfactants)

Poloxamers, also known as Pluronics, are a class of non-ionic triblock copolymers composed. These amphiphilic molecules are widely adopted in pharmaceutical and biomedical applications due to their ability to stabilize emulsions, enhance solubility, and protect cell membranes (Shirwaiker et al., 2014). Poloxamers are characterized by their unique ability to form micelles in aqueous solutions, which makes them useful as drug delivery vehicles. Among the most prominent poloxamers are P188 and P407, known for their membrane-stabilizing properties and biocompatibility (Khaliq et al., 2023). Similar surfactants include PEG (polyethylene glycol) and PPG (polypropylene glycol), which also exhibit amphiphilic properties but differ in their specific structural configurations and applications. Poloxamers are particularly valued for their versatility and effectiveness in enhancing the stability and performance of therapeutic formulations. The use of surfactants or surface-active agents in bioreactor systems, particularly in the context of mammalian cell cultures such as those involving CHO cells, has become an essential aspect of modern bioprocessing. Surfactants play a crucial role in modulating the interfacial properties of the culture medium, which in turn, can significantly impact cell viability, productivity, and overall process efficiency. Surfactants are amphiphilic molecules, possessing both hydrophilic (water-attracting) and hydrophobic (water-repelling) properties (Table 4) (Zhang, et al, 2023). This dual nature allows them to adsorb at interfaces, such as air-liquid interfaces, and reduce surface tension. In bioreactors, this reduction in surface tension is particularly beneficial for addressing issues related to gas-liquid mass transfer, foam formation, and cell protection against mechanical stress. Surfactants can also form micelles, due to their amphiphilic nature. Micelles form when surfactant molecules in an aqueous solution reach a concentration that allows them to aggregate. At a certain concentration, known as the Critical Micelle Concentration (CMC), these molecules spontaneously arrange themselves into spherical structures, with the hydrophobic tails inward and hydrophilic heads outward, minimizing unfavourable interactions with water. Micelles can even encapsulate hydrophobic substances in their core, making it easier to solubilize these substances in water. The CMC is important

because it indicates the concentration above which micelles start to form and become effective in solubilizing hydrophobic compounds (Figure 5).

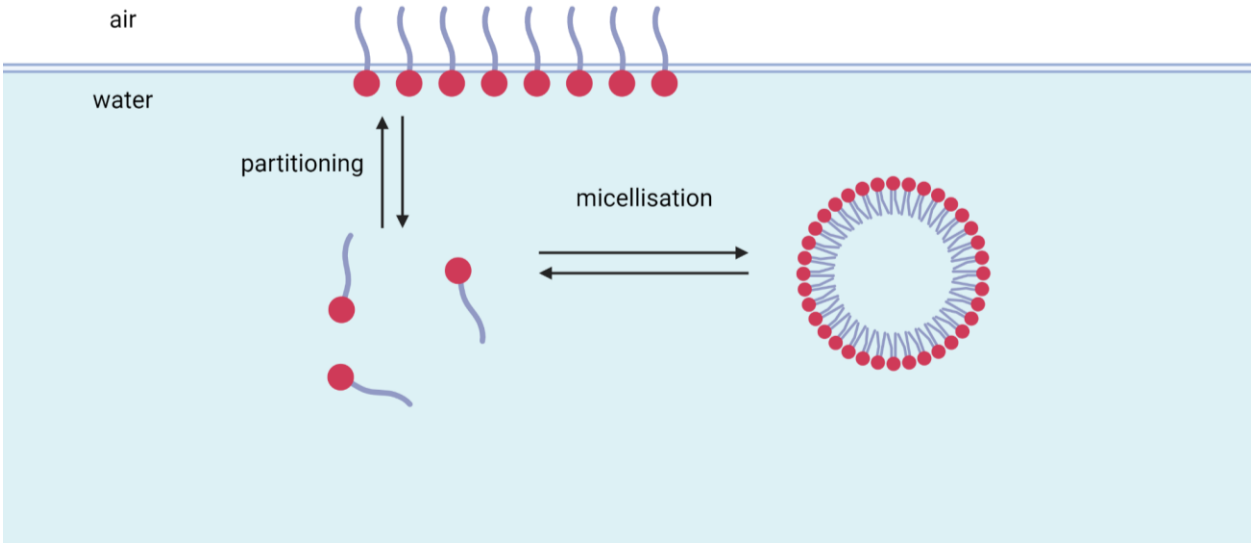


Figure 5: Micellization of polymers in liquid interface. Diagrammatic representation of the balance between surfactants on a surface, unbound surfactants, and those aggregated in micelles.

One of the primary functions of surfactants in bioreactors is to ameliorate cell stress to prevent the cell viability loss affected by sparging and agitation, which are necessary for adequate oxygen supply and mixing but can lead to the formation of foam and shear stress. Surfactants, by reducing the surface tension, help in controlling foam formation. They lower the stability of the foam, making it easier to break down, and thus minimise the risk of cell entrapment and product loss (Zhang et al, 2023).

The choice of surfactant is critical and depends on various factors, including its biocompatibility, effectiveness at low concentrations, and impact on product purity. Non-ionic surfactants, such as P188, have been widely used in mammalian cell cultures due to their low cytotoxicity and effective surface-active properties. P188, in particular, has

been shown to significantly reduce shear-induced cell damage and improve cell viability in bioreactors. However, the use of surfactants is not without challenges. For instance, surfactants can interact with proteins and other biomolecules in the culture medium, potentially affecting the quality and yield of the bioproduct (Badmus et al, 2021). Therefore, their concentration and interaction with other medium components must be carefully optimised. The interaction of surfactants with the bioreactor system extends beyond the cellular level. At the process level, surfactants can influence oxygen transfer rates, mixing patterns, and nutrient distribution within the bioreactor. The reduction in surface tension can alter bubble dynamics, affecting both the size and stability of gas bubbles. Smaller and more stable bubbles can enhance oxygen transfer efficiency but may also lead to issues with oxygen oversupply and potential oxidative stress on cells. Thus, the concentration and type of surfactant used must be optimised to achieve a balance between protecting cells from mechanical stress and maintaining optimal gas transfer rates (Fares *et al.* 2023).

Surfactant	**Molecular weight (Da)**	**Critical Micelle Concentration (CMC)**	**Common Use**
Poloxamer 188 (P188)	8,400 - 9,500	0.003 - 0.01% w/v	Stabilizing agent, cell membrane protection, drug delivery
Poloxamer 407 (P407)	12,600 - 14,000	0.002 - 0.007% w/v	Controlled drug release, topical formulations, stabilizer
Poloxamer 338 (P338)	14,600 - 16,200	0.001 - 0.002% w/v	Emulsifier, solubilizer, formulation of biopharmaceuticals

Poloxamer 237 (P237)	12,000 - 13,500	0.0015 - 0.002% w/v	Stabilization of emulsions, drug solubilization
Polyethylene Glycol (PEG)	Varies (200 - 40,000)	Varies depending on molecular weight	Drug delivery, osmotic balance, solubility enhancement
Polypropylene Glycol (PPG)	Varies (400 - 4,000)	Generally low, varies with molecular weight	Emulsifier, surfactant in personal care products

Table 4-: This table presents various types of poloxamers (P188, P407, P338, P237) and related surfactants (PEG, PPG), detailing their molecular weight, critical micelle concentration (CMC), and common uses. The information provides insight into the selection of appropriate surfactants for specific applications in pharmaceutical and cosmetic formulations.

1.7 Current gaps in knowledge – How do surfactants protect cells in bioreactors?

Understanding the underlying molecular interactions between P188 (and other surfactants) and CHO cells is essential for advancing bioprocess technology and optimising bioreactor operations. This understanding is crucial not only to enhance the efficiency of cell cultures but also to ensure the quality and safety of the biopharmaceutical products derived from these cells. Despite the widespread use of P188 as a protective surfactant in bioreactor cultures, there remains significant gaps in knowledge regarding

its exact mechanisms of interaction at the molecular level with CHO cells (Narayanappa, et al, 2019).

The primary role of P188 in bioreactor cultures is to mitigate the mechanical stress experienced by CHO cells, which can be induced by agitation and sparging. It is hypothesised that P188 adsorbs to the plasma membrane, forming a protective layer that shields the cells from the detrimental effects of shear forces. However, the detailed molecular dynamics of this interaction are not fully understood. Understanding how P188 integrates into the plasma membrane, how it affects membrane fluidity and integrity, and its impact on membrane-associated processes such as signal transduction and nutrient transport, is becoming more and more important in bioprocessing (Kwiatkowski., et al 2020). The interaction of P188 with membrane lipids and proteins, and its potential to alter the membrane structure and function, needs thorough investigation. This understanding will enable the optimization of P188 concentrations in the culture medium to maximise protection while minimising any adverse effects on cellular functions.

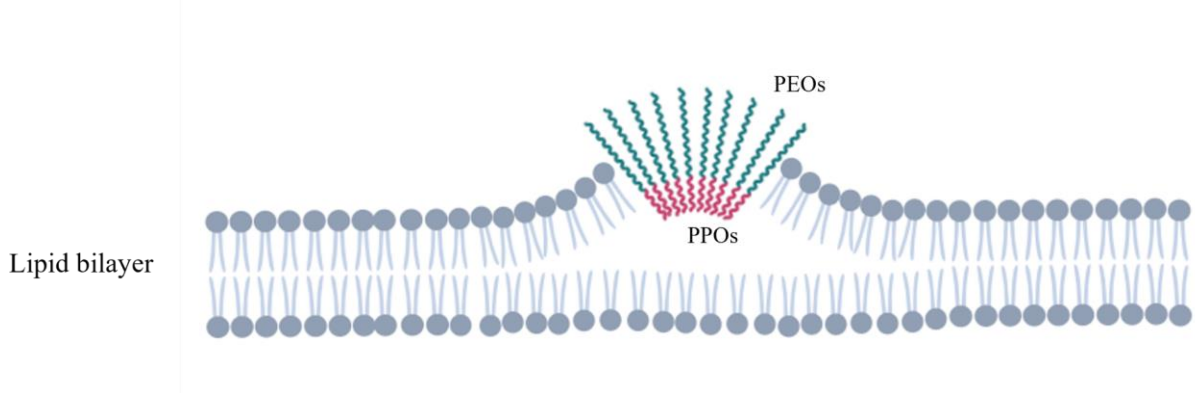


Figure 6: Theory of insertion of P188 in the lipid bilayer of a damaged cell. The PPO (Poly oxypropelene) chains preferentially adhere to the damaged plasma membrane. The hydrophilic PEO (Poly oxyethylene) chains serve as a barrier to the hydrophobic damaged plasma membrane.

Cells under mechanical stress can activate various intracellular signalling pathways leading to apoptosis, autophagy, necrosis and potentially other cell death mechanisms. The extent to which P188 influences these pathways is not well understood. Does P188

merely act as a physical barrier to mechanical stress, or does it also interact with cellular signalling mechanisms that regulate stress responses? Investigating the potential of P188 to interact with intracellular cell death signalling molecules and elucidating its role in the regulation of cellular responses to stress are critical for a comprehensive understanding of its protective mechanisms (Figure 6).

Another significant gap in knowledge pertains to the impact of P188 on the overall bioprocess, particularly its interactions with other components of the culture medium and its effects on downstream processing. Whilst it is known that P188 reduces surface tension and can control foam formation, its interactions with proteins, nutrients, and other additives in the culture medium are not fully characterised (Bandyopadhyay, *et al,* 2022). These interactions can have implications for nutrient availability, protein expression, and post-translational modifications, ultimately affecting cell growth and product yield. Additionally, the presence of P188 in the culture broth can influence downstream processes such as filtration and chromatography, potentially impacting the purity and quality of the bioproduct. A more in-depth understanding of these interactions is crucial for the holistic optimization of the bioprocess. Furthermore, the long-term effects of P188 on cell culture, such as its impact on genetic stability, cellular metabolism, and the potential for inducing cellular adaptations or resistance, remain also largely unknown. As CHO cells are often cultured for extended periods in bioreactors environments, it is important to try and understand how the prolonged exposure to P188 can potentially influence the cells genomic, transcriptomic, and proteomic characteristics. This knowledge is important not only for ensuring the consistency and quality of the bioproduct but also for understanding the evolutionary adaptations of cells in response to their microenvironment (Bailey *et al.*,2012)

While Poloxamer 188 has proven to be a valuable chemical in enhancing the viability and productivity of CHO cell cultures in bioreactors, there is a critical need to deepen our understanding of its molecular interactions with these cells. This includes elucidating its mechanisms of membrane protection, its role in modulating cellular stress responses, its interactions with other medium components, and its impact on the overall bioprocess, including downstream processing. Addressing these gaps in knowledge will enable more

precise control and optimization of bioreactor conditions, leading to improvements in the efficiency, quality, and safety of biopharmaceutical production.

1.7.1 Batch to batch variation:

The issue of batch-to-batch variation in P188 presents a known and significant challenge in bioprocessing, particularly in the cultivation of CHO cells for biopharmaceutical production. Despite its critical role in protecting cells from hydrodynamic stress in bioreactors, the lack of a clear understanding of the causes of batch variability in P188 significantly hampers the optimization and standardisation of bioprocessing protocols (Tripathi and Shrivastava, 2019).

One of the key issues is that the exact composition and molecular configuration of P188 can vary between different manufacturing batches. Poloxamer 188 is a complex mixture of polymers with varying molecular weights and block chain lengths, and even slight variations in these parameters can alter its physicochemical properties, such as hydrophobicity, micelle formation, and surface activity. All these parameters are essential with the correct interaction of the polymer with bubbles, liquid interface and plasma membranes. These variations can heavily impact its efficacy in protecting cells from shear stress and its interaction with plasma membranes and other medium components. Currently, the primary method to assess the suitability and consistency of P188 batches for cell culture applications is through empirical testing, which involves culturing cells with the P188 batch studied and monitoring for cell growth, viability, and productivity (Bareford *et al.*, 2019). This approach, while still a necessity, is time-consuming, resource-intensive, and not entirely predictive. It does not provide immediate insight into the molecular characteristics of the P188 batch that may be responsible for any observed differences in cell culture performance. This gap in rapid and precise characterization methods for P188 batches creates a big hurdle in process development and quality control. The reliance on cell culture-based assays for batch testing also introduces variability and uncertainty into the bioprocessing workflow and upstream development (Bareford *et al.*, 2019).

The response of CHO cells to different P188 batches can be influenced by multiple factors: the specific cell line, culture conditions, and the presence of other medium components. This complexity makes it challenging to isolate the effects of P188 variability and can lead to inconsistent interpretations of batch quality. Advancements in analytical techniques and a deeper understanding of the structure-function relationships in P188 would provide more direct and efficient methods for assessing batch quality. Analytical chemistry techniques such as high-performance liquid chromatography (HPLC), mass spectrometry, and nuclear magnetic resonance (NMR) spectroscopy have the potential to be a helpful means to characterise the molecular composition and structural attributes of P188 batches more precisely. Coupling these analytical methods with bioassays that specifically measure the surfactant's protective effects on cells could lead to a more streamlined and predictive approach for batch testing, although the physicochemical. Addressing these gaps in the knowledge of the underlying mechanisms of interactions of P188 in CHO cell cultures would not only enhance the efficiency and reliability of bioprocesses, but also contribute to the overall robustness and consistency of biopharmaceutical production (Gathungu, et al, 2020)

Chapter 2: Materials and Methods

2.1 Materials

Cell Lines and Culture Conditions:

- CHO-S Cell Line: Chinese Hamster Ovary (CHO-S) cells for all experiments.
- Culture Media:
    - Freestyle CHO cell media (Thermo Fisher Scientific, Cat. No. 12651022) with/without pluronic content.
    - OptiCHO and FortiCHO media (Gibco) for suspension cultures.
    - Flasks: Polycarbonate Erlenmeyer flasks of 125ml capacity (Thermo Fisher Scientific).
    - Automated Cell Counter: ViCell XR (Beckman Coulter).

Cell Preparation and Plasmid Amplification:

- E. coli Cells: Competent cells for plasmid amplification.
- Plasmid Purification: Maxi plasmid purification kit.

Reagents and Supplements:

- Poloxamers: P188, P407, P338, P237, and PEG/PPG (Croda).
- Antibiotic Solution: Antibiotic-antimycotic solution containing Penicillin/Streptomycin (Pen/Strep).
- Growth Supplement: Glutamax (Gibco).
- Transfection Reagent: Yellow Fluorescent Protein (YFP) encoding plasmid.

SDS-PAGE Materials:

- Acrylamide/Bis-acrylamide solution

- Tris-HCl buffer
- SDS (Sodium dodecyl sulphate)
- APS (Ammonium persulfate)
- TEMED (N,N,N',N'-Tetramethylethylenediamine)
- Running buffer (Tris, Glycine, SDS)
- Protein ladder/molecular weight marker

Immunoblotting Materials:

- Blocking buffer (e.g., 5% non-fat dry milk or BSA in TBST)
- TBST (Tris-buffered saline, 0.1% Tween 20)
- PBST (Phosphate-buffered saline, 0.1% Tween 20)

Antibodies:

- LC3B Antibody #2775 (Cell Signalling Technology)
- Anti-Cleaved Caspase-3 antibody [E83-77] (Abcam)
- Anti-mouse secondary antibody (Sigma-Aldrich)
- Anti-rabbit secondary antibody (Sigma-Aldrich)

Poloxamers (Croda):

- **Poloxamer 188**
- **Poloxamer 407**
- **Poloxamer 338**
- **Poloxamer 237**
- **Polypropelene glycol**
- **Polyethylene Glycol**

Machineries:

- CSC Digital Tensiometer
- Cole-Parmer Benchtop Tensiometer Kit

- Zetasizer Malvern Panalytical

Transfection and Electroporation:

- System: Neon™ Transfection System (Thermo Fisher Scientific).
- Sonicator: For cell suspension treatment (model unspecified).
- Electroporation System: Neon™ Transfection System

Particle Size and Zeta Potential Measurement:

- Zetasizer: Malvern Panalytical instrument for micelle size distribution and zeta potential analysis.

Data Analysis:

- Plate Reader: Clariostar (BMG Labtech) for fluorescence and cell growth quantification.
- Statistical Analysis: For evaluating the impact of P188 on cell viability and transfection efficiency.

2.2 Methods:

Baffled flasks

The CHO cell cultures were grown in 125ml Erlenmeyer polycarbonate flasks with 30ml working volume. Flasks with flat and baffled bottom were employed to carry out viability experiments and grow the cells. The baffled flasks have a total of 4 baffles on the base which are 1 cm high, 1 cm deep and 1.5 cm wide. The unbaffled flasks have a smooth base with no plastic baffles.

The flasks have been used as a small-scale reproduction to hydrodynamic forces occurring in large scale bioreactors.

Dynamic light scattering for calibration curve of P188:

To plot a calibration curve for the concentration of (P188) in commercially available media using Dynamic Light Scattering (DLS), standard solutions of P188 at known concentrations were prepared. Each standard was analysed using DLS to measure the average particle size or scattering intensity. The DLS measurements were plotted against the known concentrations to generate a calibration curve, fitted with a linear equation. The P188 concentration in media samples was then determined by measuring their DLS response and inputting the results using the calibration curve. The calibration curve's accuracy was validated by testing additional known concentrations of P188.

Cell Viability

Cell viability data was obtained using Vi-cell XR automatic cell counter, which automatically aspirated the sample, analyzed cell concentration and viability, and provided the total cell count and viability percentage based on Trypan Blue exclusion. The data was then exported to an Excel spreadsheet.

Immunoblot:

Proteins were electro transferred from an SDS-PAGE gel to a polyvinylidene difluoride (PVDF) membrane. This membrane was blocked in a blocking buffer prior to being incubated with primary antibodies, also in blocking buffer. The types of primary antibodies used, and their dilutions are specified in the materials section of this chapter. The blocking step and primary antibody incubation were conducted either for an hour at room temperature or overnight at 4°C. The membrane was then washed thrice with the blocking buffer, followed by incubation with a secondary antibody in the blocking buffer for one hour at room temperature. After this, the membrane was washed three times again in the

blocking buffer and another three times in PBS-T. Imaging was carried out using Immobilon HRP substrate (Millipore) and a Syngene GeneGenius ECL imaging system.

Wilhelmy Plate Tensiometer:

A Wilhelmy plate tensiometer was used to measure the equilibrium surface tension of water-based solutions with the addition of poloxamers at different concentrations. The aim of the experiment was to study if these polymers were surface active and if they influenced the liquid surface tension.

The samples consisted of purified P188 in powder form and P188 with the addition of 20% P407 both in a powder form, each compound was dissolved in distilled water, diluted and measured at following concentrations (all w/v): 0.0001%, 0.001%, 0.005%, 0.01%, 0.025%, 0.05% 0.1%, 0.5%, 1%.

Each solution was prepared and maintained at room temperature a few minutes before the measurements were taken. 50 ml of each sample were poured in a glass basin and a platinum plate would be slowly immersed in the liquid interface.The surface tension is measured using the force necessary for the platinum plate to enter the sample's surface. The platinum Wilhelmy plate was rinsed with distilled water and sterilised using a Bunsen burner between the measurements of different samples. Surface tension of distilled water was performed between experiments to ensure that the plate and the glassware had been cleaned correctly.

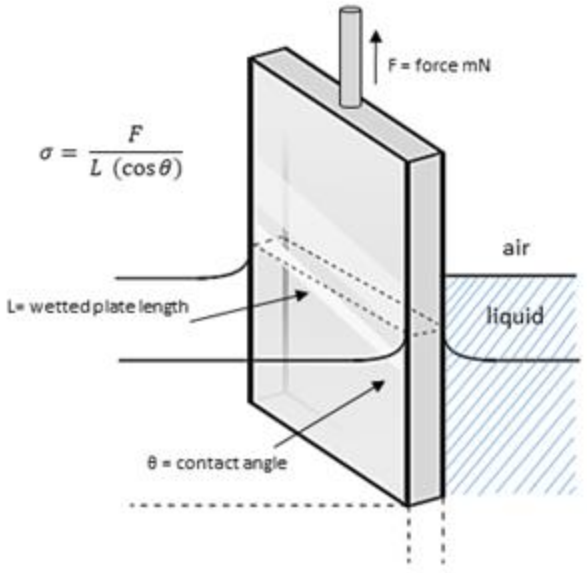


Figure1: Diagram of a Wilhelmy plate tensiometer. The thin, vertically aligned plate is immersed into a liquid interface. The plate makes contact with the liquid surface, which causes a capillary rise or depression of the liquid along the plate edges. The Wilhelmy plate method quantifies surface tension and wettability of a liquid by measuring the force changes as the plate interacts with the liquid surface. Image made with Biorender.

Bubble tension tensiometer:

The dynamic surface tension experiment with the bubble tensiometer is based on the bubble pressure dynamic in liquids. The measurements register the air pressure blown out by a capillary tube as it forms bubbles in a liquid sample immersion. The pressure in the capillary tubule is increased until the air bubbles are pushed in the liquid environment, the highest pressure is measured when the bubble is upon release, when it is a hemispherical shape before leaving the tube. The physical force applied on the

bubble in order for it to be released is determined by the surface tension of the liquid (Ménard and Kimmerle, 1973).

Dynamic surface tension was measured via a bubble tensiometer for samples in both Freestyle CHO cell media and distilled water. The samples were solutions at 0.1% w/v and 1% w/v concentrations of poloxamer mixtures where the main species was P188 with a 20% addition of either P407. P338, P237, PEG (polyethylene glycol) or PPG (polypropylene glycol). The surfactant characteristics of the compounds were analysed in distilled water and in CHO cell Freestyle media. The capillary tubes of the apparatus were immersed in 30 ml of every solution. Between measurements, the capillary tubes were wiped with disposable paper and rinsed with distilled water.

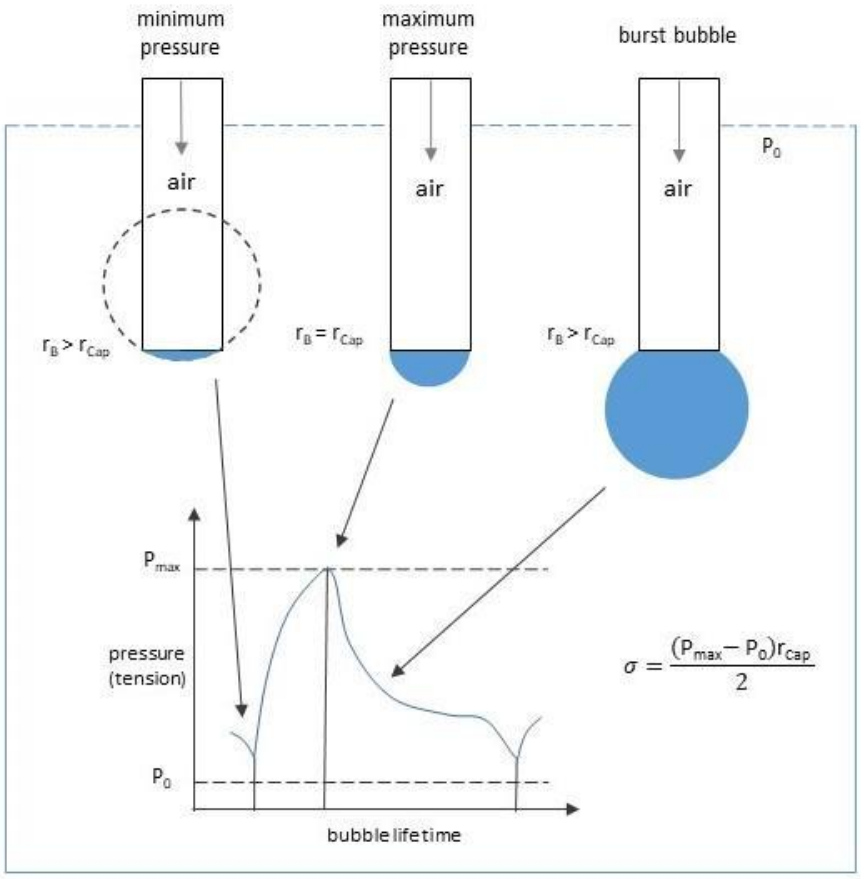


Figure 2: Mechanisms of measuring dynamic surface tension using a bubble tensiometer. This figure illustrates the process involved in measuring the surface tension of liquids using a bubble tensiometer. The key stages of bubbles include the formation of a gas bubble at the tip of a capillary tube submerged in the liquid sample, then the moment of bubble detachment. The diagram visualises the relationship between the internal pressure in the bubble and the surface tension of the liquid, calculated using the Young-Laplace equation (Sita technologies, 2024).

Dynamic light scattering (DLS):

The DLS and dynamic tension experiments were carried out on samples of sterilised water and cell media with the addition of 0.1% and 1% purified P188. To the purified P188 control were also added a range of known impurities: PEG (polyethylene glycol),

PPG (polypropylene glycol), P338, P237, and P407 at 20% concentration of the total P188. Poloxamer solutions were prepared by dissolving powder or flakes in purified water to 1% w/v and then diluted to appropriate concentrations, for this experiment we used 1% w/v and 0.1% w/v dilutions in both distilled water and CHO cell Freestyle media. 1ml of each sample was pipetted in a plastic cuvette and loaded into a Zetasizer Nano ZSP to measure differences in micelle size and intensity.

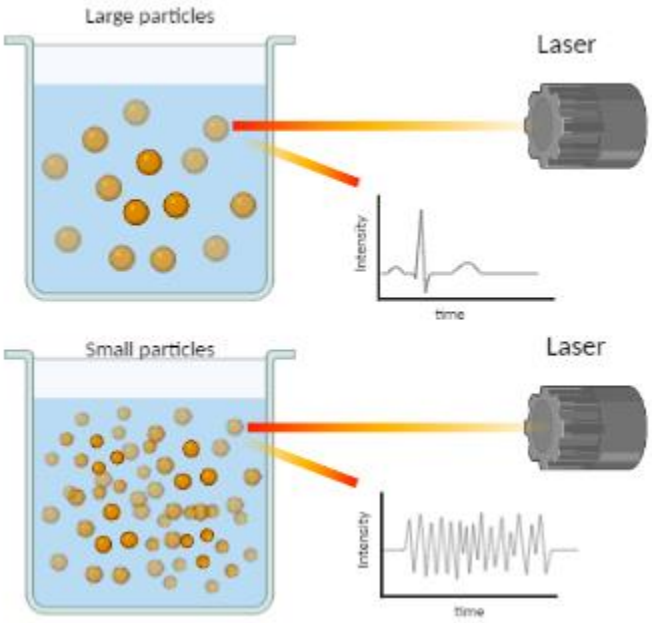


Figure 3: Diagram of the mechanisms of the DLS machinery. In samples with higher molecular sizes and density, it is possible to produce graphs with sporadic, larger peaks. In samples with smaller particles, on the graph produced by the DLS there will be more peaks as the laser bounces off the particles more times.

Adherent Cell Culture

Adherent cultures of Chinese Hamster Ovary (CHO) cells, CHO-K1, were maintained in accordance with the protocol outlined by Chatterton et al. (1999). The CHO-K1 cells were cultivated in adherent cell medium, comprising Ham's F-12 medium supplemented with 5% v/v foetal bovine serum (FBS) and 1% v/v penicillin/streptomycin (pen/strep, Gibco). The cells were incubated at 37°C in a 90% humidified atmosphere with 5% CO2 in T75

flasks, except where specified otherwise. Routine passages involved washing with Dulbecco's phosphate-buffered saline (Gibco), followed by trypsinization using trypsin/EDTA (Gibco) at 37°C for 5 minutes. The detached cells were then Reseeded in adherent cell medium.

Similarly, Mesenchymal Stem Cells (MSCs) were grown in adherent cell medium, consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS) and 1% v/v penicillin/streptomycin (pen/strep, Gibco). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO2 in T75 flasks, unless noted otherwise. For routine subculturing, cells were rinsed with phosphate-buffered saline (PBS, Gibco), then detached using trypsin/EDTA (Gibco) at 37°C for 5 minutes. The dissociated cells were subsequently reseeded in fresh adherent cell medium.

Suspension Cell Culture

Suspension CHO cells were cultured in either OptiCHO medium (Gibco), supplemented with 8mM Glutamax, 1% v/v pen/strep, and 1:1000 anti-clumping agent (Gibco), or FortiCHO medium (Gibco) supplemented with 8 mM Glutamax and 1% v/v pen/strep. Cultivation occurred in suspension T25 flasks for routine maintenance or Erlenmeyer flasks (125 mL or 250 mL) for experiments requiring a higher cell density. Flasks were placed on an orbital shaker at 125 rpm in a humidified incubator with 5% CO2. Cells were allowed to reach a density of approximately 3 x 106 cells/mL before passaging to 0.25 x 10^6 cells/mL. Monitoring involved regular assessments of cell density and viability using a Vi-Cell XR automatic cell counter (Beckman Coulter) after incubating 300 µL of cells with 300 µL TrypLE (Gibco) for 5 minutes at 37°C.

Cell Freezing and Revival

Frozen stocks of cell lines were created periodically. For adherent cells, grown in medium containing 5% FBS, cells at 100% confluency in 10 cm dishes were harvested by trypsinization and pelleted at 1,000 x g for 5 mins. Suspension cells were counted using a Vi-Cell XR automatic cell counter, and 15 million cells per vial were centrifuged at 1,000 x g for 5 minutes. Each cell pellet was resuspended in a 1.5 mL freezing medium consisting of either FBS with 10% v/v dimethyl sulfoxide (DMSO) or culture medium with 7.5% v/v DMSO for adherent and suspension cells, respectively. The cryovials were placed in a Mr Frosty™ freezing container (Gibco) at room temperature, stored at -80 °C for at least 24 hours, and then transferred to a liquid nitrogen dewar. For adherent cell revival, cryovials were thawed at room temperature and pipetted into a 10cm dish or T75 flask with 10 mL adherent cell medium. The following day, the medium was replaced with a fresh adherent cell medium. For suspension cell revival, cryovials were thawed by pipetting warm suspension cell medium onto the frozen pellet. The vial contents were gently aspirated into 5 mL medium, transferred to a 15 mL falcon tube, centrifuged at 1,000 x g for 5 mins, and the supernatant containing DMSO was discarded. The cell pellets were resuspended in 30 mL suspension cell medium and transferred into 125 mL shake flasks.

Electroporation of CHO-S cells:

To initiate the cell preparation process, cells were grown to a density of 1x10^6 cells/ml. Following this, cells were harvested with the aid of a centrifuge. Subsequently, cells were resuspended in an appropriate volume of either culture medium or electroporation buffer (Thermo Fisher Scientific). Moving on to the Neon™ Transfection System setup, adhering to the manufacturer's instructions, the system was configured, and the voltage set to 1700 volts for a single pulse. For the loading phase, CHO cells were mixed with the electroporation buffer in a sterile tube to achieve the desired cell concentration. The cell suspension was loaded into a sterile Neon™ Transfection System tube. The

electroporation step involves inserting the loaded tube into the electroporation chamber and executing the process at 1700 volts with a single pulse as specified. Following electroporation, the cells were carefully removed from the Neon™ Transfection System tube and transferred into a fresh culture dish or tube containing pre-warmed culture medium. Cells were then incubated under suitable conditions, which included a CO_2 incubator, if necessary, to facilitate their optimal growth and recovery. Subsequently, in the post-electroporation phase, cell viability and growth were monitored, making adjustments to the culture medium as needed to support the growth of transfected cells.

Chemical transfection of CHO-S cells using xtreme-gene:

In this chemical transfection protocol for introducing Yellow Fluorescent Protein (YFP) into CHO-S cells using XtremeGENE™ transfection reagent, CHO-S cells are ensured to be in logarithmic growth and have high viability. Cells were seeded to reach 70-90% viability on the day of transfection. A plasmid DNA solution containing the YFP gene was prepared using Opti-MEM. XtremeGENE transfection reagent was diluted in Opti-MEM, incubated, and mixed with the plasmid DNA solution. The transfection complex was incubated, the growth medium was aspirated from CHO-S cells and added to the transfection complex dropwise. Cells were incubated for 4-6 hours at 37°C. The transfection medium was substituted with complete growth medium and continued incubation for 24-48 hours for YFP expression. The transfection efficiency was evaluated using fluorescence microscopy.

Sonication of CHO-S cells:

In this sonication protocol tailored for CHO-S cells, the cells are prepared by ensuring they are in the desired growth phase and washed with phosphate-buffered saline before pelleting through centrifugation. The cell pellet is then resuspended in an appropriate buffer or medium for sonication, and the cell suspension is transferred to a sonication vessel. The sonication is performed using a sonicator operating at a frequency of 30 kHz for a duration of 10 seconds. Post-sonication, the sample is immediately placed on ice to

prevent excessive heating, and, if necessary, cellular debris can be separated from the supernatant through centrifugation.

Clariostar YFP fluorescence measurement of transfection efficiency:

This protocol for analysing the fluorescence absorbance of Yellow Fluorescent Protein (YFP) in transfected plates of CHO-S cells using the Clariostar plate reader, CHO-S cells are initially transfected with a YFP-containing plasmid, employing a suitable transfection method such as chemical transfection with XtremeGENE™. Following a 24–48-hour incubation period to allow for YFP expression, the transfected cells are seeded into 96-well plates and washed with phosphate-buffered saline (PBS). Subsequently, each well is filled with an appropriate volume of PBS or cell culture medium. The Clariostar plate reader is then set up, configuring fluorescence measurement settings with specified excitation and emission wavelengths for YFP (e.g., excitation: 488 nm, emission: 515-535 nm). Calibration of the plate reader using blank wells is performed before loading the prepared plate and initiating fluorescence absorbance measurements. The obtained data is exported for analysis, including normalisation and statistical assessments if necessary, using software such as Microsoft Excel. The interpreted data allows the assessment of YFP expression levels in transfected CHO-S cells.

Zeta potential - Malvern Zetasizer Nano ZSP

In this zeta potential analysis protocol, a solution is prepared by thoroughly mixing P188 with XtremeGENE™ transfection reagent in an appropriate buffer or medium, adjusting concentrations as needed. If necessary, the solution is diluted to an appropriate concentration for zeta potential analysis without compromising accuracy. The zeta potential analyzer is then initialised, calibrated using standard reference materials such as solutions containing specific ionic strengths, and set up with parameters such as measurement angle and temperature. Using disposable zeta potential cells, the mixed solution is loaded into the instrument, and zeta potential measurements are initiated, recording the values for the P188 and XtremeGENE™ mixed solution. Data analysis is performed using the instrument's software to calculate average zeta potential values and

assess the stability and charge distribution of the mixture. Results are interpreted in the context of electrostatic interactions between P188 and XtremeGENE™.

Note on Methodology:

All procedures were conducted under aseptic conditions in a laminar flow hood. Cultures were incubated in a CO_2 incubator at 37°C with 5% CO2 and humidity control. Sterile disposable pipettes, tubes, and culture dishes were used for all handling of cells and reagents.

Chapter 3: Understanding cell death mechanisms in bioreactors for enhanced monoclonal antibody production.

3.1 Introduction:

Biopharmaceutical manufacturing, especially the production of biologics using CHO cells, is testimony of the great advances made in bioengineering and cellular biology. CHO cells are one of the main production vectors in biologics production due to their ability in generating high yields of properly folded and glycosylated antibodies. However, the large-scale cultivation of these cells in bioreactors, even if it does offer controlled culture conditions and scalability, can present significant challenges, particularly concerning cell viability over time. The bioreactor environment, vital for biologics production, is designed to maintain as close as possible to optimal pH, temperature, osmolarity, and dissolved oxygen, which are critical for ideal cell growth and protein synthesis. Yet, the very environment created to enable cell growth, can paradoxically induce cell stress and cell death, primarily due to the mechanical forces generated during agitation and oxygenation processes (Chisti, 2001) (Raffals et al., 2019).

In bioreactors, oxygenation is essential for metabolic activities and is achieved through sparging, a process where air or oxygen is bubbled through the culture medium. While this process is crucial for maintaining adequate oxygen levels, the resultant bubble formation and bursting can generate shear stress, impacting cell viability negatively. Furthermore, the interface dynamics at the liquid-air boundary, characterised by constant bubble formation and collapse, can exert additional mechanical stress on the cells (Allen and Tresini, 2000) (Raffals et al., 2019). Shear stress can activate apoptotic pathways by disrupting cellular homeostasis and inducing oxidative stress, leading to mitochondrial dysfunction and the subsequent activation of pro-apoptotic signalling pathways (Goldblatt et al, 2021). Apoptosis, a form of programmed cell death, is crucial for maintaining cellular homeostasis and is characterised by a series of well-orchestrated morphological and

biochemical events. In the bioreactor environment, apoptosis can be triggered by various factors, including nutrient depletion, accumulation of metabolic by-products, and physical stress such as shear forces generated during agitation and sparging. Apoptosis induced by hydrodynamic forces in bioreactors involves the activation of specific signalling pathways.

3.1.1 Cleaved Caspase-3 (c-casp3) and LC3: markers for the fate of mammalian cells in bioreactors

As mentioned in section 1.5.3, shear stress in bioreactor can lead to mitochondrial membrane depolarization, the release of cytochrome c, and caspase activation, which are key mediators of apoptosis. The mitochondrial pathway (or intrinsic pathway) of apoptosis, is particularly relevant in bioreactor settings (Wang and Youle, 2009). This pathway is activated in response to internal stress signals, including DNA damage, oxidative stress, and disruptions in mitochondrial membrane potential, often resulting from the physical and biochemical stressors caused by bioreactor environments. The release of cytochrome c from mitochondria, a key step in this pathway, leads directly to the formation of the apoptosome and the subsequent activation of caspase-9, which then, in turn, activates caspase-3 (c-casp3) (Figure 1) (Parrish et al, 2013).

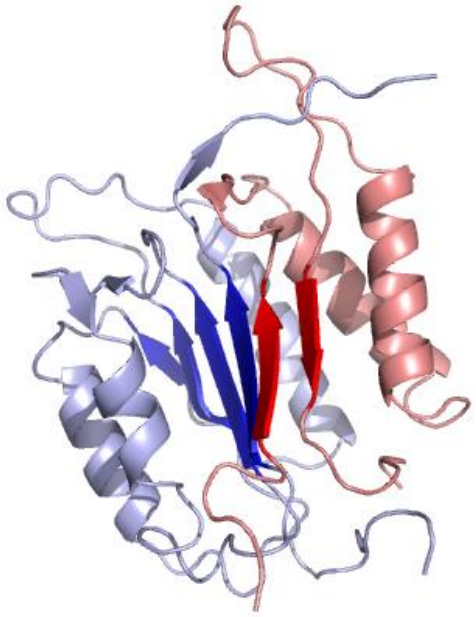


Figure 1: Structure of Cleaved Caspase-3. The p12 (pink) and p17 (light blue) subunits of caspase-3 with the beta-sheet structures of each in red and blue, respectively; image generated in Pymol from 1rhm.pdb.

Caspases are a family of cysteine proteases which play a very important role in the execution phase of apoptosis (Lavrik, 2005). The activation of initiator caspases like

caspase-8 and -9 leads to the downstream activation of executioner caspases, which include caspase-3. Caspase-3 is a protease enzyme; its active form consists of two large and two small subunits that associate to form a tetramer.

Caspase-3 is one of the executioner caspases as it is involved with the final stages of apoptosis, where it cleaves key cellular proteins and enzymes. Caspase-3 is synthesised as an inactive proenzyme called procaspase-3 (Mcilwain, 2013). The activation of procaspase-3 involves the proteolytic cleavage of the inactive enzyme into its active form. This cleavage event is typically mediated by initiator caspases, depending on the apoptotic pathway (extrinsic or intrinsic, respectively) (Figure 2), which, in turn, cleave procaspase-3 at specific sites (Parrish et al, 2013). The cleavage of procaspase-3 removes inhibitory domains and allows the large and small subunits to associate and form the active enzyme. Once activated, caspase-3 cleaves a wide range of cellular substrates, leading to cell fragmentation and death. C-casp3, is often used as a biomarker to assess the extent of apoptotic cell death in bioreactor cultures. The presence and activity level of c-casp3 serve as crucial indicators of the cellular response to the bioreactor environment and the effectiveness of cytoprotective strategies like the addition of P188 (Wurzer, 2003).

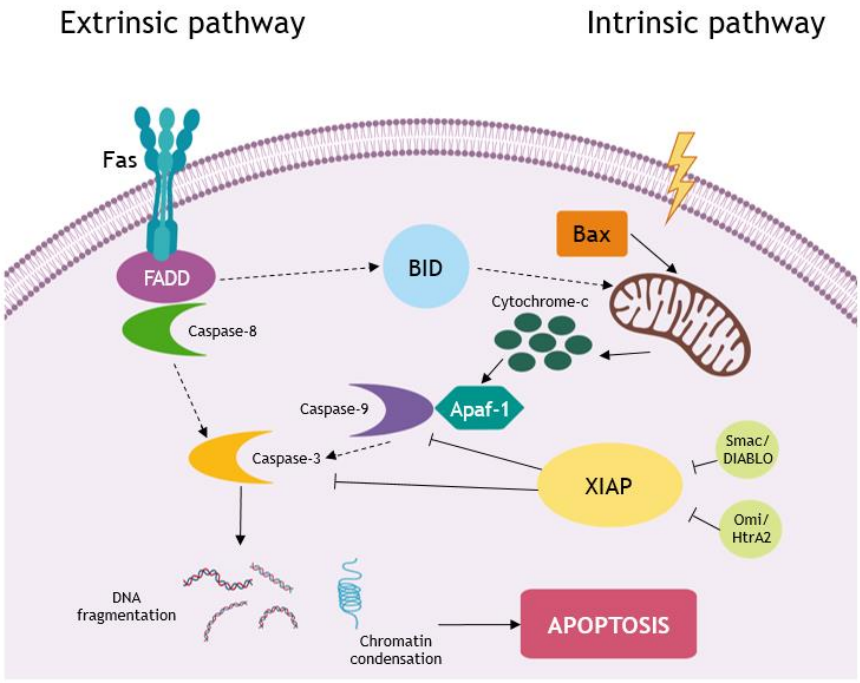


Figure 2: Diagram of apoptosis. The diagram includes intrinsic and extrinsic pathway with caspase mediation.

Another critical cellular process observed in bioreactor cultures is autophagy which serves as a mechanism for cell survival under stress conditions. This type of process involves the degradation and recycling of damaged or surplus cellular components, hence maintaining cellular homeostasis and energy balance.

Nutrient fluctuations, hypoxia, shear stress, and other bioreactor conditions, can induce the onset of autophagy. This process is often initiated as a protective response to environmental stress, which enables cells to adapt and survive under conditions which are not optimal for survivability. Autophagy can provide a temporary solution against cell death, allowing cells to withstand prolonged periods of stress (Yu and Klionsky, 2022). While autophagy primarily serves as a survival mechanism, excessive or dysregulated autophagy can lead to autophagic cell death, particularly when apoptotic pathways are inhibited or overwhelmed. In the context of bioreactors, balancing autophagy to ensure cell survival without tipping over into cell death is a delicate aspect of the study of cell viability in bioreactors.

In the process of autophagy, the protein Light Chain 3 (LC3) plays a very important role. It is a highly conserved cellular degradation and recycling process essential for cellular homeostasis and response to stress. LC3 is a ubiquitin-like protein that is involved in the elongation and closure of the autophagosome membrane. The structure of LC3 is characterised by two distinct forms: the cytosolic LC3-I and the membrane-bound LC3-II. LC3-I is synthesised as a precursor that undergoes post-translational modifications; specifically, it is cleaved at its C-terminus by the cysteine protease Atg4 to expose a glycine residue, which then forms LC3-I. When autophagy is induced, LC3-I is conjugated to the lipid phosphatidylethanolamine (PE) to form LC3-II (Mizushima and Yoshimori, 2007). The conjugation is facilitated by the E1-like enzyme Atg7 and the E2-like enzyme Atg3. The lipidation of LC3, resulting in the LC3-II form, is crucial for its insertion into the autophagosomal membrane. This conjugation is not only an essential part of autophagosome formation but also serves as a marker for the autophagy process, as the number of LC3-II correlates to the number of autophagosomes (Runwal et al, 2019).

In the context of bioreactors, the monitoring of LC3-II levels provides information about the levels of autophagic flux, which is a measure of autophagy progression. This is particularly important because the autophagic pathway, just like any other type of cell death, needs to be tightly regulated within bioreactors to ensure cell viability and optimal production yields. Autophagy can be triggered in response to various stimuli, such as nutrient deprivation, oxidative stress, and the accumulation of damaged proteins or organelles, conditions which are all common in bioreactor environments (Yun et al, 2020). The onset of autophagy in bioreactors has many implications for both cell survival and product quality. By mediating the turnover of cellular components and facilitating the recycling of nutrients, autophagy can support cellular adaptation and survival under the bioreactor's-controlled conditions. Additionally, autophagy plays a role in the quality control of proteins by degrading misfolded or aggregated proteins, which is crucial for the production of biopharmaceuticals where product integrity is crucial (Levine and Kroemer, 2008).

3.1.2 Poloxamer 188: a beacon of protection in shear-stressed environments

In bioreactor environments, P188, a copolymer composed of hydrophilic PEO and hydrophobic PPO blocks, is routinely used as a protector against cell death. Its surfactant properties enable it to reduce surface tension and interact with plasma membranes, thus offering a shield against the mechanical forces and shear stress. Studies have shown that P188 can integrate into plasma membranes, providing structural support and mitigating the damage induced by physical forces (Moloughney and Weisleder, 2012).

The mechanisms through which P188 exerts its protective effects are not well known or understood. One hypothesis state that P188 reduces shear stress and surface tension, by minimising the mechanical forces applied on cells and preventing membrane damage. This interaction is thought to stabilise the plasma membrane and enhance cell viability (Kwiatkowski et al, 2019).

Another theory suggests that P188 stabilises plasma membranes by embedding its hydrophilic PEO segments into the outer leaflet of the plasma membrane while the hydrophobic PPO segments extend into the surrounding medium. The described structural support to the plasma membrane may contribute to the prevention of membrane disruption under mechanical stresses, thus improving cell survival. Additionally, P188 is speculated to modulate the cellular response to oxidative stress, which means that it can potentially act as an antioxidant and reduce reactive oxygen species (ROS) levels, mitigating oxidative damage to the cells (Murphy et al., 2005).

3.2 Aims:

The aim of this chapter is to identify the underlying mechanisms of molecular protection that the copolymer P188 gives to mammalian cells under shear stress. Compared to a poloxamer free media and media to which known poloxamer impurities are added, the viability of the cells grown in the presence of P188 should be higher over time and the onset of known cell death mechanisms is delayed.

1. Understand the various forms of cell death that occur in CHO cell cultures when subjected to hydrodynamic forces.

- Investigate the specific triggers and molecular pathways involved in these processes, with an emphasis on how bioreactor conditions such as shear stress and oxygenation impact cell viability.

2. To Investigate the Protective Role of Poloxamer 188 (P188) in Bioreactor Environments:

- Examine how P188 can mitigate the adverse effects of mechanical forces on CHO cells during biologics production.

3. To Quantitatively Assess the Impact of P188 on Apoptotic and Autophagic Markers:

- Conduct targeted analysis to monitor the levels of key apoptotic and autophagic markers, particularly cleaved caspase-3 (c-casp3) and LC3, in CHO cells cultured in presence of shear stress.

3.4 Results and discussion:

3.4.1 Utilising baffled flasks as a scaled down version of bioreactors: Insights into shear stress effects and cellular behaviour in monoclonal antibody production:

In this study, the use of baffled flasks as a miniature model of bioreactors is driven by several key rationales, which align closely with the objectives of understanding cell behaviour and optimising biologics production in bioreactor environments.

Baffled flasks are designed to mimic the hydrodynamic conditions prevalent in large-scale bioreactors. The presence of baffles in these flasks creates a more turbulent environment compared to standard smooth-walled flasks (Figure 3). This turbulence enhances mixing and oxygen transfer, closely resembling the conditions in bioreactors where agitation and sparging are used for similar purposes. The replication of these conditions is crucial for studying the impact of shear stress and mechanical forces on cell viability and function. One of the primary challenges in bioreactor-based biologics production is the shear stress exerted on cells due to agitation and gas-liquid interactions. Baffled flasks generate increased shear forces compared to regular flasks, making them an appropriate model for studying how CHO cells respond to shear stress in bioreactors. This includes investigating stress-induced cell death pathways like apoptosis and autophagy (Zhan et al, 2020). The ability to model these stresses in a smaller and more controlled setup allows for a more detailed and manageable investigation.

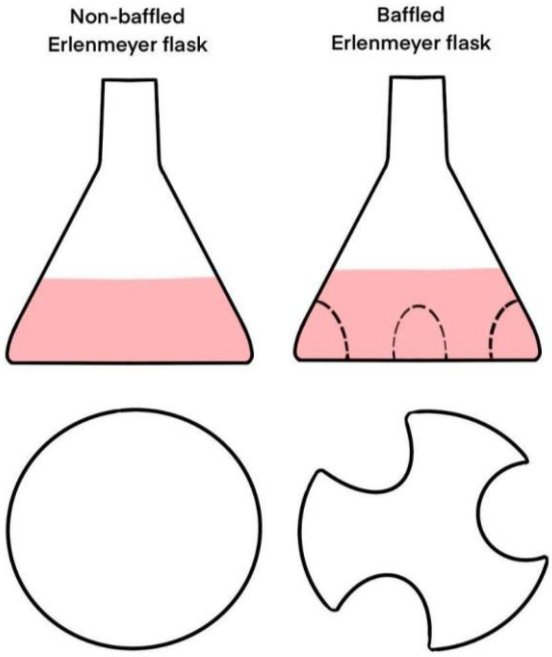


Figure 3: Differences between baffled and unbaffled flasks. The image shows different styles of Erlenmeyer flask bottom design.

Compared to full-scale bioreactors, the use of baffled flasks offers practical advantages in terms of ease of handling, manipulation, and sampling. It enables researchers to conduct multiple parallel experiments under varying conditions, such as different levels of P188 concentration, to observe its effects on cell survival and productivity (Nguyen and Wang, 2018). Moreover, baffled flasks allow for a detailed investigation of how the physical environment due to their design (Figure 3), specifically the enhanced mixing and oxygen transfer, impacts cellular processes. The flexibility of these flasks is essential for a thorough and efficient exploration of the variables involved in bioprocess optimization. For this study, employing baffled flasks is more cost-effective and time-efficient than using full-scale bioreactors for preliminary experiments. This approach is particularly beneficial in the early stages of research as it allows multiple iterations and optimizations steps (Allen and Tresini, 2000). Studies conducted in baffled flasks have already provided valuable insights into how these scaled down models work (Peng et al., 2014). The ability to translate findings from small-scale models to larger bioreactor systems is crucial for

the development of practical and effective strategies in industrial biologics production (Chisti, 2001). This understanding is key to developing strategies that enhance cell viability and productivity in industry and mitigate the negative effects of shear stress in industrial-scale bioreactor cultures (King and Miller, 2017).

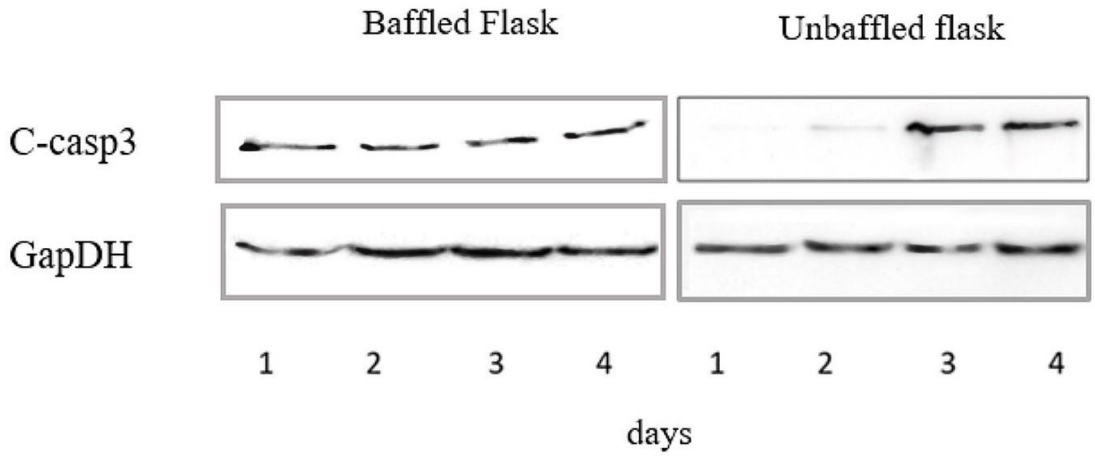


Figure 4: Immunoblot analysis of c-casp3 signalling: indicating apoptosis onset in cells subjected to increased shear forces vs. smooth-bottom flask Cultures. The samples were taken from CHO cell suspended cultures, grown in Freestyle growth medium in baffled flasks. The immunoblot shows the signal of c-casp3, which gives information about the onset of apoptosis in cells grown under an enhanced influx of shear forces compared to the ones grown in flasks with a smooth bottom.

The difference in the onset of the signal for c-casp3 between cells grown in unbaffled and baffled flasks suggests that there are factors influencing the cellular processes related to apoptosis as well as a change in cell growth and viability, in these two conditions (Figure 4).

Baffled flasks are designed to improve mixing and oxygenation of the culture medium. The increased oxygen availability and better nutrient distribution in baffled flasks might lead to faster cell growth and more efficient cellular processes. The appearance of c-

casp3 signal indicates the activation of caspase-3 (Figure 4), an enzyme involved in the latest stages of apoptosis. The earlier appearance of c-casp3 in baffled flasks suggests that apoptosis might be triggered earlier in these cells. This early onset could be due to various factors, including stress from higher cell density or faster depletion of nutrients, as well as mechanical damage caused by the enhanced turbulence in the media. The delayed appearance of c-casp3 in unbaffled flasks might imply that these cells experience less stress or progress more slowly towards apoptosis. This delay could be due to a more favourable environment in an unbaffled flasks microenvironment, better nutrient availability, or reduced cellular crowding and most importantly, less shear stress.

3.4.2 Role of surfactant P188 in mammalian cell culture media: impact on apoptosis and quantification in Gibco Freestyle CHO Expression Media

Surfactants are routinely added to mammalian cell culture media for a variety of reasons. Primarily, surfactants are aimed at improving cell culture conditions and enhancing the health of the cells with which they interact. Surfactants are compounds that have both hydrophilic and hydrophobic chains and chemical properties, which allow them to reduce surface tension and facilitate interactions between hydrophobic molecules and liquid interfaces. Surfactants can help improve the mixing and distribution of nutrients, gases (like oxygen and carbon dioxide), and other essential factors within the culture medium as they interact with the surface tension (see chapter 1). This promotes a more uniform and efficient cell growth by preventing nutrient gradients and ensuring adequate oxygenation.

Surfactants are able to inhibit the clumping or aggregation of cells, which can occur due to adhesive interactions between cells or between cells and the culture vessel and the liquid media surface. Preventing aggregation helps maintain consistent cell growth and minimises the risk of cell death due to overcrowding and, in case of foam formation, asphyxiation. Surfactants can help control the formation of foam during the agitation of culture media. Excessive foam can interfere with oxygen transfer, nutrient exchange, and

pH control, and it can also lead to cell damage or contamination. Due to these reasons, many commercially available cell media already have P188 in their composition. However, it is difficult to assess the exact concentration. To better understand the P188 mechanisms of protection it was necessary to confirm and assess the presence of P188 in commercially available media and utilise a surfactant free grade media in the cell death studies.

The onset of apoptosis was tested in CHO cells grown in the commercially available Gibcho Freestyle CHO expression media as well as a custom made special P188 free grade one.

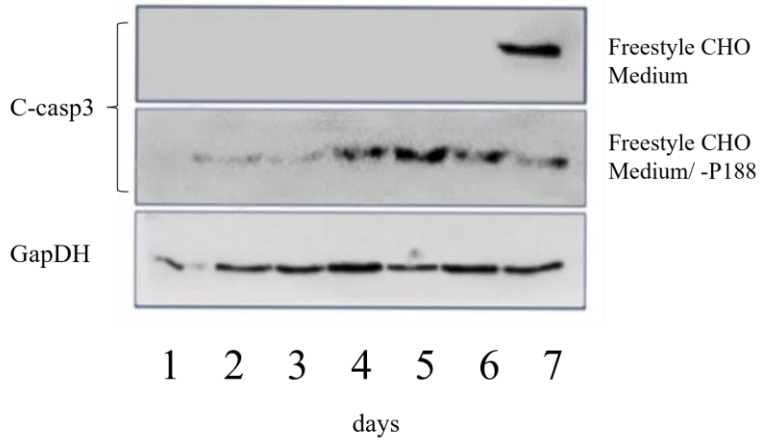


Figure 5: Immunoblot analysis: impact of P188 on apoptosis initiation in CHO cells. The impact of P188 on the apoptosis onset in CHO cells in this figure presents a representative immunoblot depicting the onset of apoptosis in CHO cells. Apoptosis is identified by visualising the signal for c-casp3 in cells grown using Gibco Freestyle CHO expression media in baffled flasks, with and without the addition of P188 surfactant. The data in the immunoblot highlights differences in the appearance of the c-casp3 signal between the two different conditions. The presence of P188 in Freestyle CHO media appears to delay the appearance of the c-casp3 signal, suggesting that P188 may play a role in inhibiting or slowing down the activation of caspase-3.

The observed difference in the cleaved c-casp3 signal between cells cultivated in Freestyle CHO media, with and without P188, implies an influence of P188 on the mechanism of apoptosis, P188 may play a role in either impeding or decelerating the activation of caspase-3 (figure 5), and thereby the apoptotic pathway, potentially pointing to a cytoprotective effect of P188. Conversely, in cells grown in the absence of P188, the detection of the c-casp3 signal from day 1 suggests an earlier execution of apoptosis. This observation might also indicate that the degree of apoptosis suppression is contingent on the P188 concentration in the media.

To aid this data, DLS was used to quantify the micelle intensity on spiking the P188 free media to create a calibration curve. These calibrations were made with both purified P188 from Croda and Kolliphor P188 and allow the quantification of P188 in the standard Freestyle media.

3.4.3 Calibration curve for P188 in commercially available CHO cell growing media.

Gibco Freestyle CHO Expression Media, designed for culturing CHO-S cells, includes the addition of P188 in its formulation. However, the precise concentration of P188 remains undisclosed due to the proprietary nature of the media recipes. This study utilises both the standard Gibco Freestyle CHO Expression Media containing P188 and a custom-prepared variant lacking any addition of P188. Determining the P188 concentration in the standard media is challenging using conventional absorbance or chromatographic techniques, largely due to the media's complex composition and the P188's intrinsic chemical structure variability.

In this experiment, DLS was employed to assess the micelle intensity by spiking the P188-free media. Calibrations were performed using both Croda's purified P188 and Kolliphor P188 from BASF. This approach enabled the quantification of P188 in the standard Freestyle media, facilitating a comparative analysis of the data.

The calibration curves were calculated by analysing the derived count rate associated with the 5 nm peak, which corresponds to P188. To isolate the intensity of this peak, the overall derived count rate was divided by the percentage intensity of the 5 nm peak (Figure 6, Table 5). A linear correlation was observed within a concentration range of 0.01% to 0.5% w/w. This correlation, aligned with the observed CHO-S cell density and viability, points to the fact that incorporating 0.15% w/w of P188 into the media yielded outcomes that were more comparable to the standard Freestyle media than the addition of 0.1% w/w to media lacking P188.

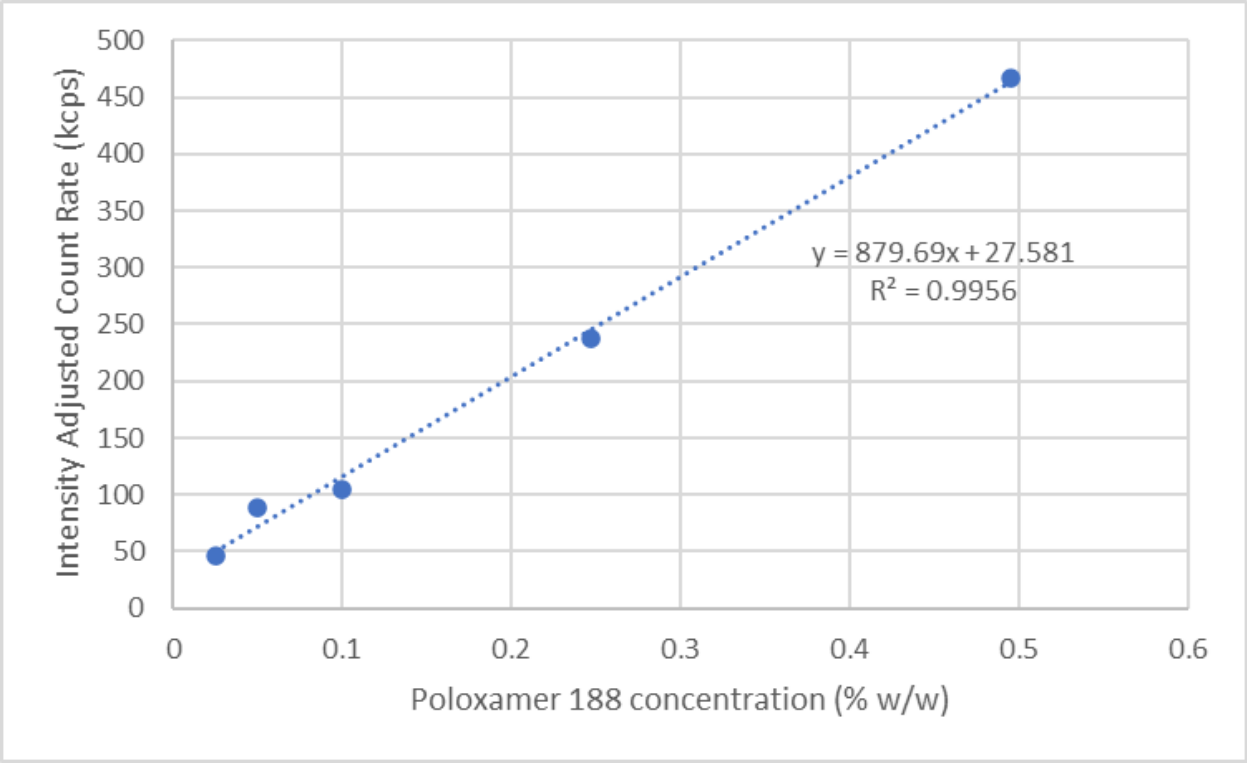


Figure 6: **Poloxamer 188 calibration curve in P188-enhanced Gibco freestyle Media.** The calibration curve is based on the poloxamer 188 peak in purified P188 spiked poloxamer free Gibco Freestyle Media.

Freestyle Sample Result	Peak Size (nm)	Peak Intensity (%)	Derived Count Rate (kcps)	Adjusted Count (kcps)	Calculated Poloxamer 188 Concentration
Set 1	Calibration based on purified Croda P188				
Run 1	6.51	81.9	189.3	155.0	**0.166**
Run 2	5.98	72.5	206.2	149.5	**0.159**
Run 3	6.19	68.9	227.4	156.7	**0.168**
Set 2	Calibration based on Kolliphor P188				
Run 1	5.763	87.6	209.1	183.2	**0.177**
Run 2	6.097	82.6	221.3	182.8	**0.176**
Run 3	5.398	87.9	208.2	183.0	**0.177**
Run 4	5.358	84.5	204.4	172.7	**0.165**

Table 5: **Peak Size, Intensity, and Adjusted Count Rate of Poloxamer 188 in Gibco Freestyle Media.**

The P188 content in the standard Gibco Freestyle Media ranged from 0.159% to 0.168% w/w when compared to Croda purified media, and from 0.165% to 0.176% w/w when compared to Kolliphor P188 as the spiking standard. Although the literature suggested an expected level of 0.1% w/w (1g/L), this finding coincides with CHO-S cell density and viability studies I carried out. Specifically, the ones which show that adding 0.15% w/w of P188 to the media produced results more akin to the standard Freestyle media compared to when 0.1% w/w was added to P188-free media.

3.4.4 Effect of surfactants on cell death mechanisms and autophagic activity in CHO-S cell cultures: implications for cellular stress responses

In order to understand the underlying mechanisms of cell protection which are supposedly granted by the addition of surfactants, particularly P188, multiple markers for cell death mechanisms were tested on CHO-S cell cultures. A selection of poloxamers was used to test different conditions in the cell cultures, other than P188. These other poloxamers represent known impurities which can occur in the industrial manufacturing process, during the polymerisation of P188. Some of these impurities are known for being detrimental to cell viability, either by triggering cellular death pathways or by failing to protect the cell as efficiently as pure surfactants such as P188.

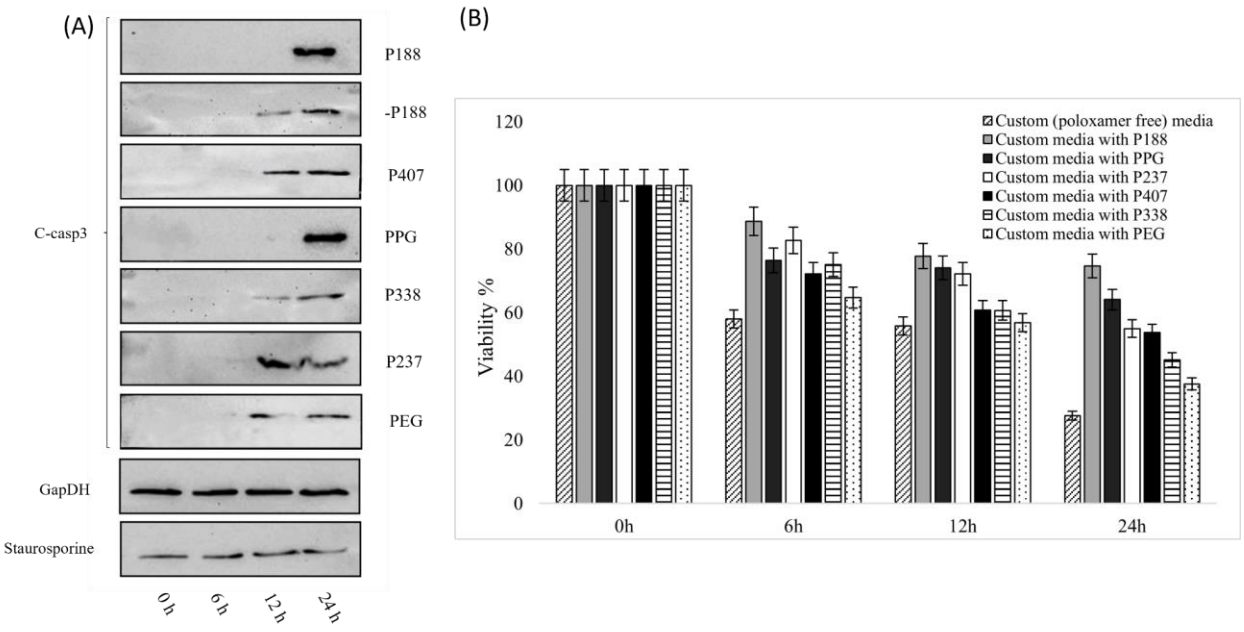


Figure 7. Onset of apoptosis in CHO-S cells under various surfactant regimens. The figure displays the results of experiments examining the onset of apoptosis in CHO-S cells grown under different surfactant regimens, including the addition of poloxamers other than P188 at 1.5g/L, which are used to represent known impurities. The immunoblot (Figure 4, Panel A) shows the activation of the apoptotic marker c-casp3 at different time

points (0h, 6h, 12h, 24h). The histogram (Figure 7, B) represents the recorded viability of the cell samples taken for the immunoblot over a 24-hour period, at the same intervals as the immunoblot. The data suggests variations in the time it takes for caspase-3 to be cleaved in response to different surfactants, indicating potential differences in how these surfactants interact with apoptosis-related mechanisms. Factors such as molecular weight and polymer composition of surfactants influence the rate of caspase-3 activation.

The immunoblot data shows the time points at which the signal for c-casp3 appears in cells grown in the presence of different known surfactants. The data suggests that there is variability in the time it takes for caspase-3 to be cleaved (activated) in response to the impurities. Surfactants like P237, P407, and P338 lead to caspase-3 activation after 12 hours, while the caspase activation in samples grown in P188 and PPG takes up to 24 hours. This suggests that different surfactants might be interacting with the mechanisms of apoptosis cell stress and apoptosis at different rates. The molecular weight and composition of the surfactants appears to dramatically influence the rate of caspase-3 activation. Surfactants with higher molecular weights (P338, P407) and those containing a higher percentage of PEO (Table 4) do not seem to protect the cell as efficiently and lead caspase-3 activation earlier. This could imply that larger or more hydrophilic surfactants might have a stronger impact on cellular processes leading to apoptosis. On the other hand, surfactants with higher percentage of PPO (polypropylene oxide) content, like P407 and P338, induce caspase-3 activation earlier despite their high molecular weight. This indicates that the type of polymer present in the surfactant might play a role in triggering cellular responses.

The variations in caspase-3 activation times could be indicative of differences in the mechanisms by which these surfactants react and interact with cellular stress and apoptosis. It's possible that surfactants with earlier caspase-3 activation times exert stronger effects on cellular membranes, cytoskeleton, or other intracellular components, leading to faster apoptotic responses.

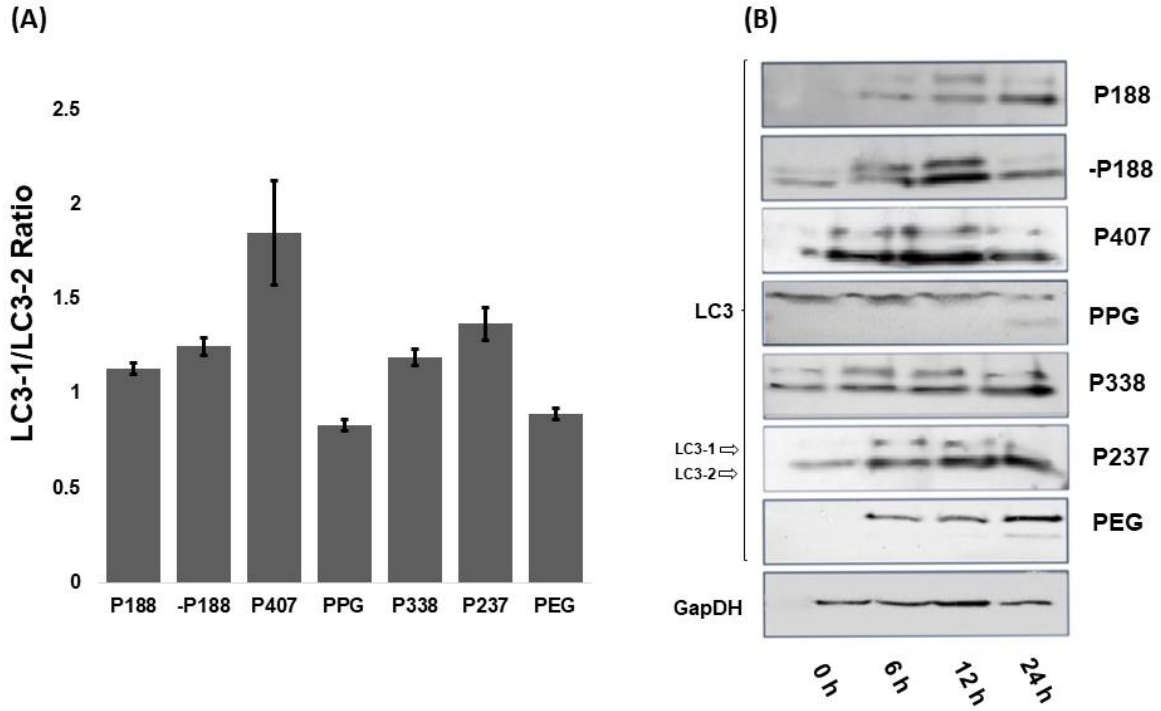


Figure 8: Autophagic activity in CHO-S cells under multiple conditions

Western blot analysis and quantification of the: (A) ratio between LC3 1 and lc3 2 signal quantification; and (B) Western blot analysis showing the expression of LC3 protein in hFOB 1.19 cells treated with 1.5g/L of study poloxamers, P188, P407, PEG, P338, P237, PPG for 0, 6,12 or 24 h. Values are means ± SEM, n = 3.

The data shows the time points at which LC3-1 and LC3-2 signals are detected in cells grown in shake flasks and in the presence of different surfactants. LC3-1 and LC3-2 are key proteins involved in the autophagy process, and their detection in immunoblots can provide information about the autophagic activity within the cells. Autophagy is a cellular process responsible for recycling cellular components and maintaining cellular homeostasis, and it can be influenced by various factors, including shear stress, which can be ameliorated by the studied poloxamers.

Surfactants with higher PEO content and high molecular weight PEO, seem to cause a delay in LC3-1 and LC3-2 signal appearance, suggesting a potential role in delaying autophagic activity. This delay could indicate prolonged autophagosome formation and maturation. Higher molecular weight surfactants (P338, P407, high molecular weight PEO) seem to have a more delayed effect on LC3-1 and LC3-2 signals, which could be related to slower diffusion or cellular uptake due to their larger size. The presence of PEO is associated with delayed autophagic responses (LC3-1 and LC3-2) compared to the presence of PPO (Figure 8, B).

The differences in the timing of LC3-1 and LC3-2 signal appearance suggest potential variations in the autophagy pathway, related to initiation, autophagosome formation, and maturation stages. LC3-1 and LC3-2 are two forms of the same protein, LC3, which plays a crucial role in the autophagy pathway. LC3 is initially synthesised as LC3-I and is subsequently converted to LC3-II through lipidation during the autophagic process. LC3-II is associated with autophagosome membranes and is commonly used as a marker for autophagy (Mintsushima and Yoshimori, 2007).

In the immunoblots, the differences in the timing of LC3-1 and LC3-2 onset help identify the stages of the autophagic pathway that are influenced by the presence of the surfactants. In some cases (P237 and P407), the LC3-1 signal is detected earlier than the LC3-2 signal, suggesting that the conversion from LC3-1 to LC3-2 might be delayed compared to the initiation of the autophagic pathway. This could imply that the formation of autophagosomes is initiated before the full maturation of autophagosomes with lipidated LC3-2. In other cases (P338 and -P188), the LC3-2 signal is detected earlier than or simultaneously with the LC3-1 signal, indicating that the conversion to LC3-2 could be rapid upon initiation of autophagy. Overall, the differences in LC3-1 and LC3-2 signal appearance times may reflect variations in the kinetics of autophagosome formation, LC3-1 to LC3-2 conversion, and autophagosome maturation influenced by the presence of different surfactants.

The LC3 quantification data (Figure 8, A) shows that surfactants with higher PEO content and molecular weight, such as P407 and P338, exhibit elevated LC3-1/LC3-2 ratios,

indicating a delay in the conversion of LC3-I to LC3-II. This delay suggests a slower progression through the autophagy pathway, particularly in the formation and maturation of autophagosomes. In the context of cells under shear stress, such as those found in bioreactors or in this case baffled flasks, this delay could have important molecular implications. Autophagy plays a critical role in cellular homeostasis by removing damaged organelles and proteins, especially under stress conditions. A delayed autophagic response could lead to an accumulation of damaged cellular components, as the slower formation and maturation of autophagosomes would impair the cell's ability to efficiently recycle and remove these components (Braasch et al., 2021).

This accumulation might exacerbate cellular stress and could potentially lead to increased cell death, particularly in a high-stress environment like a bioreactor. Cells under shear stress already face mechanical challenges, and an impaired autophagic response might hinder their ability to cope with additional stressors, leading to reduced cell viability and productivity. On the other hand, surfactants like PPG and PEG, which show lower LC3-I/LC3-II ratios, suggest a more efficient autophagic process. In these cases, cells might be better equipped to manage and survive shear stress by promptly clearing damaged cytosolic components, and therefore maintaining cellular function and viability.

These findings could show that the different surfactants might affect specific stages of the autophagic pathway. However, further experiments and detailed analyses are necessary to confirm these hypotheses and to understand the precise mechanisms by which the surfactants influence autophagy and its various stages. The observed variations in signal appearance times may reflect differences in autophagy regulation, including the formation of autophagosomes and their subsequent maturation.

3.4.5 Other markers of cell death in CHO cells under shear stress:

In addition to assessing markers for apoptosis and autophagy, I also attempted to detect the involvement of the necroptosis and apoptosis pathways by blotting for MLKL (Mixed Lineage Kinase Domain-Like Protein), caspase-9, and caspase-8. MLKL is a executor of necroptotic pathway, a form of programmed necrosis triggered by cellular stress, leading to inflammatory cell death (Martinez-Osorio et al., 2023). Caspase-9 is an initiator of the intrinsic apoptosis pathway, which is subsequently activated by internal signals such as DNA damage, oxidative stress, or other intracellular stressors, leading to mitochondrial outer membrane permeabilization and subsequent activation of downstream caspases, like caspase-3. Caspase-8 plays a vital role in the extrinsic apoptosis pathway, which is initiated by external signals, such as shear stress or ligand binding to death receptors on the cell surface (Van Opdenbosch & Lamkanfi, 2019).

Examining these pathways is important because cell death in CHO-S cells under stress conditions could involve multiple mechanisms. The intrinsic apoptosis pathway is particularly relevant because it is directly triggered by internal cellular damage, while the extrinsic pathway is pertinent due to potential shear stress in bioreactor conditions. Identifying activation of caspase-9 and caspase-8 would help confirm that the cell death observed is due to intrinsic and extrinsic signals, respectively.

Despite these efforts, the results for MLKL, caspase-9, and caspase-8 were inconclusive. This lack of clear data does not necessarily indicate that these pathways are not activated or relevant, studies have found that necroptosis and other non-apoptotic cell deaths can occur to CHO cells grown in bioreactors (Mentlak et al., 2023). These challenges highlight the complexity of cell death mechanisms and suggests that further investigation is necessary to fully understand the roles of necroptosis and both intrinsic and extrinsic apoptosis in this setting. Notably, apoptosis was only corroborated with the detection of cleaved caspase 3, indicating downstream activation of apoptotic pathways without pinpointing the exact initiating signals. The findings highlight the need for more sensitive techniques or alternative approaches to investigate the potential contributions of these pathways to cell death in CHO-S cells under stress conditions, as understanding these

mechanisms could lead to better strategies for protecting cells and enhancing the efficacy of biopharmaceutical production.

3.5 Conclusions:

The aim of the chapter was to identify mechanisms through which the addition of P188 affects the cell survivability in cultures when they are under mechanical stress. The reason for this investigation is to try and discover and characterise the mechanisms of cell protection afforded by poloxamers in presence of hydrodynamic forces, specifically in bioreactor environments. In order to do so, a scaling down model of bioreactors has been used, through the employment of baffled Erlenmeyer flasks, which geometry induces more sparging and shearing forces into the media. The presence of baffles in the flasks appears to enhance mixing and oxygen availability, leading to faster cellular processes such as apoptosis. Baffled flasks might be more suitable for cultures that require enhanced nutrient distribution and oxygenation but in the scope of these experiments they provide a useful way to add shear forces to CHO cell cultures.

The data (Figure 4) suggests that the cellular response to triggering apoptosis is influenced by the culture environment. Cells which are grown in baffled flasks exhibit a more rapid apoptotic response, which can be explained by the increased sparging. Researchers should take into account the influence of culture vessel design (baffled vs. unbaffled) when designing experiments related to cell growth, viability, and apoptosis. The choice of flask design can impact the timing and extent of specific cellular processes. The data suggests that the presence of baffles in the flasks affects the timing of the onset of c-casp3, indicating differences in apoptosis dynamics as well as growth and viability, rendering the flask a suitable model for the reproduction of hydrodynamic forces on a small scale.

When looking at commercially available media, the presence of p188 in Freestyle CHO media delays the appearance of c-casp3 (Figure 5), indicating a potential role in the inhibition of the apoptotic pathway, which explains the polymer's wide use in biotechnologies. These findings have implications for understanding the role of p188 in cellular processes and might have relevance for both basic research and potential uses in industry. When investigating cell death mechanisms, especially apoptosis, the data indicates that different surfactants, characterised by their molecular weight and PEO/PPO

content, induce caspase-3 activation in cells at different time points with surfactants with a higher MW and PPO% contributing to an earlier onset of apoptosis. This information aids to the understanding on how P188 affects cellular viability and apoptosis, and further research is required to fully explore the underlying mechanisms and potential applications. This data suggests correlations between surfactant characteristics and cellular responses. However, more research is needed to understand the exact mechanisms by which these surfactants induce apoptosis and how the molecular weight, PEO/PPO content, and other factors contribute to these effects.

The LC3 immunoblot (Figure 8) data strongly suggests that the presence of different surfactants can influence the dynamics of autophagy within cells. The observed variations in the timing of LC3-1 and LC3-2 signal appearance indicate that surfactants have the potential to affect different stages of the autophagic pathway. Surfactants containing higher percentages of PEO, such as P188, P237, P338, and P407, tend to exhibit delayed LC3-1 and LC3-2 signal appearance. This suggests that the PEO component might play a role in slowing down autophagy-related processes. The molecular weight of the surfactants also seems to correlate with the delay in autophagic responses. Higher molecular weight surfactants like P338 and P407 show an earlier onset of LC3-1 and LC3-2 signals compared to lower molecular weight surfactants. The different timing of LC3-1 and LC3-2 signal appearance suggests that surfactants might influence different stages of autophagy. Some surfactants seem to affect autophagosome initiation, while others might impact autophagosome maturation and LC3-1 to LC3-2 conversion. Understanding how surfactants influence autophagy could not only have implications in the maintenance of cell viability in biologics production but also therapeutic implications. Autophagy dysregulation is associated with various diseases, including neurodegenerative disorders and cancer. Manipulating autophagy through surfactant treatment might offer a way to modulate cellular responses in certain contexts. It would be still necessary to conduct more experiments to confirm these findings and to investigate the specific mechanisms by which surfactants impact autophagy. Additionally, investigating the effects of surfactants on other autophagy markers and pathways could provide a more comprehensive understanding.

P188 appears to positively contribute to the maintenance of high levels of cell viability in environments where cells are subjected to enhanced hydrodynamic forces. From both the viability and the immunoblot data of autophagy and apoptosis it is evinceable that the addition of 1.5g/L of purified P188 helps sustain cell viability over time and delays the onset of apoptosis compared to a poloxamer free media. It is also clear that other surfactants which can even occur as contaminants during the synthesis of P188, fail to protect cell viability with the same efficiency of P188 and do not delay the onset of cell death mechanism in the same way.

Even if the data shows promise, it is clear that more than one cell death pathway is taking place at the same time in the cultures. In the future it would be beneficial to analyse other types of cell death, specifically necroptosis or ferroptosis as to further the knowledge in the mechanisms of cell protection granted by the addition of P188.

Chapter 4: Surfactant quality assessment: the impact of impurities on the surface tension of liquid interfaces and their micellization of P188

4.1 Introduction

4.1.1 Poloxamer 188 in monoclonal antibody production: impurities and manufacturing challenges

Monoclonal antibodies are an incredibly valuable class of therapies that can be produced in industry using biological vessels, one of the most popular being CHO cells.

Biologics and mAbs represent a significant breakthrough in both therapeutic and diagnostic applications in biomedicine. This therapeutic class of drugs is produced by identical immune cells cloned from a single parent cell; the antibodies produced by these cells are highly specific to their target antigens. The production of biologics in bioreactors is a complex and carefully controlled process (Vulto and Jaquez, 2017). It begins with the selection of a specific cell line, typically derived from mammalian cells like CHO cells, which are genetically engineered to produce the desired antibody. These cells are then cultivated in bioreactors, sophisticated stirring tanks that provide an optimal environment for cell growth and antibody production (Li et al, 2013). The bioreactors maintain precise control over conditions such as temperature, pH, oxygen levels, and nutrient supply, ensuring high yield and quality of the antibodies. The process is closely monitored and adjusted to maximise the cell efficiency and the purity of the products. After cultivation, the antibodies are harvested and purified through several filtration and chromatography steps (Liu et al., 2010). The ability to produce monoclonal antibodies in bioreactors has revolutionised the field of biotechnology, enabling the mass production of these critical components for a variety of medical and research applications.

Bioreactors' environments have been studied and optimised for many years in order to maximise production of monoclonal antibodies. One of the main challenges remains the maintenance of high viability in CHO cells over time. In the bioreactor environment, mammalian cells are subjected to multiple stresses, including stress from hydrodynamic shear forces, foam formation and gas bubbles bursting on the culture's liquid surface. A certain degree of hydrodynamic forces is inevitably used to circulate oxygen supply and feeds. This leads to a need for consistent sparging and mixing forces to be applied to the culture (Frahm et al, 2009). To ameliorate the mechanical stress that the mammalian cells are subjected to in large scale production, a range of additives can be added to the media. One of the most popular additives used to control shear damage is P188 (Figure 1) (Chen *et al.*, 2022). P188 has for many years proven to be beneficial for the support of high cell viability in industry, although the underlying mechanisms of protection are still largely unknown (Peng et al., 2016). Current evidence suggests that P188 provides a mechanical, chemical and biological protection of the cells, when they come in contact with this polymer (Peng et al., 2014). P188 is thought to lower the surface tension between the plasma membrane and the turbulent liquid environment of the bioreactors as well as physically inserting in damaged plasma membranes, protecting them from further shear damage and bubble burst damage (Chang et al., 2017).

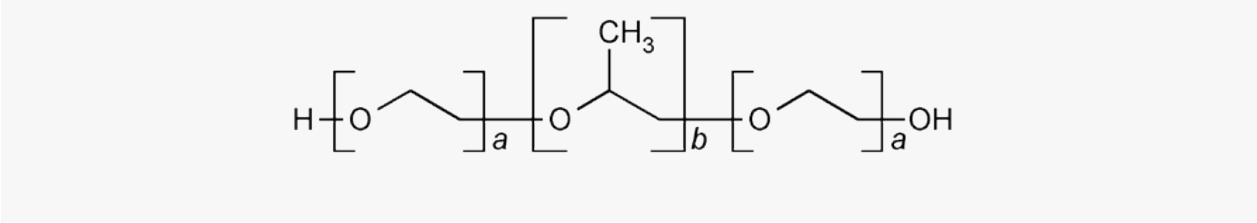


Figure 1: General chemical structure of Poloxamers. a. represents flanking chains of hydrophilic polyoxyethylene and b. represents the central chain of hydrophobic polyoxypropylene.

P188 belongs to a class of non-ionic copolymers known as poloxamers. Poloxamers are triblock copolymers composed of three main polymeric chain blocks, a chain of hydrophobic polyoxypropylene (PPO) flanked by hydrophilic polyoxyethylene (PEO) chains. The poloxamer group of surfactants contains more than 50 different compounds, all built from the same triblock (Figure 1). These different poloxamers can have a

drastically diverse range of molecular weight distribution and percentage of PPO which influences their surface tension reduction and water solubility (Alexandridis et al., 1994) (Steiner et al., 1964). Poloxamer physical and chemical properties are largely defined by the polymerisation process and can impact their performance on cell culture. Poloxamer polymerisation is a complex process which makes it difficult to assess each batch's molecular weight distribution and allows for non-specific polymerisation of contaminants: the variation of ratio between the two main chemical chains that make up the polymer can cause batch-to-batch variation in P188 protective effectiveness.

The first step in the P188 manufacturing process is the use of sodium and potassium hydroxide to catalyse polymerisation of propylene glycol through alkoxylation. Propylene oxide (PO) is then added to the reaction for propagation, and it will start increasing the molecular weight (MW) of the compound until the required MW is reached. The other building polymer of P188, ethylene oxide (EO) is then added, and the reaction comes to an end. Due to the process of polymerisation, it is hard to purify P188 and also to assess the Gaussian distribution regions of MW, which are heavily reliant on the manufacturing process (Emanuele and Balasubramaniam, 2014) (Figure 2).

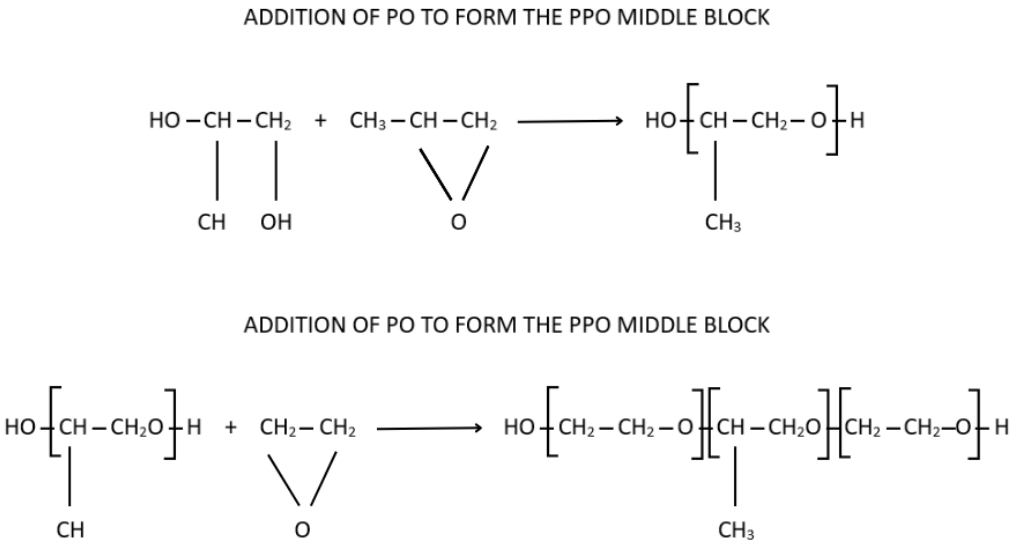


Figure 2: The diagram illustrates the general synthesis process of poloxamers, highlighting the formation of the polypropylene oxide (PPO) middle block. The process begins with the addition of propylene oxide (PO) to a hydroxyl-terminated initiator, typically a polyethylene glycol (PEG) derivative. The reaction proceeds via the opening of the PO's epoxy group, resulting in the sequential extension of the polymer chain with PPO units. This forms the central hydrophobic segment of the poloxamer, flanked by hydrophilic polyethylene oxide (PEO) blocks, creating the characteristic amphiphilic block copolymer structure.

The propensity to develop impurities can be attributed to several factors related to the chemical structure of P188 and poloxamers in general and their synthesis. Since the synthesis of P188 typically involves the anionic polymerization of ethylene oxide and propylene oxide, the process can lead to variations in the molecular weight and composition of the polymer chains, resulting in batch-to-batch inconsistencies. Impurities can also arise from incomplete reactions, side reactions, or the presence of catalyst residues. The polymerization reaction is sensitive to conditions such as temperature, pH, and the purity of the monomers (Šrom et al., 2022). Even a slight deviation in these conditions can lead to the formation of by-products or incorporation of unreacted monomers, contributing to the formation of impurities. Post-synthesis, P188 can also adsorb impurities from the environment or containers due to its surfactant-like properties. This can introduce impurities even after successful synthesis and purification (Chen, W., et al. 2022).

Like other poloxamers, P188 has a broad molecular weight distribution, the common molecular weight of commercial P188 ranges from 8,400 to 9,500 Daltons. This inherent characteristic means that there are molecules of various sizes and compositions within the same batch, which can be considered impurities depending on the specific application and required purity standards. Removing impurities from P188 can prove to be challenging due to its amphiphilic nature; traditional purification methods like distillation or crystallisation are less effective for such polymers, and specialised techniques may be

required, which can be complex, time consuming and not always fully effective (Wang *et al.*, 2019).

4.1.2 P188 and Impurities: implications for cell viability in bioreactor environments

While the benefits of P188 addition to bioreactors is widely welcomed and accepted, the reported batch-to-batch variation in maintaining cell viability hinders the reliability of the product. The batch-to-batch variation likely results from varying proportions of impurities in the P188 preparation. These impurities (also referred to as contaminants) in final P188 preparations can be unsaturated monohydroxy polymers, or byproducts of autoxidation. Either can arise from the polymerisation protocol through which P188 is manufactured. The manufacturing process of P188 polymerisation makes it difficult to assess each batch's molecular weight distribution: the variation of ratio between the two main chemical chains that make up the polymer can lead to changes in its properties such as water solubility, hydrophobicity and micellization temperature, which can decrease the P188 protective efficacy (Peng et al, 2016).

One of the best procedures currently available to assay for effects of undesired species in P188 is use of small scale cell culture models to recreate shear forces that cells are subjected to in the turbulent environment of sparged bioreactors (Peng et al. 2014), however this is resource heavy both in terms of time and cost. In this study we have assessed the effects of commonly found impurities on cell viability under shear stress conditions both in the presence and absence of P188. In the earlier chapter, it was reported a direct correlation between effect on cell viability, surface tension and micelle radius present in the media to which various impurities have been added and propose that this could serve as the basis of a rapid screening method for batches of P188. Furthermore, the efficiency of protection provided by P188 can be heavily influenced by various percentages of different impurities. High molecular weight impurities in P188 batches, such as P407, result in low protection from viability drops of mammalian cells in bioreactor environments. P407 is a surface active poloxamer of higher molecular weight

and a lower % of PEO, which is known to have low shear protection in mammalian cell bioreactor cultures (Chen et al., 2022).

Surface tension at the liquid-air interface can influence cell behaviour and viability. High surface tension can lead to plasma membrane rupture and cell death. Understanding the relationship between surface tension, interfacial forces, and cell response is crucial for optimising bioreactor conditions and minimising cell damage (Figure 3). The chemical composition of the poloxamer is thought to interfere not only with the hydrodynamic forces affecting the cell but also with the way they get taken up in the plasma membrane. Poloxamers have been used in nanomedicine and as therapeutics to restore plasma membrane integrity in ischemia and respiratory distress syndrome (Houang et al., 2015) (Plataki et al., 2011). A similar phenomenon, in which P188 physically inserts in damaged plasma membranes (PM) of CHO cells, is thought to be one of the main mechanisms of the surfactants in bioreactors (Goliaei *et al.* 2016) (Image 11). The functioning of the mechanism of insertion and release of the P188 seems to be strongly correlated with the chemical components of the poloxamer. Theoretically, small changes in the molecular weight and interfacial characteristics can determine the manner and rate of insertion and release of the poloxamers in and from the PM (Chang et al., 2017).

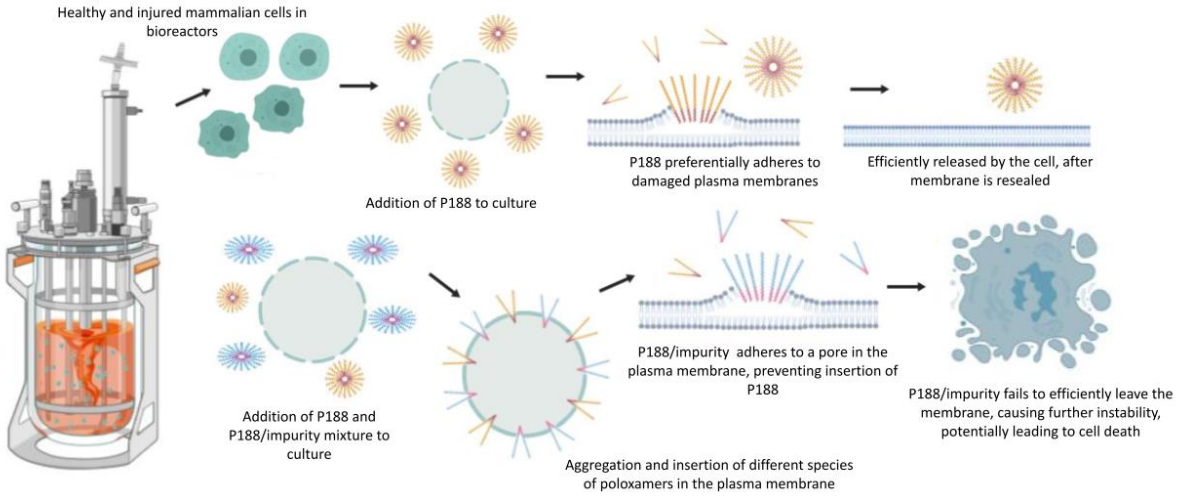


Figure 3: Sequential interaction of P188 surfactant with mammalian cells in a bioreactor: Therapeutic processes and complications. The diagram presents a sequential overview of the interaction between a surfactant molecule, P188, and mammalian cells within a bioreactor, detailing the therapeutic process and potential complications. Initially, healthy and injured mammalian cells are shown in the bioreactor environment, where cell health is maintained or restored via the bioreactor's-controlled conditions. Upon the addition of P188 to the culture, it preferentially adheres to damaged plasma membranes, suggesting a targeting mechanism for membrane repair. Two pathways are depicted following the addition of P188: one where P188 acts beneficially and another where impurities in P188 preparation leads to adverse outcomes. In the beneficial pathway, P188 integrates into the plasma membrane and is eventually released after the membrane is sealed, implying a successful self-repair process facilitated by P188. Conversely, the introduction of a mixture of P188 and impurities results in a different interaction. Here, the impurity binds to a pore in the plasma membrane, blocking P188 from performing its function. This leads to an aggregation of different poloxamer species within the membrane, causing further instability and potentially leading to cell death due to the inability of P188 to dissociate effectively from the membrane.

4.2 Aims:

The aim of this chapter is to investigate the impact of impurities on the surface tension and micellization of P188. This study seeks to develop a method for quickly identifying low-performing contaminants in P188 batches, anticipating potential variations in performance across manufacturing batches, without necessitating extensive and expensive viability studies in cell culture.
To achieve these overall aims, the following objectives were identified:

1. Identify and analyse P188 surface tension patterns:

    - Assess and identify distinctive patterns in surface tension between purified P188 and P188 contaminated with known impurities.
    - Explore how variations in surface tension manifest under both turbulent and static liquid environments containing P188.

2. Micelle Formation Analysis Investigation:

    - Investigate and compare micelle formation patterns of pristine P188 with those formed in the presence of additional surfactant impurities with the aim of use micelle size profiles as a screening tool for batches of poloxamers.
    - Examine the influence of impurities on the stability and characteristics of P188 micelles.

3. Analysis of Batch-to-Batch variability:

    - Develop a predictive method to screen for the presence of impurities influences batch-to-batch variability in the surface tension and micellization behaviour of P188.
    - Evaluate the potential of surface tension and micellization assessments as indicators of overall surfactant performance in diverse manufacturing batches.

4. Practical Implications:

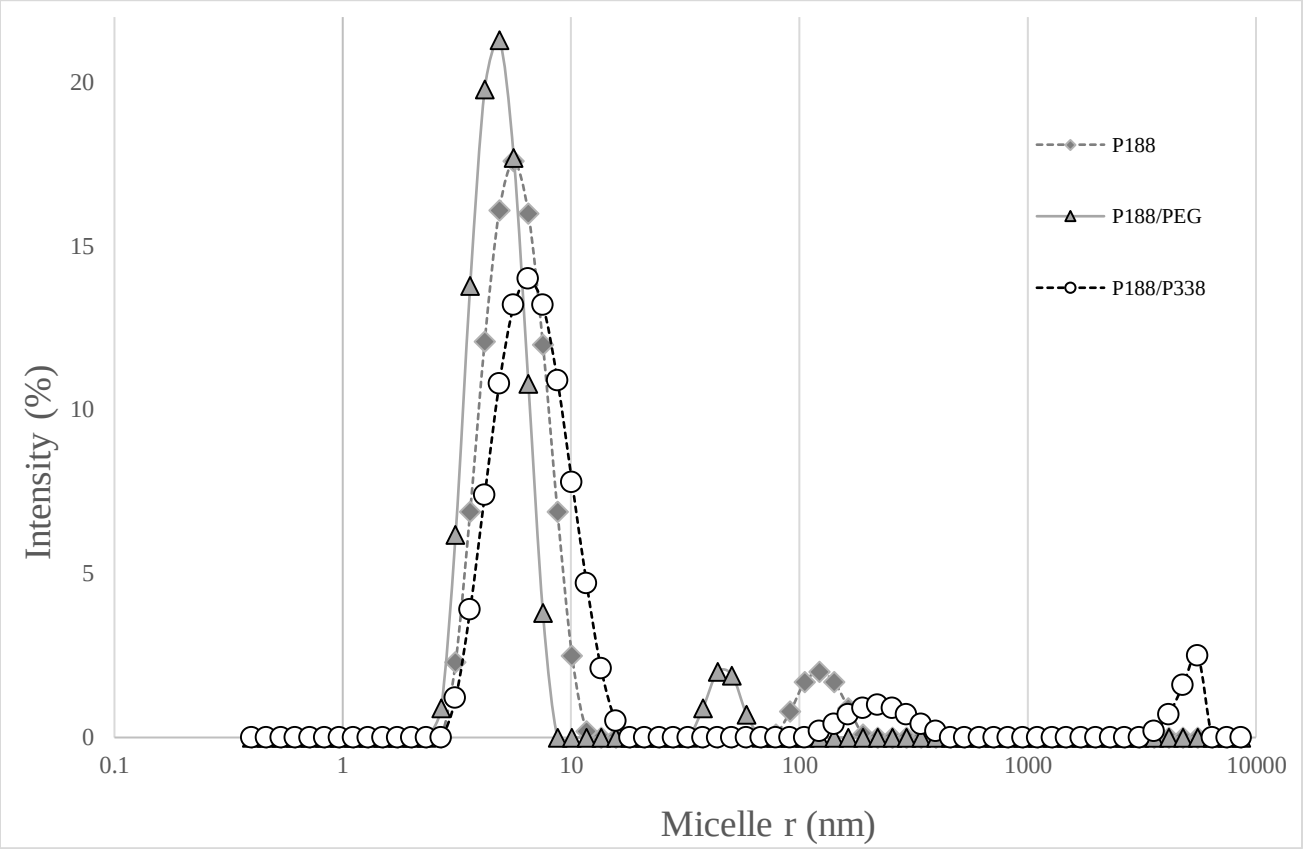
    - Provide insights into the physicochemical interactions between P188 and other impurities, contributing to the development of an efficient and streamlined quality assessment of surfactant batches.
    - Offer a practical approach to identify low-performing batches without resorting to cell culture studies.

4.4 Results:

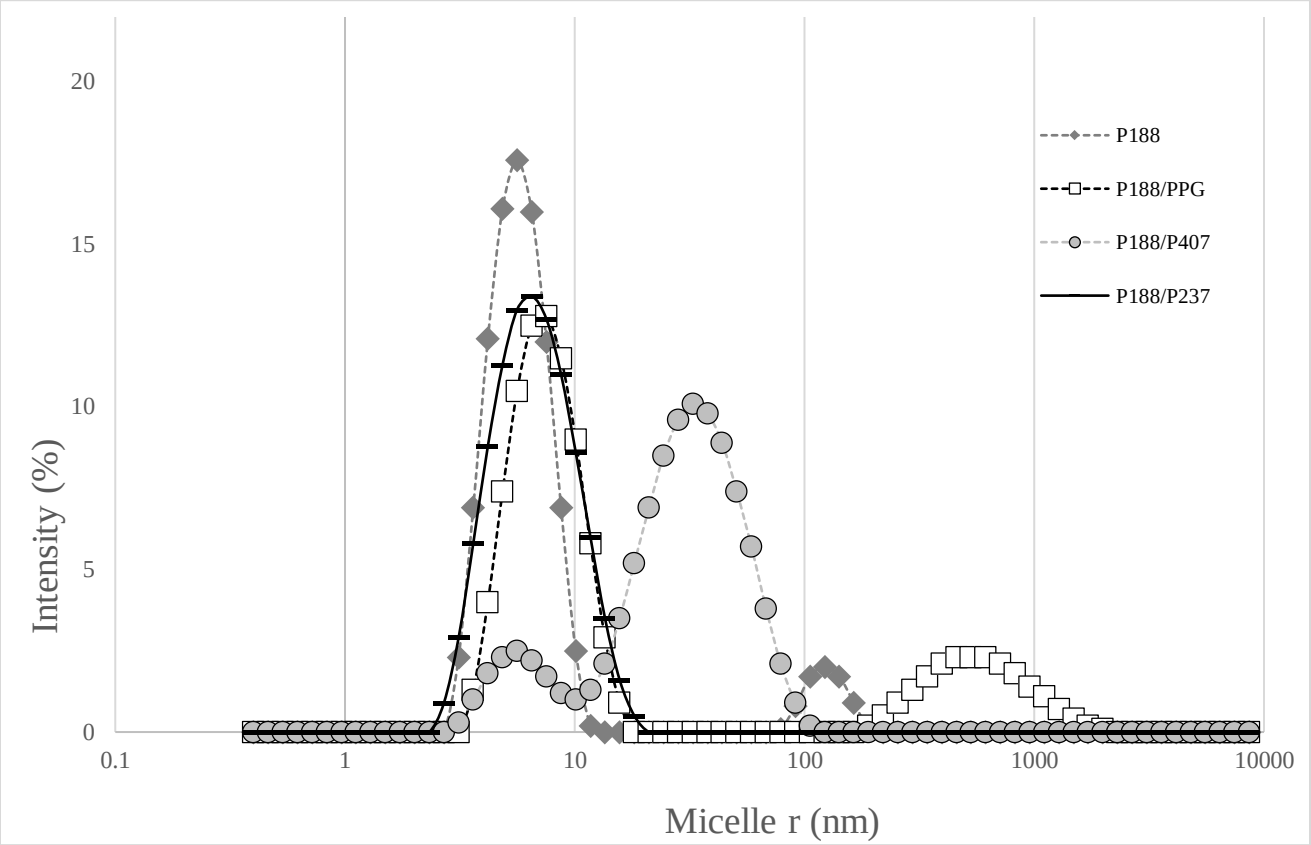
4.4.1 Dynamic light scattering (DLS) of P188 and P188 mixed with common impurities:

DLS can measure the size distribution of micelles in P188 solutions, helping to look into how impurities affect micelle formation and stability. This process can prove crucial for understanding the impact of impurities on the micellization behaviour of P188 and since DLS can help identify the presence of contaminants by detecting changes in micelle size distribution, it can contribute to the methods in which low performing batches of P188 can be identified. DLS offers a quick, non-destructive method and not cell-culture based assay to analyse the micelle properties in P188 solutions.

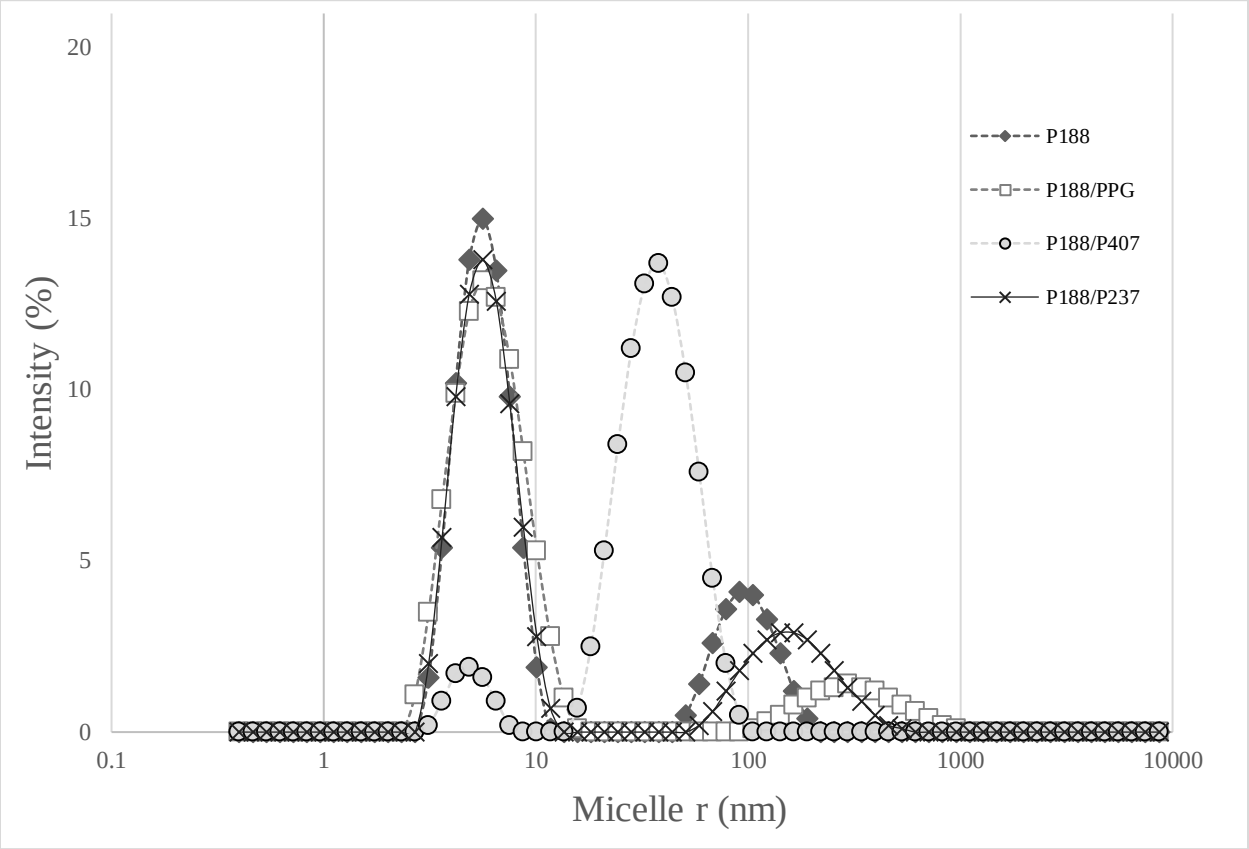
(A)


20
15
10
5
0
Intensity (%)
0.1
1
10
100
1000
10000
Micelle r (nm)
P188
P188/PEG
P188/P338

(C)


20
15
10
5
0
Intensity (%)
0.1
1
10
100
1000
10000
Micelle r (nm)
P188
P188/PPG
P188/P407
P188/P237

(B)


20
15
10
5
0
Intensity (%)
0.1
1
10
100
1000
10000
Micelle r (nm)
P188
P188/PPG
P188/P407
P188/P237

(D)

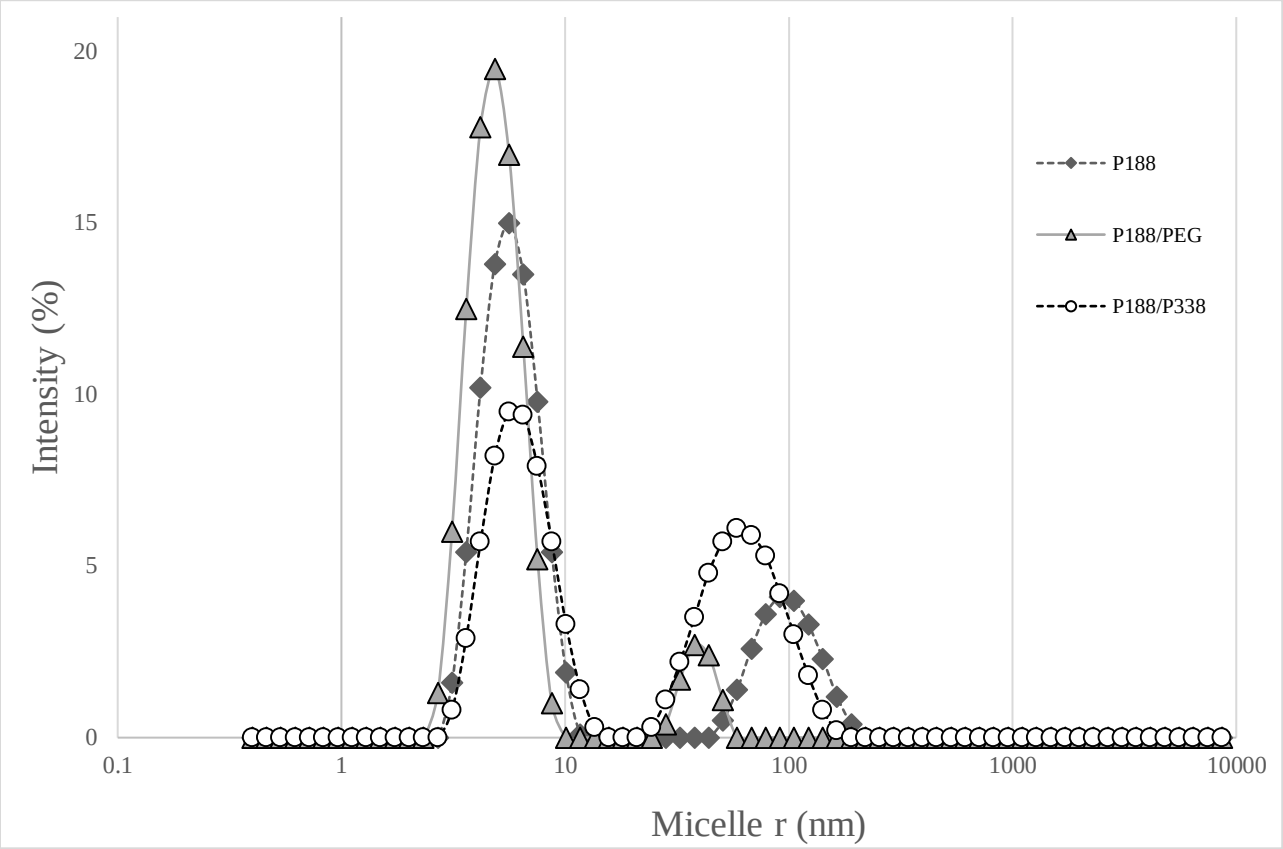
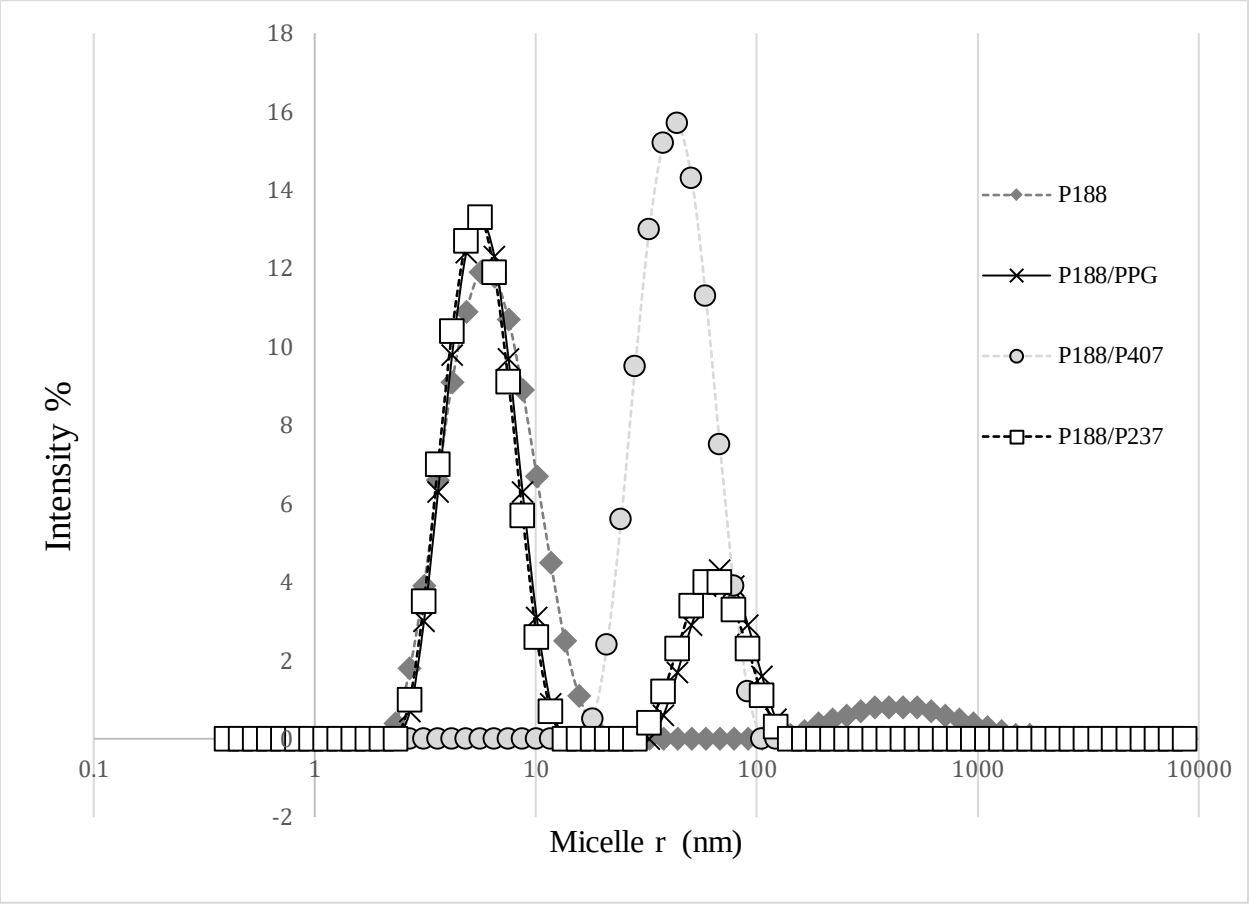


Figure 4: Representation of micelle size and intensity of samples of P188 and P188 with impurities in distilled water at 1% (A, C) and 0.1% (B, D) concentration of poloxamer mixtures. Results from DLS of 1% w/v mixture of P188 with mixed with high MW (A) and low MW poloxamers (C). poloxamers with lower MW and lower % of PPO (A) and (C) IN H_2O. The other two graphs show results from DLS of 0.1% w/v mixture of P188 with mixed with high MW (B) and low MW poloxamers (D) in H_2O.

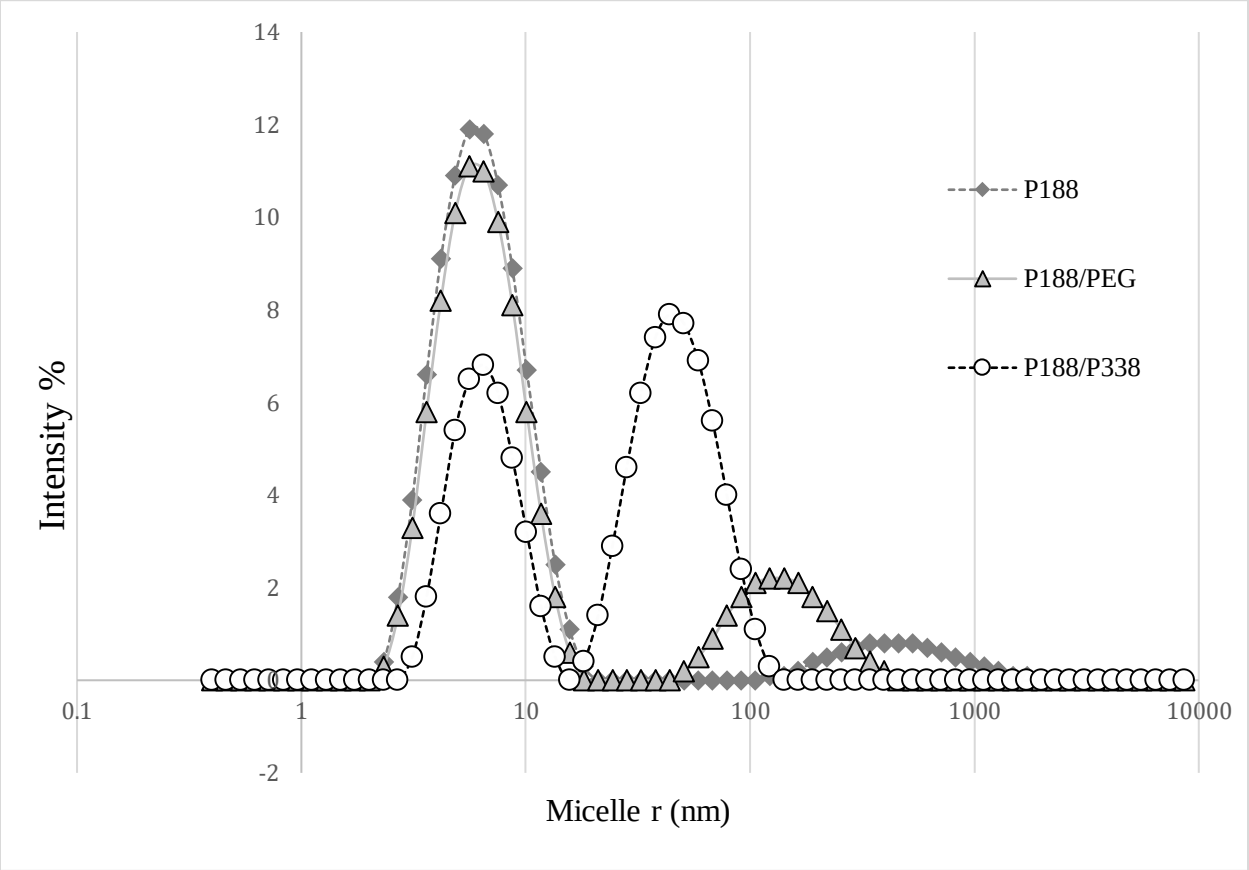
The first results show that a purified P188 at 1% w/v in water has a micelle size distribution gathered around the range of 5 to 10 nm (Figure 4, A, C), with a small spike towards the 100 nm size which is possibly saturated monomers. When the P188 is contaminated with other surfactants, the micelle pattern shifts and most of the micelles are, on average, slightly larger in nm, apart from P188 with an added 20% w/v of PEG. The presence of 20% of P338 and P237 causes two distinct micelle sizes to form in the interface, one akin to the P188 at around 10nm, and another group of micelles, much bigger at ≥ 100 nm. The latter are not dissimilar to purified P188 but at a higher intensity for P338 and on a broader size range for P237.

When the concentration of poloxamers is lower in the interface, the micellization behaviour of the compounds remains like the previous results; pure P188 maintains a peak of small micelles at around 8 nm radius. The addition of high MW impurities still causes a shift in the larger micelle sizes of the second species and the biggest shift occurs again with the presence of 20% P407 (Figure 4, B). Compared to pure P188 Mixing 20% of lower MW impurities to purified P188 at 0.1% w/v to water causes the formation of a larger spike of micelles at 8 nm radius for the mixture of P188/P338 and a smaller number of larger micelles. However, the addition of PEG lowers the spike of smaller micelles and shows another larger peak at around 80 nm.

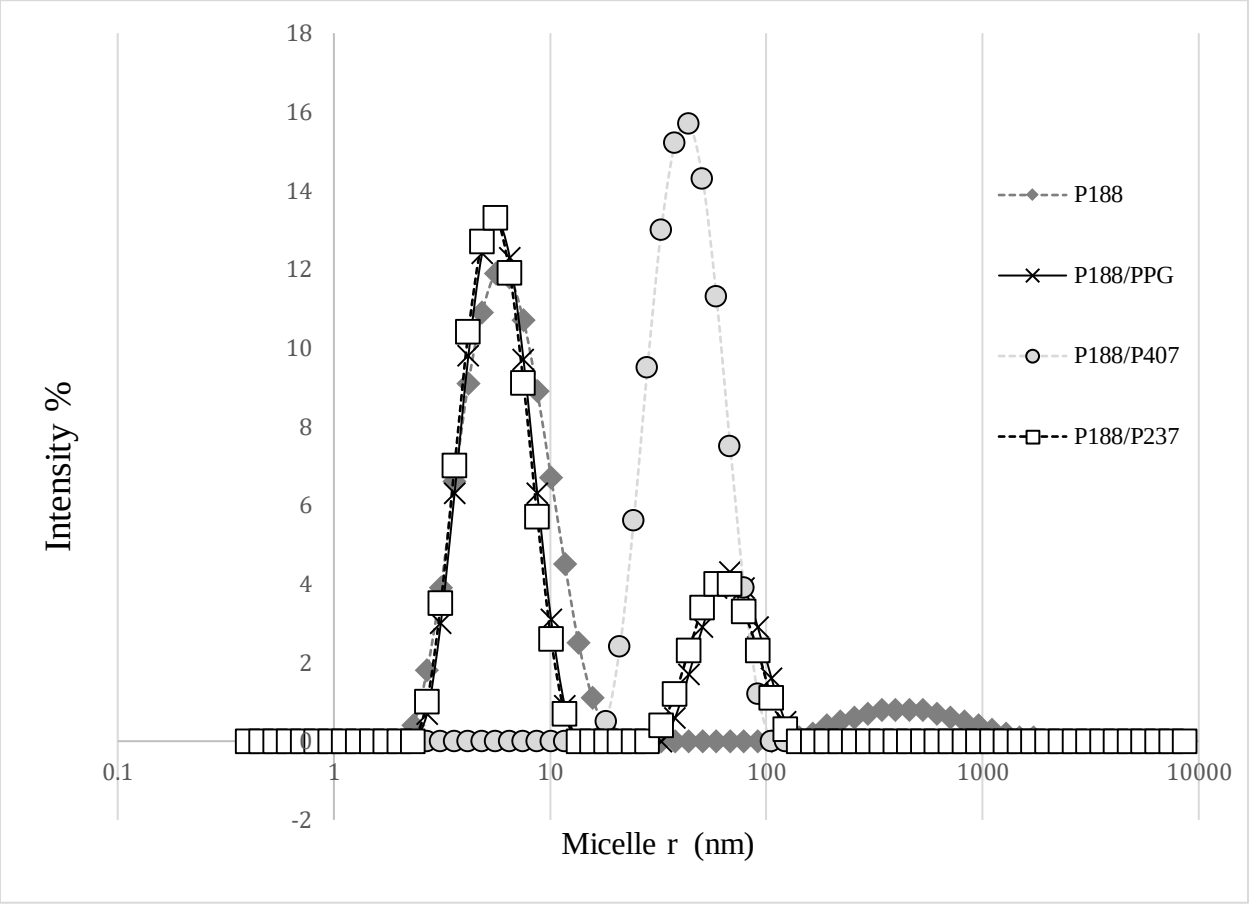
(7,A)


18
16
14
12
10
8
6
4
2
0
-2
Intensity %
0.1
1
10
100
1000
10000
Micelle r (nm)
P188
P188/PPG
P188/P407
P188/P237

(7, C)


14
12
10
8
6
4
2
0
-2
Intensity %
0.1
1
10
100
1000
10000
Micelle r (nm)
P188
P188/PEG
P188/P338

(7, B)


18
16
14
12
10
8
6
4
2
0
-2
Intensity %
0.1
1
10
100
1000
10000
Micelle r (nm)
P188
P188/PPG
P188/P407
P188/P237

(7, D)

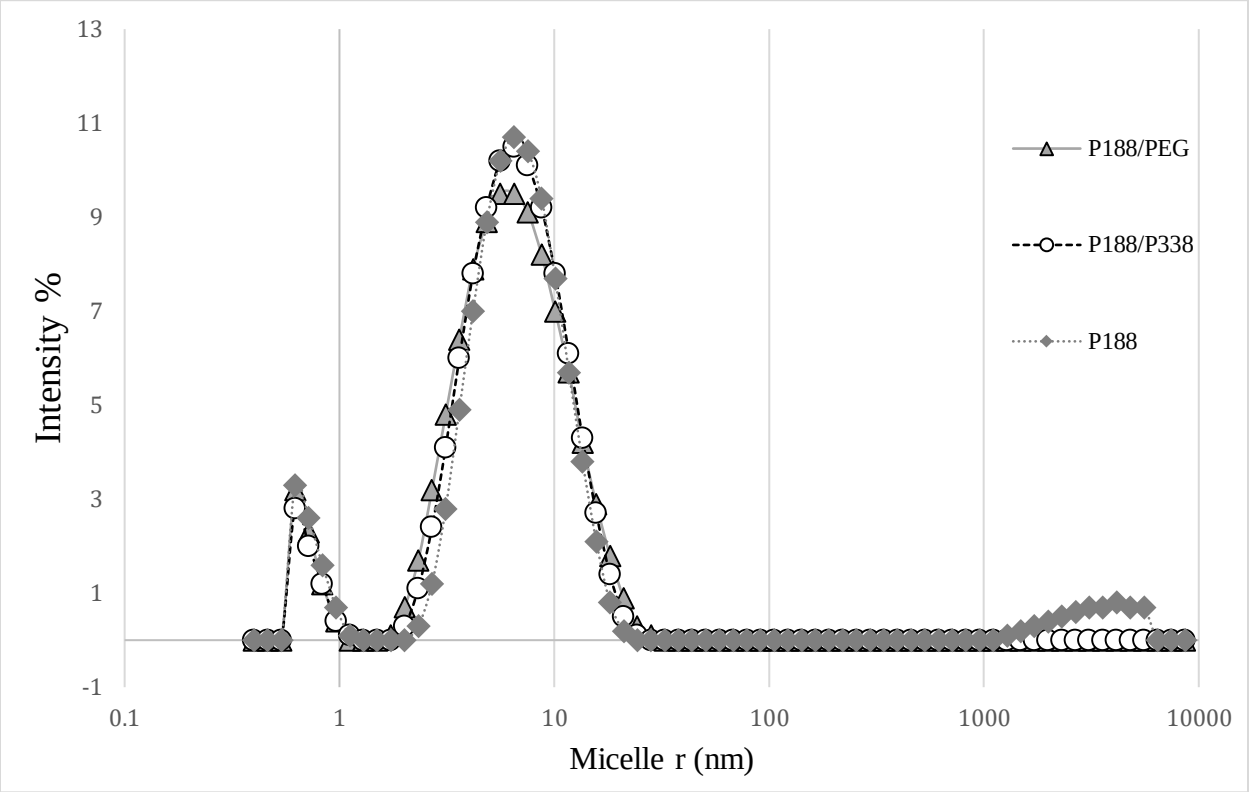


Figure 5: Representation of micelle size and intensity of samples of P188 and P188 with impurities in Freestyle CHO cell media at 1% (A, C) and 0.1% (B, D) concentration of poloxamer mixtures. Results from DLS of 1% w/v mixture of P188 with mixed with high MW (A) and low MW poloxamers (C). poloxamers with lower MW and lower % of PPO (A) and (C) in Freestyle CHO media. The other two graphs show results from DLS of 0.1% w/v mixture of P188 with mixed with high MW (B) and low MW poloxamers (D) in Freestyle CHO media.

The next DLS experiment was carried out in Freestyle CHO cell media. These measurements were taken in order to assess whether the micellization behaviour of P188 and P188 with impurities would be influenced in a different liquid surface, more akin to the one used in bioreactors. The aim was to assess whether micellization is influenced by common mammalian cell media additives as well as impurities in the poloxamer. Not dissimilar to 1% purified P188 sample in distilled water, the micellization of the same

concentration of surfactant in cell media produces a strong peak from micelles of the size between 3 and 10 nanometres. The second peak is much broader and at lower intensity than the P188 sample in water, showing that larger micelles are not formed as efficiently in a liquid surface containing the media components. The addition of P237 and PPG to purified P188 in media yields very similar micelle sizes in both intensity and distribution.
As seen in the previous experiment, higher molecular weight impurities create a completely shifted pattern of micelle size, compared to the purified P188 control. P338 contributes to the formation of two distinct micelle sizes at lower intensity than the control, one averaging between 3 and 10 nm, one other much larger between 10 and 100 nm. The P407 impurity causes a much more significant shift in the average micelle size, as previously observed in distilled water. The range between 10 and 100 nm in size and at a much higher intensity compared to the P188 control. This shift in micelle size in presence of CHO cell media can be explained by the lower water solubility of P407 as well as its higher molecular weight. Addition of 20% PEG doesn't seem to influence the micelle size and intensity of P188 the only difference is a distribution of larger micelle sizes represented by a peak between the 100 and the 1000 nm region. Higher molecular weight poloxamers, such as P407 and P338, have larger PPO blocks compared to P188. This increased hydrophobicity can result in the formation of larger micelles as the hydrophobic cores aggregate more extensively to minimize exposure to the aqueous environment. The increased size and altered HLB can also lead to less stable micelles, causing a broader distribution in micelle size. Lowering the concentration of poloxamers in the media (figure 7, B-D) shows that most contaminations are not detectable by micelle size and intensity pattern, the most significant shift occurs with the addition of P407, which shifts the main peak to much larger micelles than the purified P188 control. Understanding these shifts can guide the selection and purification of poloxamers for specific applications, ensuring consistent product quality and performance.

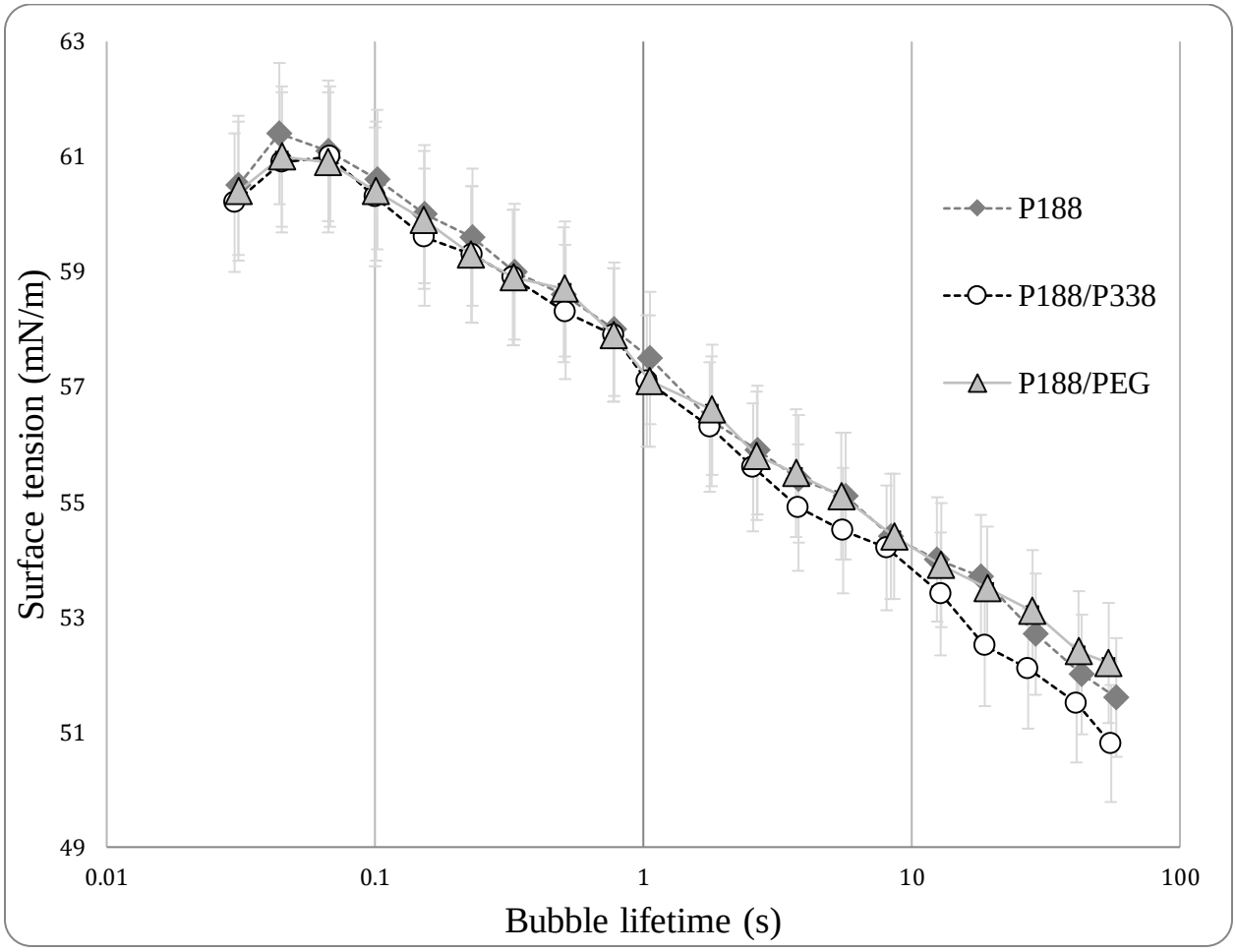
4.4.2 Dynamic surface tension analysis of surfactants and impurities in liquid interfaces: implications for bubble formation and stability in cell culture media:

Surfactants, or surface-active agents, are molecules that are used to lower the surface tension of a liquid, thereby promoting the spreading and wetting of the liquid on solid surfaces or interfaces. Dynamic surface tension measurements are important in understanding how well a surfactant functions under dynamic conditions, in this case the turbulence created by the creation and lifetime of the bubble. The dynamic force tension is measured through the release of an air bubble in the liquid interface and the bubble stability in the tested interface. The surface tension in a liquid environment can be greatly influenced by the addition of surface-active compounds. In surfactants studies, it is important to measure physical characteristics of the changes that the polymers attribute to the system they are in. Bubble formation and retention is a phenomenon which can interest cell culture viability and health. Oxygen bubbles in particular are a common presence in shaking flasks and any bioreactor, borne of sparging hydrodynamic forces caused by feeding and oxygenation. The dynamic surface tension experiment with the bubble tensiometer is based on the bubble pressure dynamic in liquids. The measurements register the air pressure blown out by a capillary tube as it forms bubbles in a liquid sample immersion.

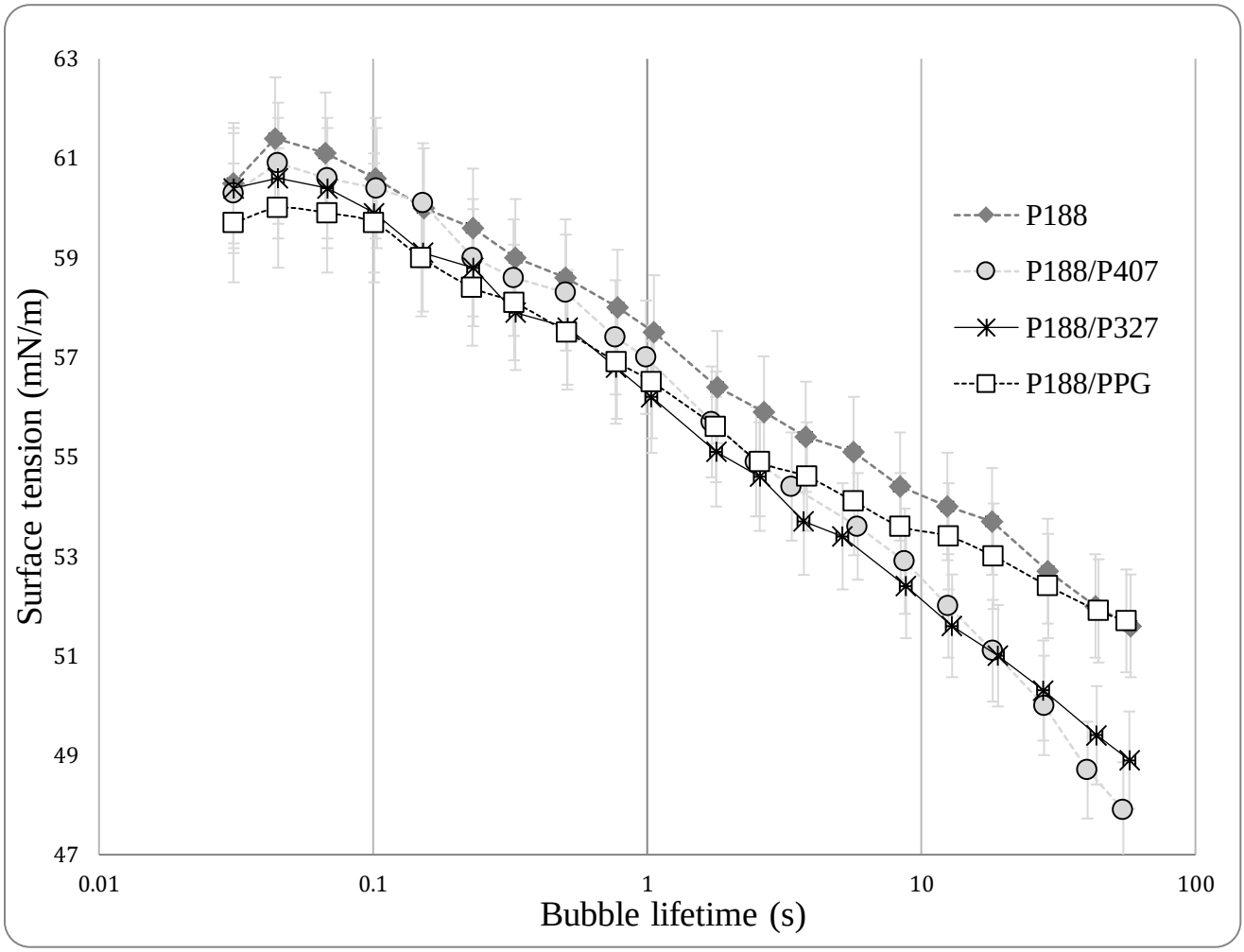
P407 and other higher molecular weight surfactants are known to produce more structurally stable foams and bubbles than their lower molecular weight counterparts (Chang et al., 2017). The results show that bubble stability in samples with purified P188 and P188 with impurities can have a high degree of variation, depending on the interfacial characteristics of the impurities (Tran et al., 2016) (Cherry and Hulle, 1992).
For this study four sets of data were produced, two sets of data adding surfactants at 1% and 0.1% concentration in distilled water, two adding surfactants at 1% and 0.1% concentration to Freestyle media. In these regimes, the samples were made up of purified P188 and purified P188 with the addition of 20% impurity. In the case of this study, the impurities were P407, P237 and PPG for the compounds with a lower percentage of PEO

in their structure; P338 and PEG were instead used as examples of surfactants which have comparatively higher percentage of PEO (not dissimilarly to P188). These impurities were selected in order to obtain data regarding species which are known to contribute to low efficacy in cell protection, and the ones that didn't have a significant effect on cell viability (chapter 3, figure 7, figure 8).

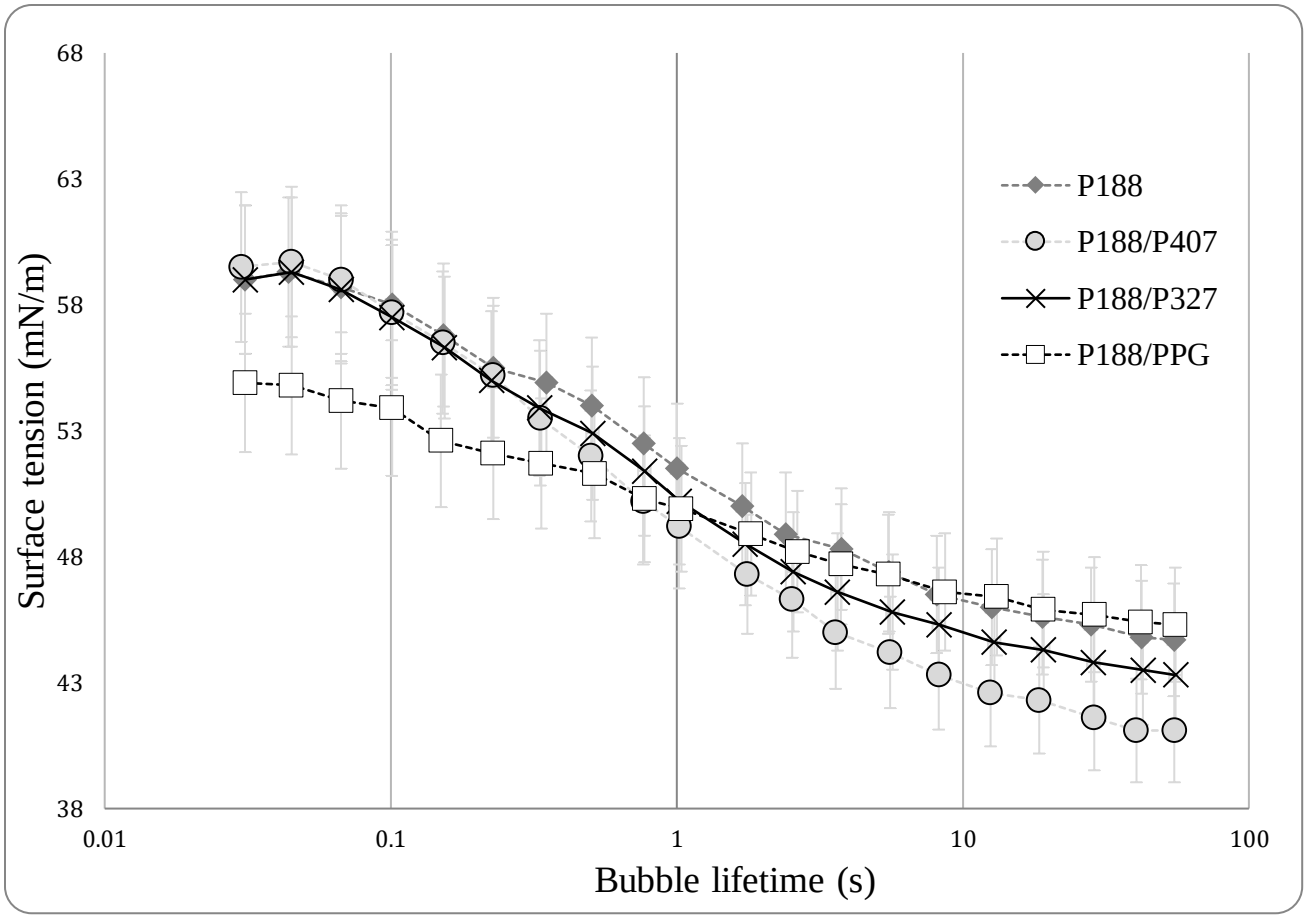
A1



A2


63
61
59
57
55
53
51
49
47
Surface tension (mN/m)
0.01
0.1
1
10
100
Bubble lifetime (s)
P188
P188/P407
P188/P327
P188/PPG

B1


68
63
58
53
48
43
38
Surface tension (mN/m)
0.01
0.1
1
10
100
Bubble lifetime (s)
P188
P188/P407
P188/P327
P188/PPG

B2

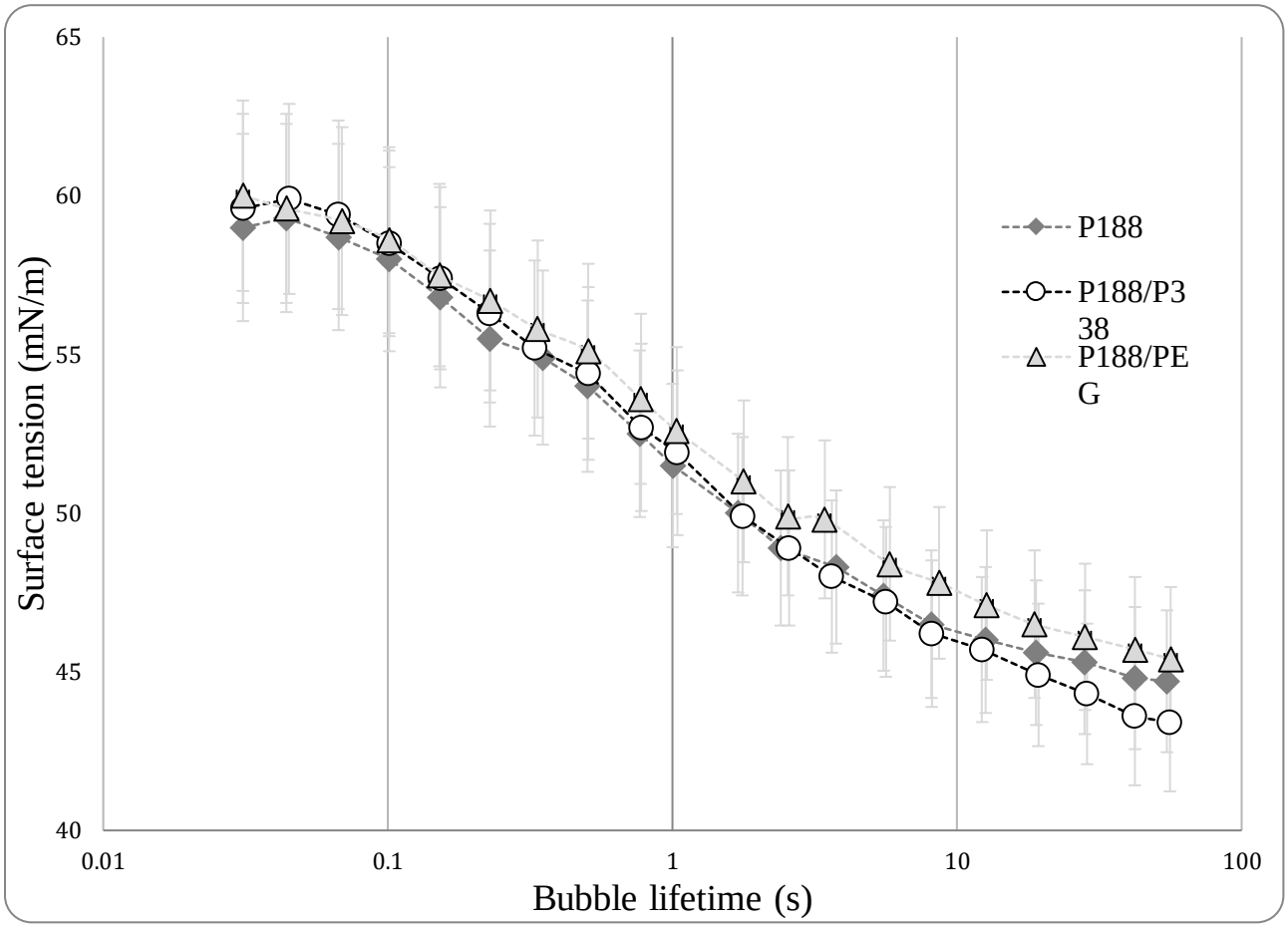
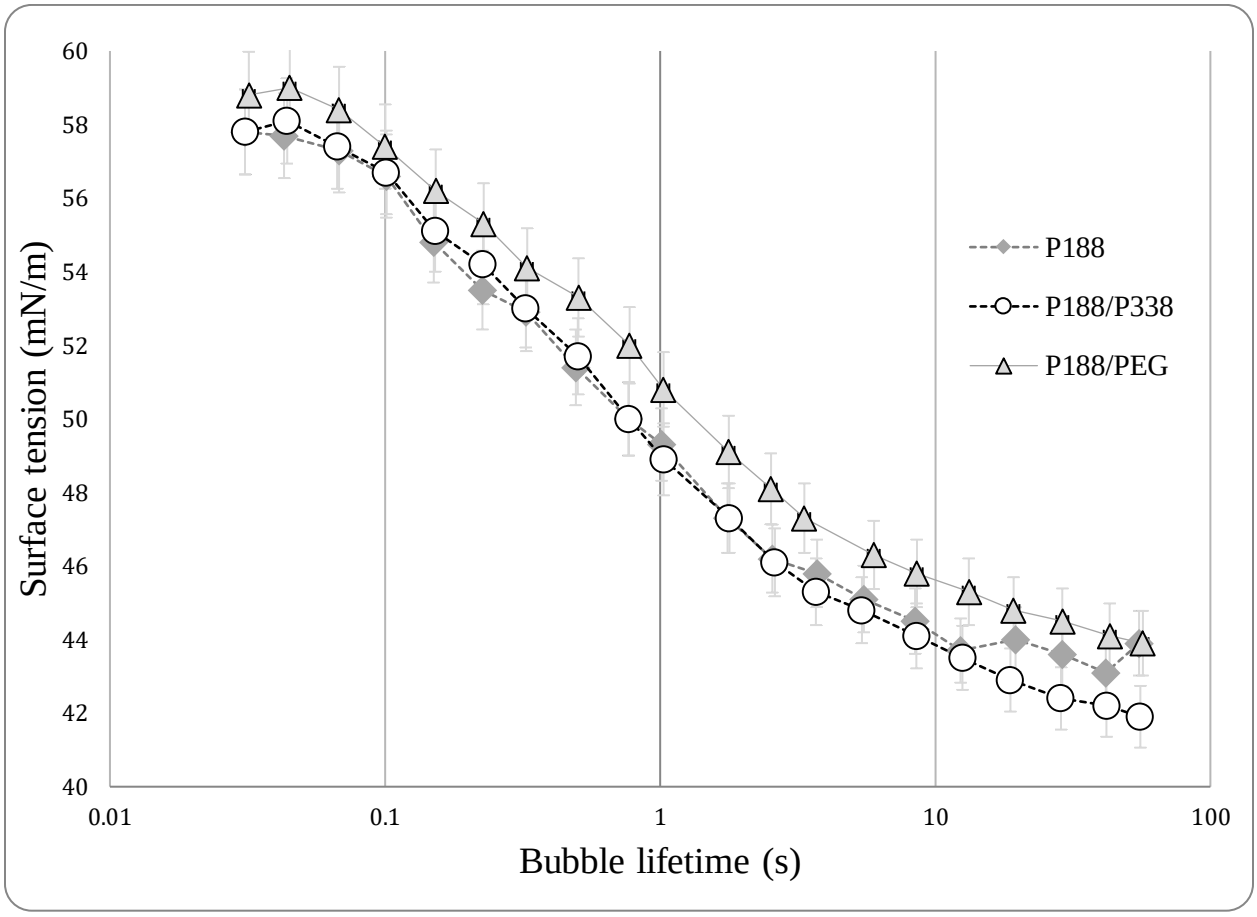


Figure 6: Dynamic surface tension measurements of distilled water samples with the addition of pure P188 and P188 with a 20% addition of known contaminants, PPG, PEG, P407, P338 and P237 at 1% (A1, A2) and 0.1% w/v (B1, B2). The measurements at the beginning of the bubble lifetime indicate surface tension for bubble formation and towards the end of the bubble lifetime gives an indication on bubble stability over surface tension. Samples with lower % of PEO, P188-P407 and P188-P237 at both concentrations are more surface-active leading to a loss of surface tension towards the end of bubble life.

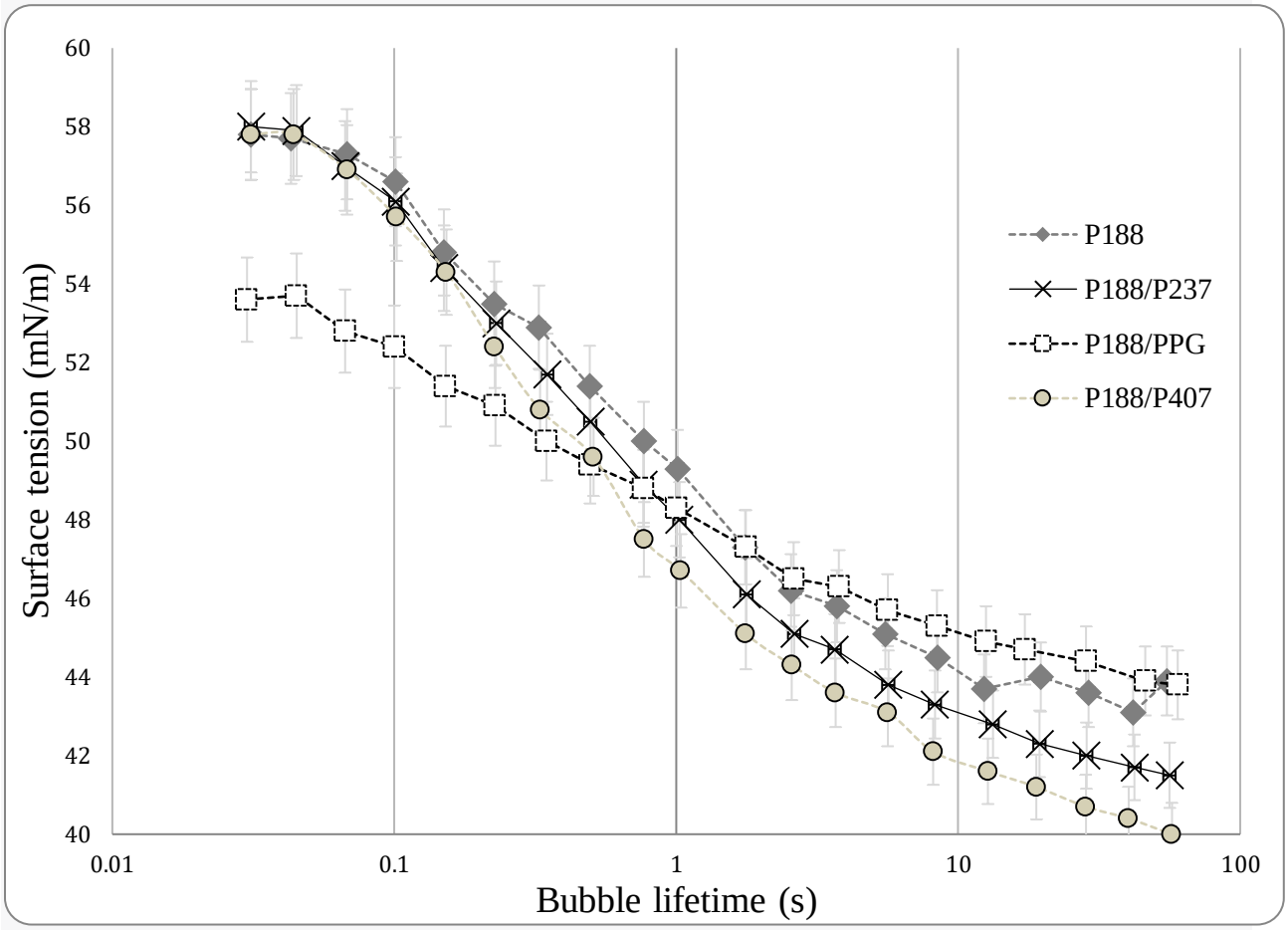
Bubble formation is supported the most in samples of P188 with the addition of hydrophobic PPG (Figure 6.). However, the surface tension at the end of bubble lifetime is close to the P188 control, indicating that it wouldn't support the bubble's stability over

a prolonged period of time, producing a less stable foam and theoretically causing less of a loss of cell viability. The other sample which deviates from the purified P188 control is P188/P407. Surface tension to support bubble formation does not seem to be affected by the high molecular weight impurity at the beginning of the bubble lifetime. Over the course of the experiment, the surface tension is gradually lowered by this sample, suggesting that its high surface-active characteristics lead to a more stable bubble. Foam and bubble stability is often linked to poloxamers with low shear protection, which P407 is an example of (Chang et al., 2017).

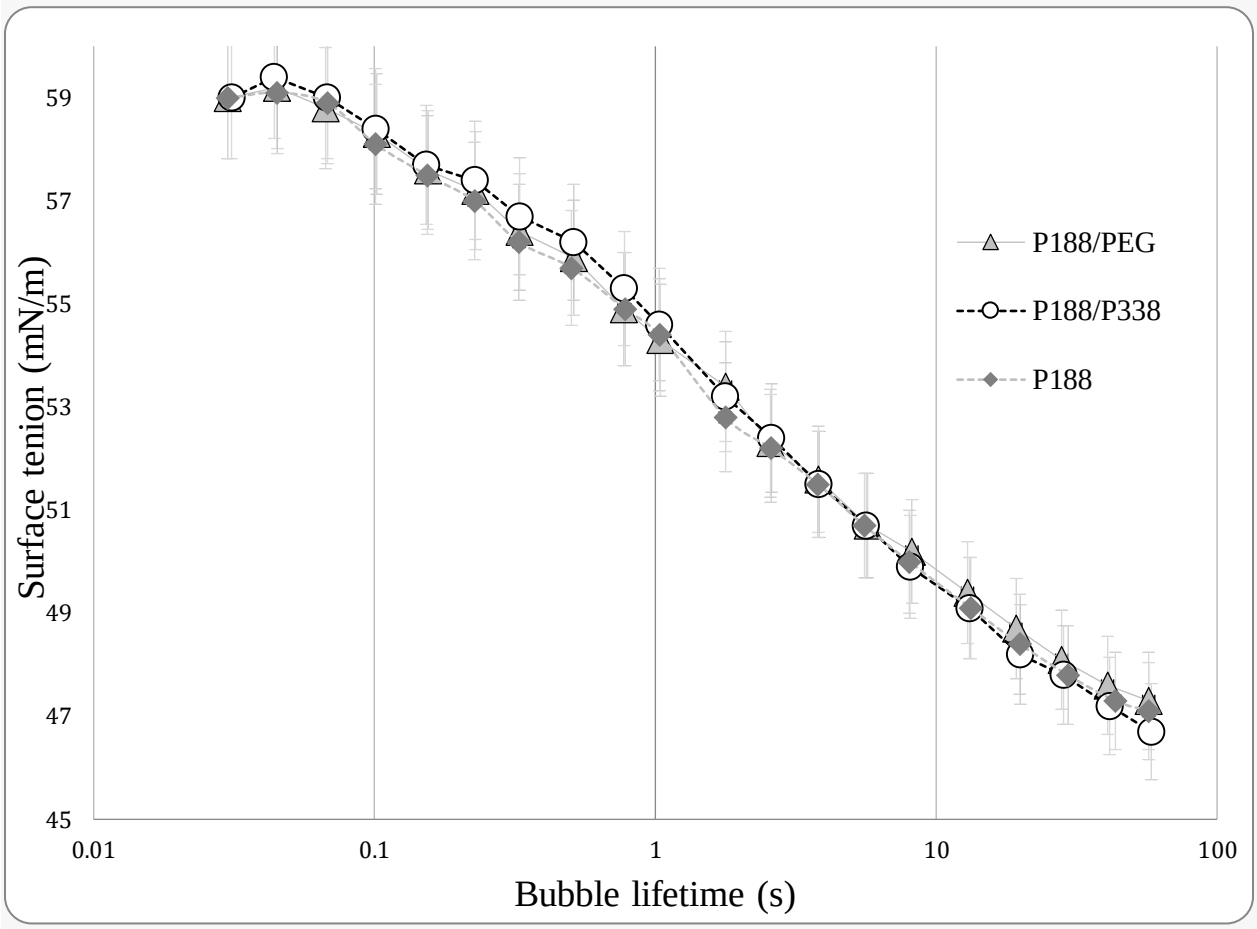
C1



C2


60
58
56
54
52
50
48
46
44
42
40
Surface tension (mN/m)
0.01
0.1
1
10
100
Bubble lifetime (s)
P188
P188/P237
P188/PPG
P188/P407

D1


59
57
55
53
51
49
47
45
Surface tenion (mN/m)
0.01
0.1
1
10
100
Bubble lifetime (s)
P188/PEG
P188/P338
P188

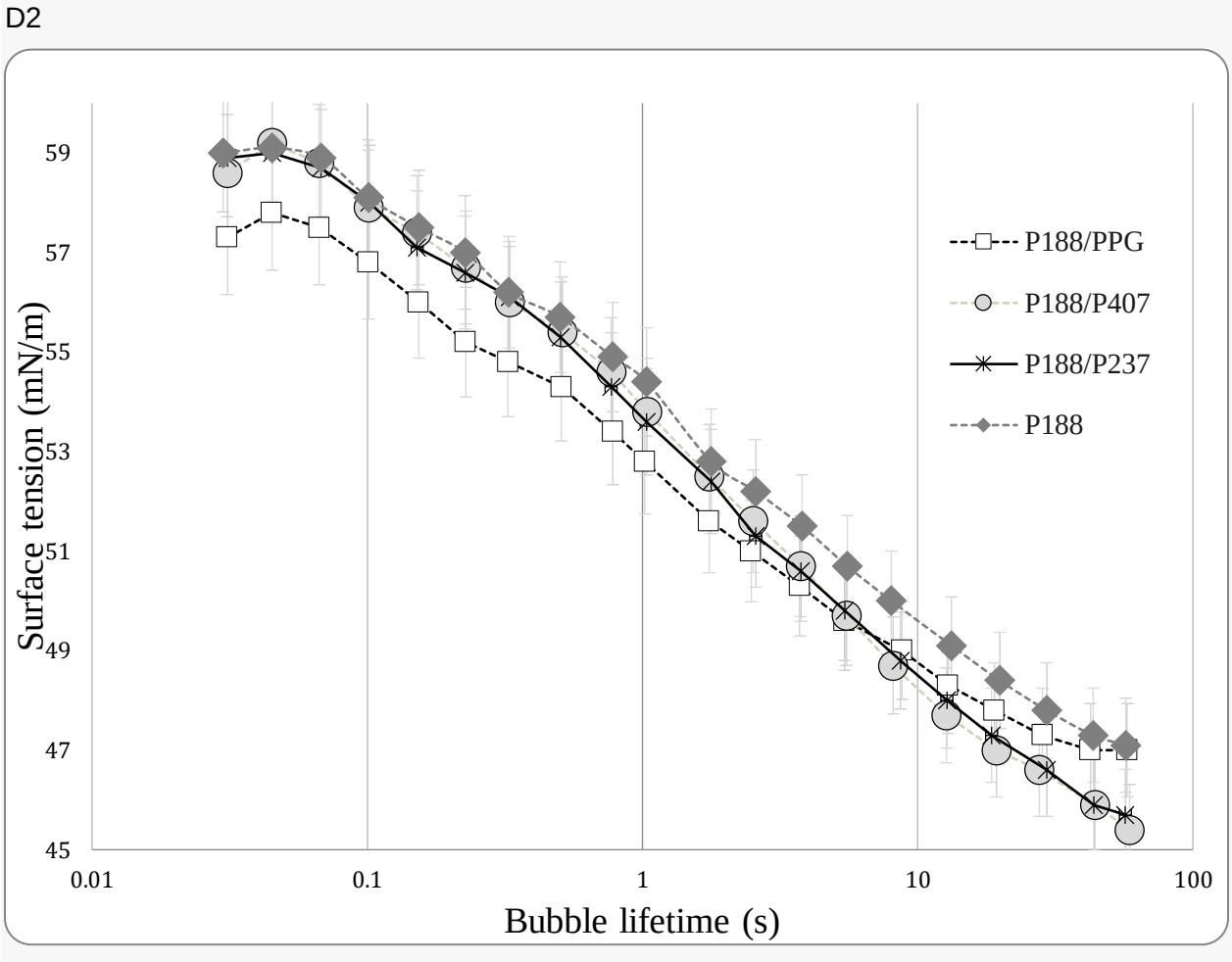


Figure 7: Dynamic surface tension measurements of Freestyle media samples with the addition of pure P188 and P188 with a 20% addition of known contaminants, PPG, PEG, P407, P338 and P237 at 1% (C1, C2) and 0.1% w/v (D1, D2).

In cell media (Figure 7), the low concentration of higher molecular weight impurities seems to drastically lower the overall surface tension compared to the addition of purified P188 as it can be seen in the bottom part of the graph (figure 7, B). PPG addition supports bubble formation more efficiently than the rest of the samples, although the retention of bubble stability does not seem to be varied compared to the purified P188 control. The sample with higher water affinity P188/PEG, does not seem to lower the dynamic surface tension as much as the rest of sample as, at this concentration, the sample adsorbs more efficiently to the liquid interface resulting in less bubble formation and retention.

At lower concentration, the addition of contaminants to P188 to the liquid interface, doesn't have a different effect on the bubble formation and retention compared to the samples at higher concentration w/v. The main differences are observed in samples with higher MW than P188, where even when the mixture is at a 0.1% w/v bubble formation is supported by the lower surface tension caused by a 20% addition of p407 to P188.

4.4.3 Impact of P407 Contamination on the Surface Activity of P188: analysis of the concentration-dependent surface tension

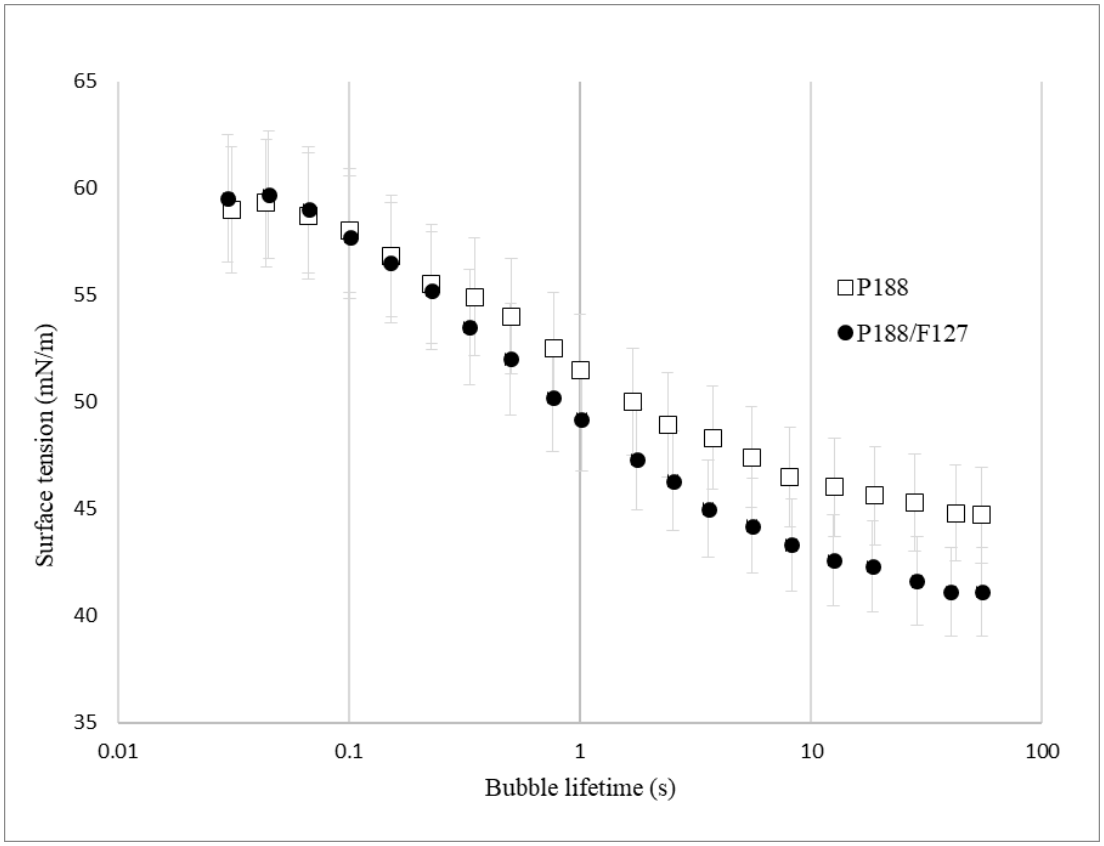


Figure 8. Equilibrium surface tension measurements of 1% purified P188 and 1% P188/P407 in purified H_2O at room temperature.

Equilibrium Surface Tension reflects the lowest surface tension achievable once the surfactant molecules have fully equilibrated at the interface. This measurement provides insight into the maximum effectiveness of a surfactant in reducing surface tension, which is important for stabilizing emulsions, dispersions, or foams. Understanding EST is vital for maintaining stable conditions over time in bioreactors, ensuring consistent product quality and preventing issues such as protein denaturation or aggregation. The contaminated P188 shows an overall higher surface activity than purified P188 at both higher and lower concentration regimes (Figure 8).

Compared to the purified P188, substituting 20% of the poloxamer concentration with P407 makes the compound more surface active which is indicated by the overall lower surface tension in figure 5. The results highlight that even the addition of a small concentration of P407 has an impact on the air/water tension.

The surface tension lowers the higher the concentration of surfactant in the solution. At higher density, the poloxamer molecules form a denser layer on the surface transitioning from a horizontal configuration to a vertical one, where the polyoxypropylene chain attaches at the interface (Chang et al., 2017). The P188/P407 solution continues decreasing the surface tension reaching 41.57 mN*m at CMC of 0.05% concentration, compared to a much higher equilibrium surface tension at CMC of 0.05% concentration of purified P188 at 47.39 mN*m.

4.5 Discussion

4.5.1 Dynamic light scattering (DLS) of P188 and P188 mixed with common impurities:

Micellization behaviour and other chemical characteristics of P188 have been a matter of great interest for scientists involved with the industrial production of monoclonal antibodies in recent years. This interest aims not only to answer the question of mechanisms of mammalian cell defence in bioreactors, but also to approach possible techniques of identifications of low performance batches of purified P188. Changes in the concentration of polymers in the media can cause the repulsion and attraction forces within molecules to encourage aggregation and therefore formation of micelles. The critical micelle concentration (CMC) is the concentration of poloxamer, or any surfactant, required in order to create a micelle monolayer (Sehgal et al., 2008).

The peculiarities of micelles can be influenced by small changes in the chemical structure of the poloxamer molecules or by conditions in the liquid interface in which they reside. Changes in pH, in temperature, in pressure, ionic strength and hydrodynamic forces are all influencers of the micellization process. Importantly, micellization and micelle size can be influenced by the concentration of the surfactant (Alexandridis and Alan Hatton, 1995) (Figure 4, Figure 5). In industry, a concentration range of 1% to 3% w/v is added to the bioreactors, we looked at 1% P188 concentration in the samples for DLS and dynamic surface tension in order to assess if differences were visible at the lowest concentration.

Both in water and in Freestyle media the measurement of the P188 with the addition of 20% P407 caused the biggest shift in micelle size compared to the purified P188. A 20% addition of P407, causes the micelles to be much larger and wider, with an average radius which is 10x larger than the control. P407 (approx. 12000 Da) has an overall higher molecular weight than P188 (approx. 8350 Da) but a lower percentage of PEO, which is the hydrophilic part of the co-polymer. The poloxamers adsorb on air-water and solid-

water interfaces; those adsorbed at hydrophobic interfaces, it is expected that the polyoxypropylene block would be located at the interface while the polyoxyethylene block extends into the solution (Sehgal et al., 2008). Higher PPO is normally associated with a higher water solubility, but higher molecular weight can lead to a more emulsifier and dispersant compound, which could explain the inefficiency of P407 as a protective surfactant for mammalian cells (Alexandridis and Alan Hatton, 1995). The increased micelle size of the P188/P407 compound can be explained by taking in consideration the weaker chemical bonds in the polyoxypropylene which P407 is richer of, compared to purified P188.

Although at 0.1% concentration, the addition of contaminants doesn't lead to an obvious shift in micelle size and intensity of the compounds, neither with the addition of high MW poloxamers or low MW poloxamers.

In Distilled Water, purified P188 shows a clear and consistent micelle size distribution with a primary peak around 5-10 nm and occasional larger aggregates (~100 nm). When impurities (e.g., P338, P237, PEG) are added, the micelle size distribution shifts, showing larger micelles or multiple size distributions, indicating a disturbance in micellization caused by the impurities.

While the general micellization pattern is similar, the micelles formed in the presence of Freestyle CHO media are generally smaller and less intense, especially for larger micelles. This suggests that the cell culture media components interfere with the micellization process, potentially reducing micelle formation or stabilizing smaller micelles. Larger micelles, which are prominent in water (especially with higher MW impurities like P407), are less pronounced or shift to lower intensities in the media. This could be due to interactions between media components and micelles, which impact micelle stability.

It is interesting to note, though, that even at low concentrations, in both water and Freestyle media, indicating the significant effect of these contaminants. However, in the media, the intensity of these larger micelles is lower than in water, suggesting that media components might reduce micelle aggregation or stabilize smaller micelles. The shift in micelle size is still detectable, but the intensity patterns change, which is important for

understanding how impurities affect performance in bioreactors. In the previous chapter it was observed how P188/P407 mixture can cause a more rapid loss of cell viability in culture, probably due to its inability to provide plasma membrane stabilisation as well as pure P188. This assay can provide a fast and reliable way to identify higher molecular contaminants in P188 samples.

4.5.2 Dynamic surface tension analysis of surfactants and impurities in liquid interfaces: implications for bubble formation and stability in cell culture media:

The measurements in the dynamic surface tension show that even at low concentrations, impurities can cause great shifts in surface tension dynamics.
However, the surface tension at the end of bubble lifetime is close to the P188 control, indicating that it wouldn't support the bubble's stability over a prolonged period of time, producing a less stable foam and, theoretically, causing less of a loss of cell viability. The other sample which deviates from the purified P188 control is P188/P407. Surface tension to support bubble formation does not seem to be affected by the high molecular weight impurity at the beginning of the bubble lifetime. Over the course of the experiment, the dynamic surface tension is gradually lowered by this sample, suggesting that its high surface-active characteristics lead to a more stable bubble. Foam and bubble stability is often linked to poloxamers with low shear protection, which P407 is an example of (Chang et al., 2017). The measurements in the dynamic surface tension show that even at low concentrations, impurities can cause great shifts in surface tension dynamics.

It is noticeable (Figure 6, 7) that samples that had a higher MW and lower PEO% display a different pattern of surface tension even if they make up only 20% of the additive sample. I believe that this analytic technique, together with viability assay can help identify batches of P188 which carry impurities which lower the surface tension compared to pure P188 and are known to be not as effective in aiding cell cultures in retaining high viability % over time. The experiment was repeated with samples dissolved in Freestyle CHO cell media. The overall surface tension for every sample was lower compared to the experiment carried out in distilled water. This result would indicate that the components which enrich the media tend to lower the surface tension and support bubble formation.

4.5.3 Impact of P407 Contamination on the Surface Activity of P188: analysis of the concentration-dependent surface tension

Surface tension is caused, on a molecular level, by the forces that cohesively interest liquid molecules. In a liquid environment, molecules interact equally with each other in every direction. The only exception is for the molecules that gather on the surface of the liquid interface, which aren't completely submerged. This way, a net inward force pulls together the molecules. These molecules together form a layer on the liquid surface, when an object goes through this interface it will need more force applied than when it executes motions submerged (Muñoz et al., 2000).

If the surface area of the liquid is increased it means that more surfactant molecules are present at the surface, therefore the liquid surface has more Gibbs energy compared to the rest of the liquid bulk. Surfactants do not tend to change in aqueous environments, but they normally comprise a polar region and develop a positive or a negative charge depending on the qualities of the liquid they are dissolved in such as pH and temperature. When at low concentrations, surfactant molecules can result in unassociated monomers. If the concentration of the poloxamers increases, the forces of repulsion and attraction between the molecules bring about aggregation between them, which leads to the formation of micelle layers. The concentration of poloxamer required for these monolayers of micelles to form is called critical micelle concentration (CMC) (Sehgal et al., 2008). These measurements indicate not only that even a low concentration of higher surface activity surfactants can yield a considerable difference to surface tension dynamics, but also gives an insight into the molecule configuration in the liquid interface of interest (Chang et al., 2017) (Figure 8). We know that a saturation of the surface tension by these polymers at the CMC will trigger a more accelerated formation of micelles. A change in surface tension after CMC break can therefore be attributed to micelle formation and also the natural distribution of MW in the poloxamer molecules, promoting the lowering of H2O surface tension when the concentration of higher MW, lower PEO% molecules, are introduced.

The observation that contaminated P188 exhibits higher surface activity compared to purified P188 suggests a better understanding of surfactant behaviour. Surface activity refers to the ability of a substance to lower the surface tension of the liquid interface it is found in. In this case, the presence of impurities or the mixture with P407 seems to increase the surfactant's capacity to lower surface tension. The higher surface activity of contaminated P188 could be attributed to several factors. Firstly, the presence of impurities, or the inclusion of P407, may alter the molecular arrangement of the surfactant molecules at the air/water interface. The presence of additional components could lead to different interactions with the air or water molecules, promoting an increase in surface tension.

Additionally, the mixed P188/P407 system might create synergistic effects. P407, which is a different type of poloxamer with distinct molecular properties, could interact with P188 in a way that enhances its surface-active properties, which are shown to be detrimental to cell viability as they promote the earlier onset of cell death (chapter 3). The mixture may allow for a more complex arrangement of molecules at the interface, further reducing surface tension. Impurities or interactions between different poloxamer types could affect the packing behaviour of the surfactant molecules at the interface. This might lead to a greater exposure of hydrophobic portions of the surfactant molecules to the air, resulting in a stronger reduction in surface tension. The enhanced surface activity could be explained by the differences in molecular structure and properties of these two poloxamers. P407 has a higher polyethylene oxide (PEO) content compared to P188 which could create a denser and more organised layer, effectively pushing the air and water molecules apart and thereby reducing the surface tension to a greater extent. The enhanced hydrophilic characteristics, allow P407 to more effectively orient itself at the air/water interface, reducing the surface tension.

It's worth noting that these observations can bring up practical implications. The ability to manipulate and enhance the surface activity from surfactants is crucial in various industrial applications. Understanding the factors that contribute to increased surface activity can aid in the design of more effective surfactant formulations for tasks such as

emulsification, wetting, and dispersion. In cell culture, poloxamers with higher molecular weight and higher PPO have shown not to be able to sustain cell viability as long as P188 and other lower, comparatively, lower MW polymers. This simple screening could prove useful for a cell culture free screening for contaminants in P188 products.

4.5 Conclusion and future work:

Three main experiments were carried out in order to assess the chemical and interfacial properties of purified and impure P188 in water and cell media. The first experiment assessed micelle size and distribution using DLS. The second intended to investigate the surface tension changes in the presence of different concentrations of P188 in a dynamic liquid environment using a bubble tensiometer. The last surfactant experiment aimed to assess the CMC and equilibrium surface tension using a Platinum Wilhelmy Plate tensiometer.

These results can help describe the surfactant characteristics of P188 batches, indicating that ones with surface active impurities, which result in lower shear protection compared to purified P188, could be screened using surface tension parameters.

Contaminated P188 displays higher surface activity compared to purified P188. This observation shows interesting implications for surfactant behaviour. The presence of impurities or a mixture with P407 enhances the surfactant's capacity to reduce surface tension. Several factors could contribute to this enhancement, including altered molecular arrangements at the air/water interface due to impurities or P407. The interaction between P188 and P407 might also create synergistic effects that result in a more efficient reduction of surface tension. The ability to manipulate and enhance surface activity has practical implications for various industrial applications, such as emulsification, wetting, and dispersion. In cell culture environments, enhanced surface activity is linked to a faster loss of cell viability and therefore it is useful to easily recognise compounds which comprise surfactants which lower the surface tension and have an identifiable shift in physical patterns in surface tension and micelle concentration and intensity. So far the most reliable method to screen for batch to batch variation, has been culture based. This study suggests a novel approach which could save valuable time and effectively screen out batches of P188 with expected low performance.

The use of the bubble tensiometer corroborates the Wilhelmy plates finds which point to the fact that even a minimal presence of impurities in pure P188, especially the ones which have a higher MW and PPO % than P188, lead to an overall lowering of the surface tension as well as the support of bubble lifetime over time. This analytical method is another fast and reliable way to screen batches of P188 which does not involve cell-culturing.

Chapter 5: Enhancement of transfection efficiency by P188: mechanisms and interactions in gene delivery protocols

5.1 Introduction

5.1.1 Transfection in biotechnology

Transfection is a very common and essential technique in biotechnologies which promotes the introduction of exogenous nucleic acids, such as DNA, RNA, and siRNA, into cells, allowing for the manipulation of gene expression enabling researchers to manipulate gene expression, study protein function, model diseases, and develop therapeutic strategies (Yin et al., 2014). In transfection experiments, the scientist's goal is to achieve efficient delivery of nucleic acids while maintaining high cell viability. Optimal transfection conditions are typically determined through empirical optimization, where various settings are meticulously tested to find an advantageous balance between transfection efficiency and cell survival (Li and Huang, 2000)

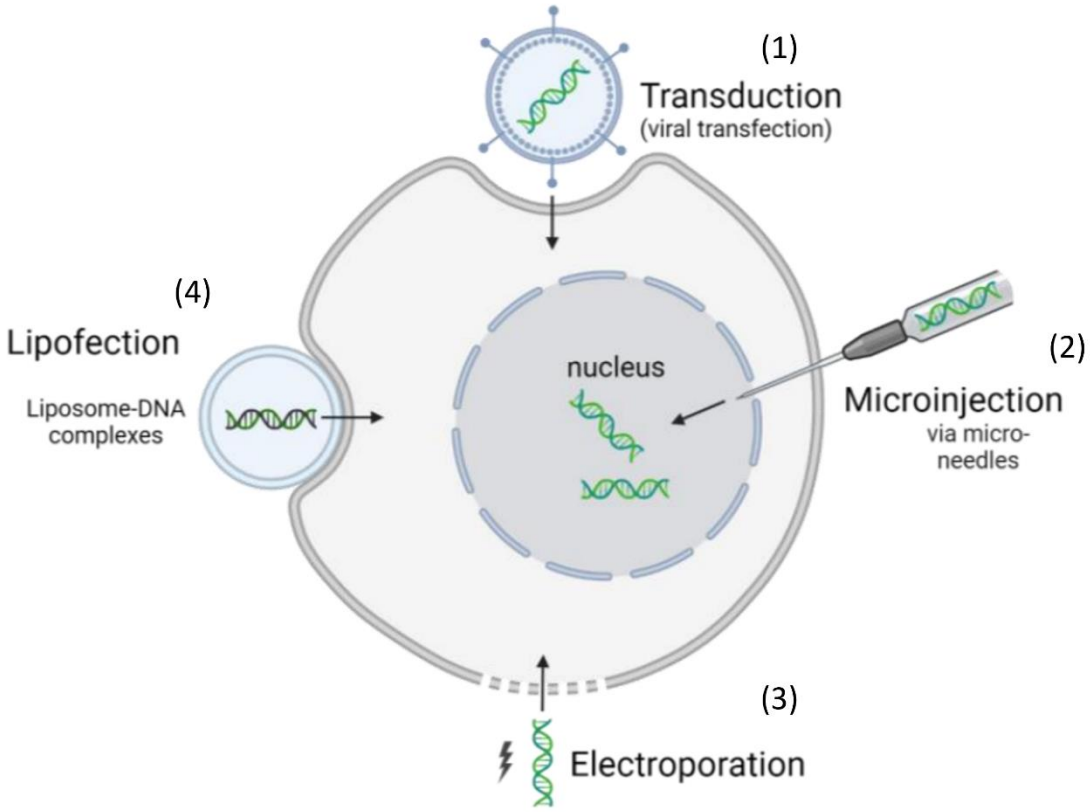


Figure 1: Diagram which represents various transfection methods in eukaryotic cells. On the left, (4) the particle is shown forming a complex with nucleic acids (DNA/RNA), indicated by intertwining strands. The complex is depicted merging with the plasma membrane, symbolising the process of chemical transfection facilitating the entry of nucleic acids into the cell. On the top (1) modified viral vectors are depicted, these vectors, characterised by their distinctive capsid structures, are shown carrying genetic material and entering the cell. At the bottom (3), electroporation is represented by high-voltage electrical pulses directed towards the plasma membrane, the plasma membrane is illustrated with temporary pores, signifying the entry points for nucleic acids into the cell through electroporation. On the right (2), a fine needle, representing the microinjector, is shown penetrating the plasma membrane and directly injecting nucleic acids into the cytoplasm or nucleus.

There are several different commonly used techniques to achieve the transfection of nucleic acids into eukaryotic cells: in viral vector-mediated transfection viral vectors, which are derived from viruses such as lentivirus, adenovirus, or retrovirus, are modified to carry the desired genetic material (Image 15) (Yin and Eltoukhy, 2014) . These vectors can efficiently deliver nucleic acids into cells, making them widely used in gene therapy and gene expression experiments and studies. Nanoparticle-mediated transfection uses, as the name suggests, nanoparticles, often made of materials like gold, silica, or magnetic nanoparticles, can be functionalized to bind to nucleic acids and deliver them into cells. Gold, for instance, can be chemically modified to attach nucleic acids. This is typically achieved by coating the AuNPs with cationic compounds like polyethyleneimine (PEI) or thiol-modified molecules, which can bind to the negatively charged backbone of the nucleic acids.

This chapter will be concentrating mainly on enhancing the efficiency of chemical transfection, sonoporation and electroporation.

Chemical Transfection involves the use of chemical reagents to facilitate the uptake of nucleic acids into the plasma membrane. Liposomes are cationic polymers, such as polyethyleneimine and calcium phosphate precipitates, are common reagents used to form complexes with nucleic acids, enhancing their cellular internalisation in transfection (Zabner J et al. 1995).

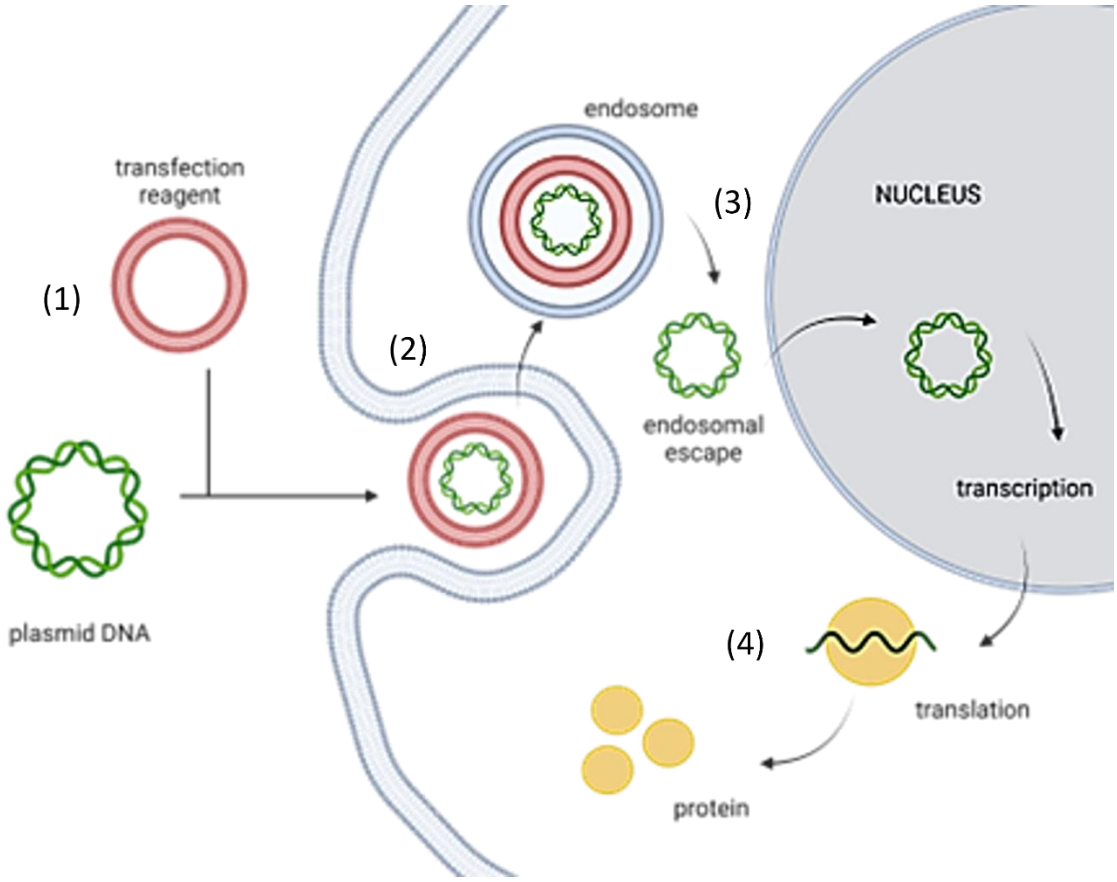


Figure 2: Schematic Representation of chemical transfection in eukaryotic cells. It represents transfection agents, like liposomes and cationic polymers, binding to nucleic acids to form complexes (1). These complexes are then interacting with the plasma membrane, their attachment and subsequent internalisation into the cell, likely via endocytosis (2). Inside the cell, the nucleic acids are released from the complexes and proceed to the nucleus (3). The final part of the figure demonstrates the expression of the introduced nucleic acid, symbolising the achievement of transfection (4).

Sonoporation can be achieved using several types of sonication devices, the sonoporator generates ultrasound weaves, typically in the frequency range of 20 kHz to several MHz. The ultrasound waves are transmitted into the medium in which the cells are suspended. This is usually done in a liquid environment to facilitate the propagation of sound waves. The sound weaves cause cavitation, which is a process which involves the formation,

growth, and implosion of microscopic bubbles in the liquid medium. These bubbles are created when the negative pressure phase of the ultrasound wave stretches the liquid, forming voids or cavities. The pores in the plasma membranes allow extracellular substances, such as drugs, DNA, or other therapeutic molecules, to enter the cells. This is particularly useful for molecules that cannot easily cross the plasma membrane under normal conditions (Suzuki et al. 2006).

In electroporation (Figure 2), cells are harvested and washed to remove culture media and any residual reagents that may interfere with the electroporation process, as voltage might struggle to travel in a liquid interface which has more molecules (Heller LC, Heller R. 2006). The nucleic acids, such as plasmid DNA or RNA, are prepared in an appropriate buffer or solution, often containing salts or other components to optimise transfection efficiency. The cell-nucleic acid mixture is transferred to a cuvette or an electroporation chamber, which typically consists of two electrodes. The cuvette is placed in the electroporation system and an electric field is applied to the cell-nucleic acid mixture by delivering a high-voltage pulse. This pulse transiently disrupts the plasma membrane, creating a temporary permeabilization which allows the entry of foreign nucleic acids. However, excessive or too high voltage strength and dispersion can cause irreversible damage to the membrane, leading to cell death (Sukharev and Anishkin, 2006). Electrochemical reactions occur at the electrodes during electroporation, which can generate reactive oxygen species (ROS) and other toxic byproducts. The presence of these reactive species can induce oxidative stress, alter cellular redox balance, and potentially damage the plasma membrane and intracellular structures (Neumann E, et al, 1999). Following the electroporation pulse, the cell suspension is immediately transferred to fresh growth media to allow cells to recover and the expression of the transfected genes to occur (VandenDriessche et al, 1999).

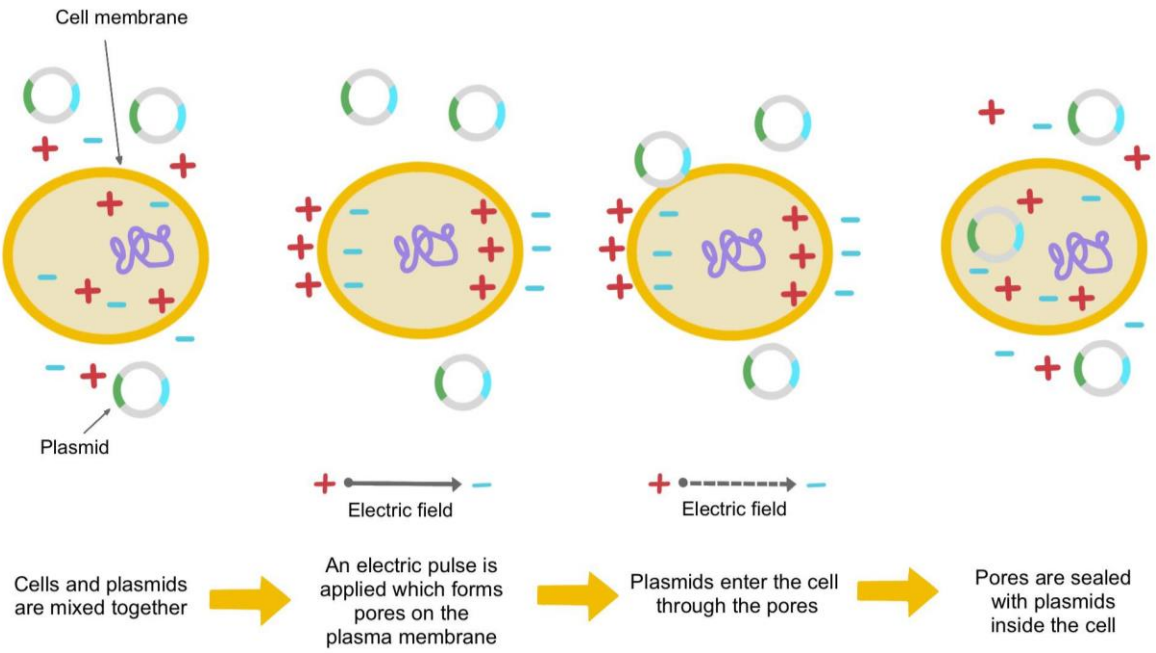


Figure 3: Four stages of electroporation. The image illustrates the of electroporation, a technique used to introduce foreign nucleic acids into a cell. In the initial stage, cells and plasmids are mixed together in suspension, allowing close proximity for the transfer of plasmids into the cells. Following up, an electric pulse is applied, which disrupts the plasma membrane's integrity, forming temporary pores. During the third stage, the plasmids are able to enter the cell through these pores. In the final stage, if the cell survives the permeabilization, the plasma membrane reseals, trapping the plasmids inside the cell, allowing for the potential expression of plasmid genes by the host cell's machinery.

Technique	Advantages	Disadvantages
Sonoporation	- Higher cell survivability - Single cell transfection - Automated by high throughput system	- Lower transfection efficiency - Complex, expensive instrumentation
Electroporation	- Higher transfection efficiency - Works for many different types of eukaryotic cells	- Greater loss of cell viability - Low transfection efficiency in primary cells - Higher cost for reagents
Lipid mediated	- Better for adherent eukaryotic cells - Low cost - High transfection efficiency for nucleic acids	- Medium loss of cell viability - Not always suitable for suspension cells - Low transfection efficiency in primary cells
Viral transduction	- Recommended for primary cell lines	- Greater loss of cell viability

		- Time consuming - More biosafety necessary

Table 6: Advantages and disadvantages of commonly used transfection techniques

5.1.2 The role of surfactants in enhancing transfection efficiency and cell viability:

Surfactants, such as P188, have emerged as potential candidates to ameliorate cytotoxicity during transfection. These compounds exhibit various mechanisms that aid in safeguarding cells from the adverse effects of transfection processes. They create a protective layer around cells, shielding them from mechanical damage caused by physical stresses like shear forces or sonication. Moreover, surfactants can stabilise the plasma membrane, reducing the risk of osmotic imbalances, ion fluxes, or leakage of intracellular components (Allen and Cullis., 2004). Some surfactants possess antioxidant properties that counteract reactive oxygen species (ROS) generated during transfection, thereby mitigating cytotoxicity and maintaining cell viability.

Given P188's routine use for sustaining cell viability in cell cultures, it stands as a promising candidate for transfection studies. Its potential physical capabilities in interacting with the lipid bilayer of plasma membranes underscore its significance in facilitating the uptake of nucleic acids, making it a compelling subject for further exploration. There is little amount of research on the role of poloxamers in the enhancement of transfection efficiency, especially in chemical transfection (Zhang et al, 2013).

Surfactants have the potential to enhance transfection efficiency by promoting the interaction between nucleic acids and plasma membranes and facilitating their uptake. Additionally, surfactants can indirectly promote transfection efficiency by helping protect

cells from external damage caused by the transfection process. Surfactants can interact with nucleic acids to form complexes, commonly known as lipoplexes (Felgner et al. 1985). These lipoplexes can protect nucleic acids from degradation and facilitate their entry into cells. By improving the interaction between nucleic acids and plasma membranes, surfactants can enhance transfection efficiency and reduce the need for high concentrations of nucleic acids or other potentially cytotoxic components (Opanasopt et al, 2011).

Furthermore, surfactants like P188 have demonstrated direct enhancement of transfection efficiency by facilitating the interaction between nucleic acids and plasma membranes. These interactions promote the uptake of genetic material into cells and can even lead to the formation of complexes, such as lipoplexes, that protect nucleic acids from degradation and facilitate their entry (Felgner et al. 1985). This enhancement in the interaction between nucleic acids and plasma membranes not only improves transfection efficiency but also reduces the necessity for high concentrations of potentially cytotoxic components.

The choice of surfactant should consider the specific requirements of the transfection experiment and the cell type being used. Factors to consider include the charge and structure of the surfactant, as well as its compatibility with the nucleic acids and target cells (Attia, et al, 2018). It is essential to optimise the surfactant concentration and transfection conditions to achieve a balance between transfection efficiency and cell viability.

Throughout this chapter, the investigation focuses on investigating P188's impact on transfection efficiency and looking into the underlying mechanisms behind its enhancement. Methodologies include experiments in electroporation, chemical transfection, zeta potential analysis, and an exploration of avenues for further research. These aim to contribute important data which will be able to optimise gene delivery strategies and advance biotechnological applications.

5.2 Aims:

The aim of this chapter is to investigate the potential of P188 as a transfection enhancer and explain its mechanism of action when it interacts with the cells and the transfection reagents. This investigation is based on the hypothesis that P188's unique physicochemical properties could facilitate and enhance successful delivery of genetic material into target cells. To achieve the overall aim of this chapter, the following specific aims were pursued:

1. Characterising the effects of sonication and P188 on cell viability:

    - Determine the impact of sonication on cell viability by assessing cell survival and damage after mechanical disruption of the plasma membrane.

    - Investigate the potential protective role of surfactant P188 in preserving plasma membrane stability following sonication.

2. Evaluating transfection efficiency with different membrane disruption methods:

    - Assess the transfection efficiency when using electroporation and chemical transfection techniques in cells with disrupted plasma membranes.

    - Compare the effects of surfactant P188 in enhancing transfection efficiency for these two different membrane disruption methods.

3. Exploring molecular interactions between P188 and chemical transfection reagent:

    - Investigate the molecular interactions between surfactant P188 and the chemical transfection reagent Xgene.

    - Analyse these interactions using techniques such as zeta potential and dynamic light scattering, aiming to understand the physicochemical aspects of their interaction.

5.4 Results

5.4.1 The effects of P188 on CHO-S cell recovery and plasma membrane resealing after mechanical damage:

P188 can be considered a membrane sealant which also acts as a surface stabiliser (Spurney et al., 2011), which interacts with the plasma membrane without crossing the outer phospholipid barrier, making it a valuable tool for plasma membrane stabilisation and repair. Studies on the mdx mouse heart lacking dystrophin revealed that administering P188 during stress prevented acute cardiac failure, suggesting its potential as a membrane sealant (Martindale & Metzger, 2014) (Czeiszperger et al, 2020).

In order to test this theory, cells were treated with poloxamer after being subject to controlled mechanical damage with the use of sonication and other types of controlled damage (voltage, lipofectamine).

As a preliminary experiment, a sonoporation without the addition of a plasmid had been carried out in high density CHO-S samples in order to assess a viability change in the cells immediately after sonication and observe the potential difference between cell samples treated with P188 and the poloxamer free control media over three days (Figure 4).

5.4.1.1 Does P188 aid cell viability of CHO-S cells in recovery after sonication?

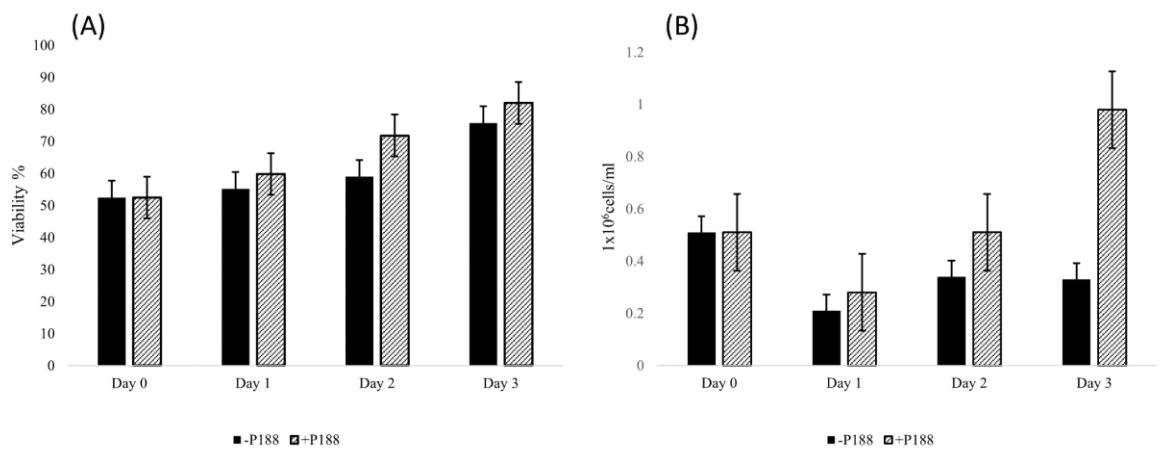


Figure 4. Cell viability and viable cell density of CHO-S samples after sonication at 30 kHz for 10 seconds. The samples were resuspended in media with and without addition of 1g/L P188. (A) Represents the cell viability of CHO-S cells on the day of sonication (day 0) until day 3 of recovery. (B) Represents the viable cells density of CHO cells from day 0 to day 3 after sonication. The graphs show the median results from the experiment performed in triplicates.

On day 0, $1x10^6$ cells per condition were sonicated at 30kHz for 10 seconds. After sonication, the viability was halved for both samples, and they were immediately incubated in pluronic free-Freestyle media. The study sample differed from the control by addition of 1g/L purified P188. The study sample showed a faster recovery of viability on day 1, 2 and three compared to the control sample, which lagged in viability by 8% (Figure 11, A). The cell density was also faster to recover in the study sample, which on day three shows a threefold increase in cell density (Figure 4, B).

Based on the preliminary sonoporation experiment results, indicates that the test sample, treated with P188, exhibited a faster recovery of viability and cell density compared to the control, the three-fold increase in cell density in the test sample further supports the theory that P188 facilitated cellular repair and growth, potentially sealing damaged membranes and allowing cells to proliferate more rapidly. Given the promising outcomes of the experiment, conducting a transfection using electroporation would serve as a

complementary experiment to further validate P188's role as sealant for the plasma membrane.

5.4.1.2 Transfection efficiency in CHO-S using different electroporation conditions:

Electroporation is a technique which involves the use of electrical pulses to introduce molecules into cells, which would allow testing whether P188 enhances the sealing of membranes post-transfection (Sokołowska and Błachnio-Zabielska, 2019). If cells treated with P188 show improved viability and exhibit less damage or stress post-electroporation compared to the control group, it would reinforce the notion that P188 effectively acts as a membrane sealant. The electroporation experiment serves to strengthen the findings from the sonoporation experiment, providing a more comprehensive understanding of P188's role in preserving plasma membrane's integrity. It would also provide additional data points supporting the hypothesis that P188 aids in sealing cellular membranes, validating its potential utility in cellular repair processes and its value in bioprocessing.

In order to assess the best electroporation conditions for CHO-S cells, a regime of three experiments had been carried out using three experimental conditions. Electroporation is capable of causing significant issues in the cell viability of samples as it involves the creation of pores on the plasma membrane. Researchers typically optimise voltage and pulse conditions through trial and error, considering factors such as type of cell, the nature of the transfection reagent, and the desired outcome. Optimization can ensure the best possible results for a particular experiment taking into consideration cell viability and transfection efficiency. The study focuses on how the addition of surfactants in the recovery period after electroporation can further improve successful transfections, by potentially increasing the survivability of cells after the voltage has damaged their plasma membrane integrity (Figure 5).

For the experiment cells had been electroporated at the following conditions: 1 pulse at 1700 V, 2 pulses at 1700 volts and 1 pulse at 2000 volts.

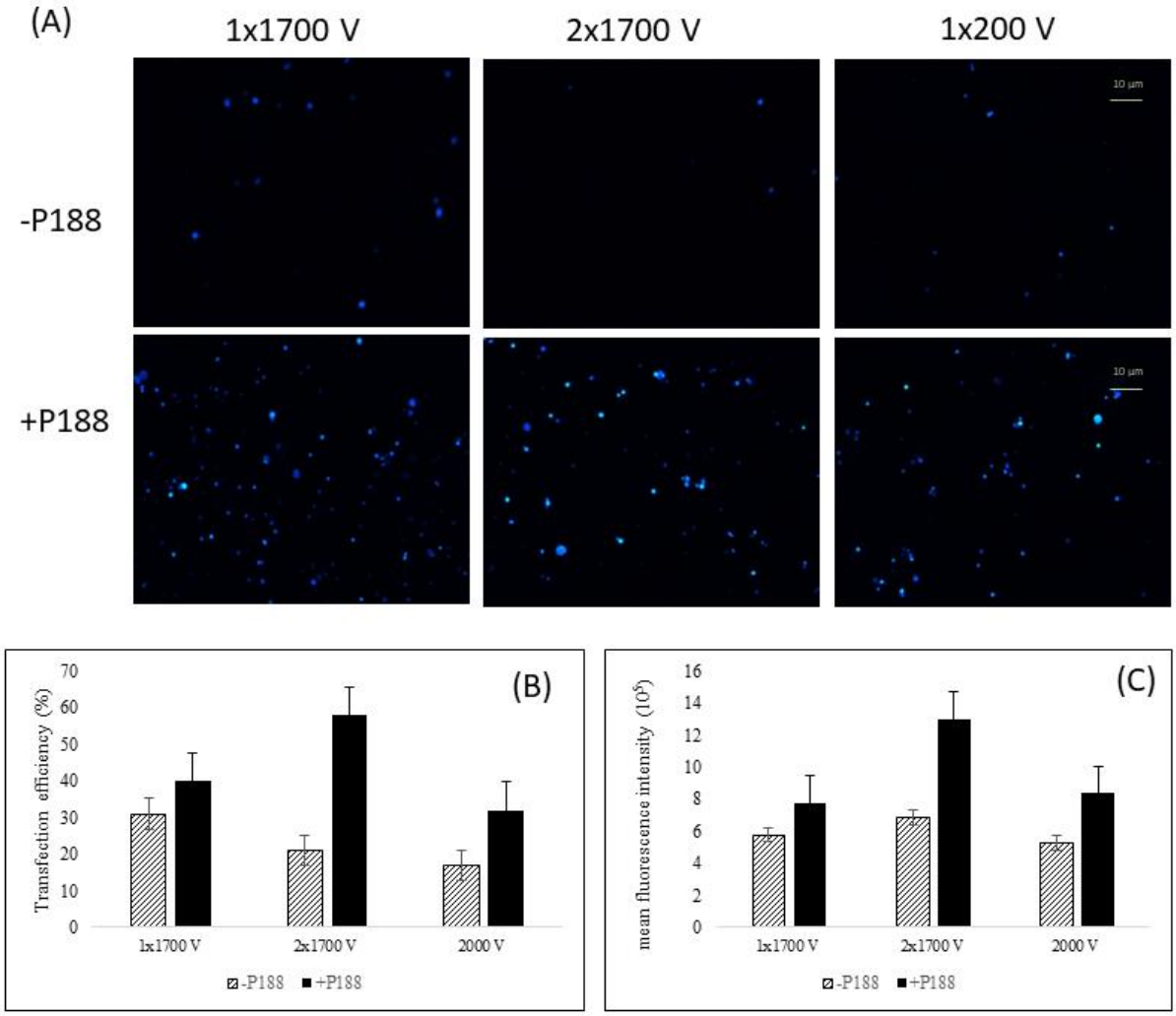


Figure 5: Fluorescence microscopy of YFP protein in transfected CHO-S cells after 48 h of transfection with different voltage regiment. (A) Micrograph of electroporated cells at different voltages and pulses with and without the addition of P188. (B) Cell transfection efficiency was detected using YFP by image analysis. (C) Mean fluorescence intensity of both cell samples after 48h from electroporation. Experiment was carried out in triplicates.

The results indicate that increasing the electroporation voltage from 1700 volts to 2000 volts under the same conditions (1 pulse) resulted in a decrease in transfection efficiency of YFP. This suggests that higher voltage might be detrimental to cell viability and may possibly cause more significant damage to the cells during electroporation (Figure 5, A). Two pulses at 1700 volts resulted in a significantly higher transfection efficiency compared to one pulse at the same voltage. This suggests that the number of pulses can have a substantial impact on transfection efficiency and the plasmid DNA uptake. More pulses might allow for better permeabilization of the plasma membrane, increasing the uptake of the transfection reagent or DNA. Each electroporation pulse creates temporary pores or openings in the plasma membrane, allowing external molecules, such as DNA or transfection reagents, to enter the cell. Using multiple pulses can even help ensure a more uniform distribution of the transfection reagent or DNA throughout the cell population. This minimises the risk of only a subset of cells being transfected and increases the overall transfection efficiency across the entire cell population, compared to less pulses and higher voltage.

The most significant observation in these results is the positive impact of surfactant P188 on transfection efficiency. In all cases, the presence of P188 enhanced transfection efficiency. The increase in transfection efficiency with P188 suggests that this surfactant plays a crucial role in improving either the delivery of genetic material into the cells or the cell viability after electroporation.

5.4.1.3 Transfection efficiency in CHO-S with P188 addition during recovery time after electroporation:

Following the determination of optimal electroporation conditions for CHO-S cells, the subsequent experiment aimed to investigate the impact of adding the surfactant P188 during the recovery period on transfection efficiency. The experiment involved electroporating cells under the previously established optimal conditions and then assessing the transfection efficiency with and without the addition of 1g/L P188 during the recovery phase.

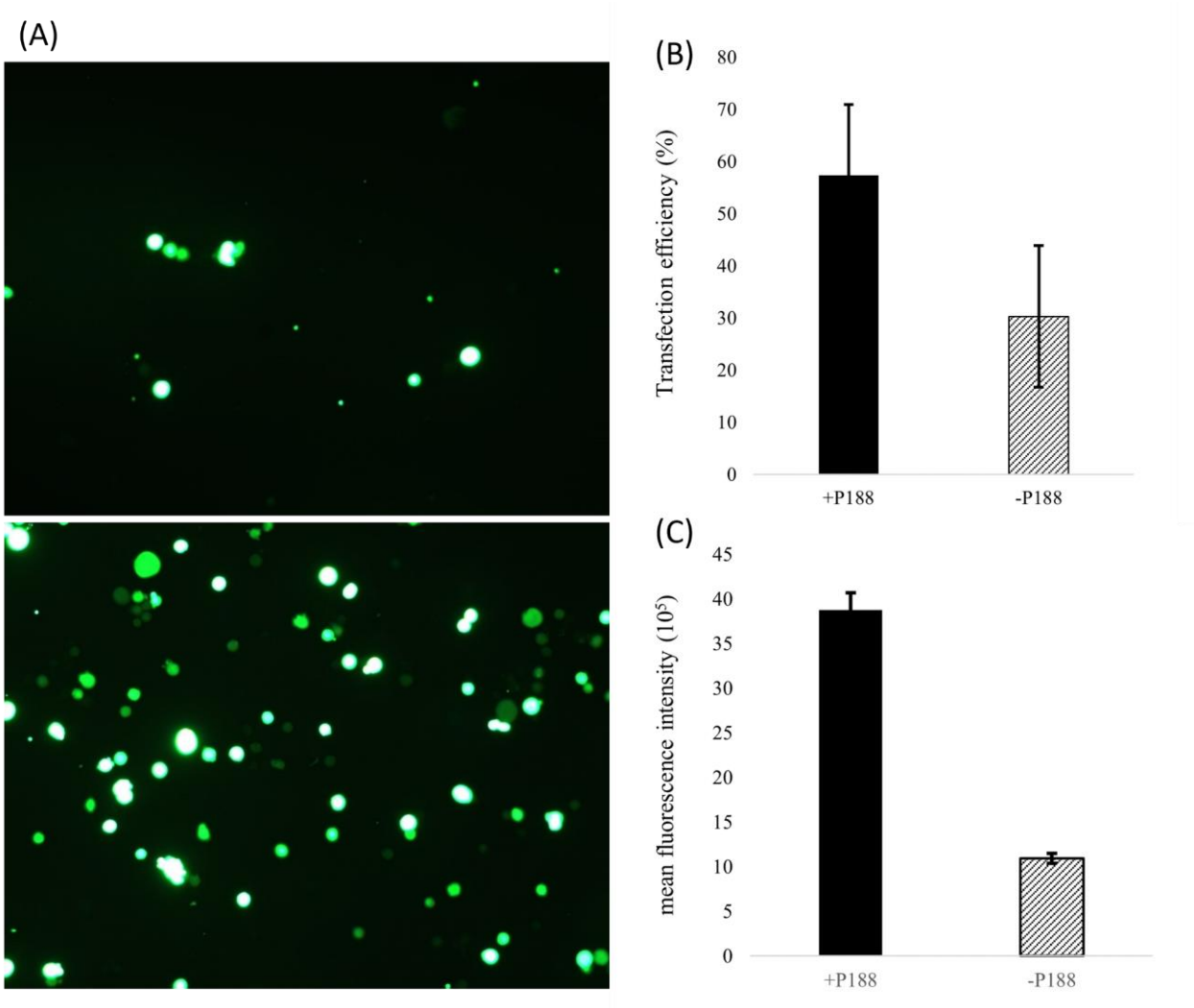


Figure 6: Fluorescence microscopy of YFP gene in transfected CHO-S cells after 48 h of transfection through electroporation at 1700V. (A) Micrograph of cells transfected without the addition of P188 in the post-transfection incubation, and cells transfected with the addition of 1g/L P188 in the post-transfection incubation. (B) Cell transfection efficiency was detected using YFP by image analysis. (C) Mean fluorescence intensity of both cell samples after 48h from electroporation. The results are based on triplicate repeats of the experiment.

The presented data (Figure 6) demonstrates that the utilisation of P188 confers a substantial boost in transfection efficacy within CHO-S cells. Specifically, post-transfection treatment with 1g/L of P188 resulted in a noticeable rise in transfection efficiency, increasing from 30.3% in non-treated cells to 57.4% in P188-treated cells. This enhancement, nearly doubling the proportion of successfully transfected cells, indicates the potential of P188 on the transfection process. Additionally, the augmented mean fluorescence intensity observed in P188-treated cells signifies a heightened abundance of expressed YFP protein. This quantitative measure of protein expression within individual cells not only substantiates the efficacy of P188 in facilitating gene delivery but even highlights its role in effectively translating the delivered genetic material into substantial protein products within the cell.

In chapter one, it was evidenced that the addition of P188 delays the onset of apoptosis and other cell death mechanisms. The mechanism by which P188 enhances transfection efficiency and increases the translation of transfected YFP in CHO-S cells likely involves several factors. P188 stabilizes and repairs cell membranes by sealing pores created during electroporation, reducing cell death and improving cell survival. It also helps mitigate stress responses, potentially decreasing apoptosis, and oxidative damage, thereby facilitating quicker cellular recovery.

The data strongly support the notion that P188 treatment elevates both the rate of successful gene delivery and the subsequent expression of the desired protein in CHO-S cells, proving that P188 has the potential to be a significant reagent in enhancing transfection efficiency and protein production.

5.4.2 Transfection efficiency after P188 treatment pre and post lipid-mediated chemical transfection:

The observations in the electroporation-based transfection experiment, where P188 has been found to significantly enhance transfection efficiency, provided a robust rationale to explore its impact on other commonly used transfection methods, such as lipid-mediated chemical transfection methods. Extending this investigation to chemical transfection allows for a broader examination of P188's influence across distinct modes of genetic material introduction into cells. Such an analysis could validate its consistent efficacy and potential, which can offer a complementary and/or alternative method to enhance transfection outcomes. Confirming P188's effectiveness in chemical transfection could expand its applicability and even provide researchers with additional tools to augment transfection efficiency in various experimental contexts. Ultimately, this exploration aims to support P188's role as a useful transfection enhancer and extend its potential utility across a spectrum of transfection techniques.

The aim of the experiment is to evaluate the impact of P188 treatment and electroporation on YFP fluorescent intensity in different lipid mediated, chemical transfection conditions. The four experimental groups were designed to investigate the combined effects of preincubation with P188 and recovery conditions, both with and without P188. The measured YFP fluorescent intensities at 48 hours post-electroporation were as follows: Group 1, Group 2, Group 3, and Group 4 (figure 7). Group 1: preincubation without P188, recovery without P188. Group 2: preincubation without P188, recovery with P188. Group 3: preincubation with P188, recovery without P188. Group 4: preincubation with P188, recovery with P188.

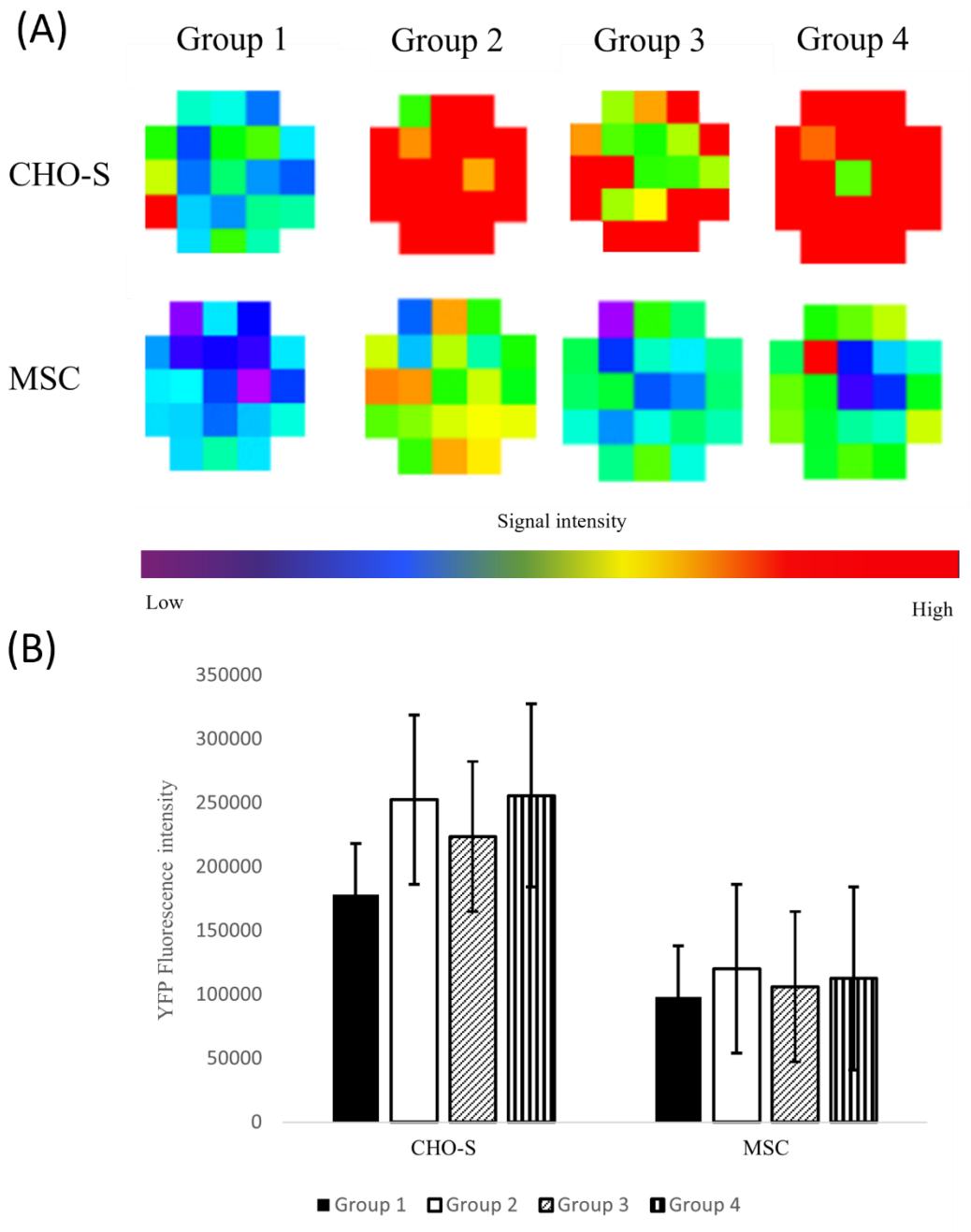


Figure 7: (A) Representation of chemical (Xtreme-gene) transfection efficiency in CHO-S cells and MSc using fluorescence intensity on Clariostar plate reader. The samples with the most intense signal are the ones obtained from experimental conditions group two

and four, CHO-S. (B) represents the same data of YFP Fluorescence intensity in a histogram to compare the transfection efficiency in CHO-S cells and in MSCs.

The results indicate that preincubation with P188, as seen in Groups 2 and 4, tends to promote higher YFP fluorescence intensity compared to groups where P188 was not included during preincubation (Groups 1 and 2, Figure 14-B). Group 4, which received both preincubation and recovery with P188, exhibited the highest YFP fluorescence intensity at 255,709. This suggests that P188 might play a role in enhancing transfection efficiency or intracellular YFP accumulation, possibly by facilitating cellular uptake of the YFP expression vector or contributing to cell resealing after chemical damage. Comparing Groups 1 and 3, where recovery was carried out without P188, it is possible to observe a noticeable increase in YFP fluorescence intensity in Group 3 (223,741) compared to Group 1 (178,145). This suggests that the presence of P188 during recovery might have a positive effect on YFP expression. Group 2, which had preincubation without P188 but recovery with P188, exhibited a higher YFP intensity (252,590) than both Group 1 and Group 3. This could imply that P188 supplementation during recovery has a pronounced effect on enhancing YFP expression, regardless of whether it was present during preincubation. Comparing Groups 2 and 4, which had identical recovery conditions but different preincubation conditions, we notice that the presence of P188 during both preincubation and recovery (Group 4) led to a slightly higher YFP intensity compared to only having P188 during recovery (Group 2). This suggests a cumulative effect of P188 when used throughout the experiment, supporting the notion that P188 may play a role in enhancing the transfection or expression process, as well as cell viability.

5.4.3 Zeta potential behaviour of Xgene in presence of surfactant P188

Zeta potential, also known as electrokinetic potential, it's a measurement which describes the electrical charge present at the interface between a solid surface and a liquid medium, such as a solution which can contain colloids, surfactants and other dispersed particles.

It is an important measurement in surfactant science as it can provide information about the stability and the behaviours of particles in a solution (Elgegren M., et al, 2023).

X-tremeGENE HP DNA (Xgene) is a commonly used and ready available transfection reagent from Merk which forms a non-liposomal complex with the DNA and then transports it into mammalian cells. The manufacturer claims that it is claimed to work with a variety of cell types and that it can support the increase of transfection efficiency. Investigating the zeta potential of the Xgene with the addition of P188 can shed light on how P188 facilitates or enhances transfection efficiency. Understanding the electrostatic properties and potential interactions between P188 and the transfection reagent can help understand the mechanisms underlying the observed increase in YFP fluorescence intensity in samples chemically transfected with the addition of P188.

The zeta potential distribution analysis has shown the interaction of Xgene in the presence of water (H2O) and subsequent interaction with the 1:1 P188 surfactant. The zeta potential measurements, reflecting the surface charge distribution, look into the molecular interactions occurring within the system (Figure 8).

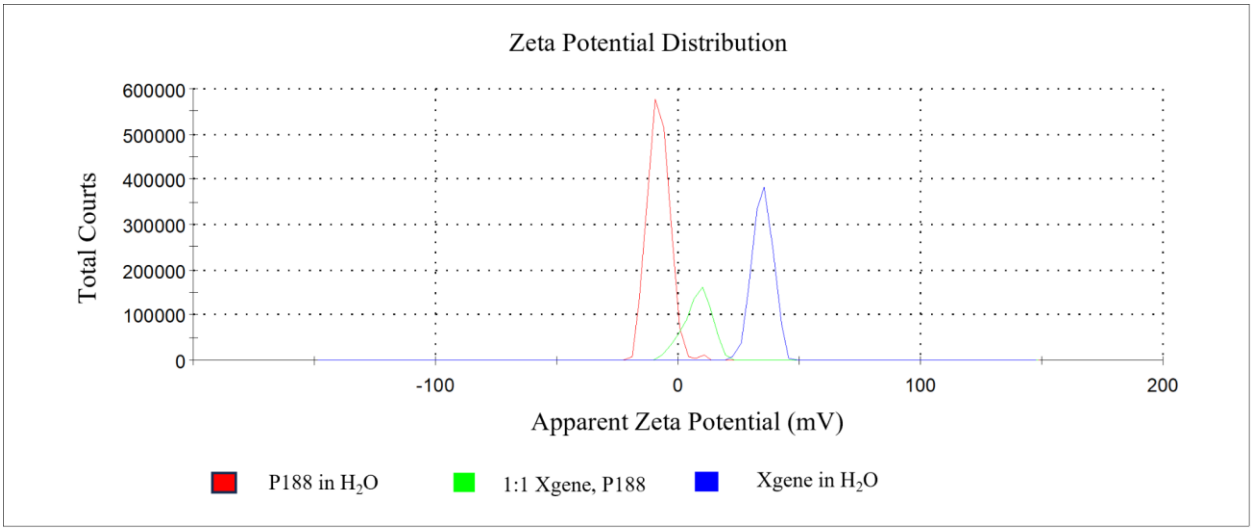


Figure 8: Distribution of the zeta potential of Xgene in water as well as P188 in water and a ratio 1:1g of Xgene and P188. The zeta potential distribution for the 1:1g mixture

of Xgene and P188 reveals the combined surface charge behavior of the mixture. This data can show how P188 influences the stability of Xgene particles at room temperature.

The zeta potential distribution analysis has shown the interaction of Xgene in the presence of water (H2O) and subsequent interaction with the 1:1 P188 surfactant. The zeta potential measurements, reflecting the surface charge distribution, look into the molecular interactions occurring within the system (Figure 8).

The initial zeta potential distribution of Xgene in H2O is displayed at a prominent peak at approximately 40 mV, which is indicative of a substantial positive surface charge. This high zeta potential value suggests robust electrostatic repulsion between Xgene particles, reflecting a stable colloidal dispersion. This observation aligns with expectations of good colloidal stability associated with higher zeta potentials. Upon the introduction of the 1:1 P188 surfactant into the system, a shift was picked up in the zeta potential distribution. The peak, initially at 40 mV, made a noticeable reduction to around 20 mV. This shift in the apparent zeta potential reflects a marked alteration in the surface properties of Xgene particles upon interaction with P188.

The consistent zeta potential values at both room temperature and physiological cell culture condition conditions (37 degrees Celsius) suggest that the mixture of P188 with the chemical transfection reagent remains stable even above room temperature (37 C). (Figure 9). This is an important characteristic as maintaining stability under physiological conditions is crucial for applications involving mammalian cell transfection. The stability of the mixture at these temperatures is a promising sign for its use in transfection applications. A stable mixture can facilitate the uniform distribution of the transfection reagent and improve its ability to interact with plasma membranes, potentially leading to a more efficient transfection.

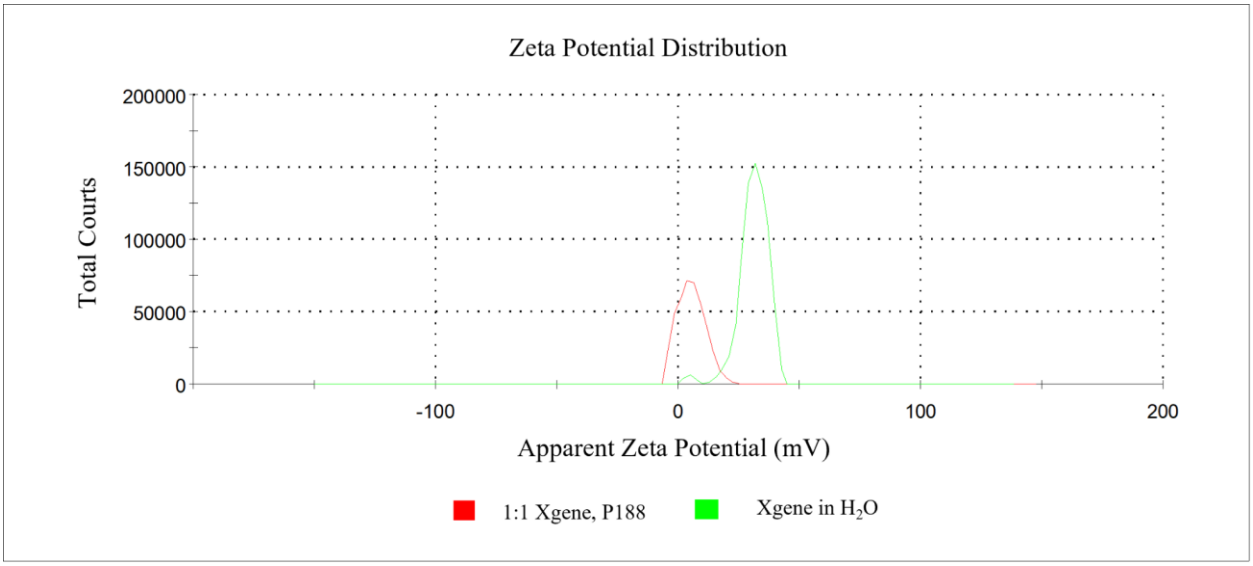


Figure 9: zeta potential of Xgene in H20 as well as the zeta potential of 1:1g Xgene and P188 compound at 37 C.

In this experiment (figure 17), the zeta potential of 10 mV remains constant for both ratios of P188, suggesting that the zeta potential is not significantly influenced by the ratio of P188 to Xgene in the above conditions (2g and 1g of P188). This indicates that the charge characteristics of the mixture are consistent, regardless of the relative proportions of the components. The stable zeta potential values of 10 mV suggest that the mixture maintains a relatively constant surface charge. This stability is important as it implies that the mixture should not aggregate or destabilise if the concentration of the poloxamer and the Xgene changes, which is crucial for transfections. The differences in total counts are notable. The mixture with a 2:1 ratio of P188 to Xgene has a higher total count (approximately 500,000) compared to the 1:1 ratio (approximately 150,000). The total count reflects the number of particles in the solution. This increase in total counts suggests a higher number of dispersed particles in the 2:1 ratio, which could be attributed to differences in the composition of the mixture at different ratios.

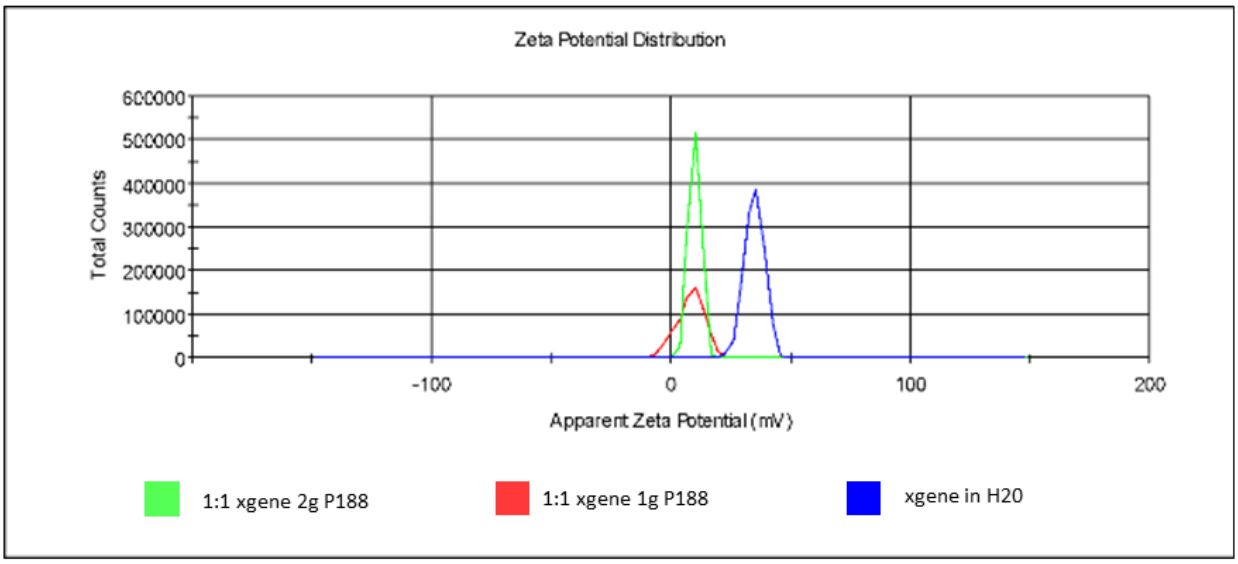


Figure 10: Zeta potential of Xgene in H20 as well the zeta potential of molecules of Xgene and P188 at respectively 1g and double.

5.4.4 Discussion

5.4.4.1 The effects of P188 on CHO-S cell recovery and plasma membrane resealing after mechanical damage with sonoporation and electroporation:

Sonoporation and electroporation were used to test the potential of P188 of aid in the resealment of plasma membrane and promote the genetic uptake. The improved transfection efficiency in P188-treated CHO-S cells may suggest that P188 might aid in cellular uptake and internalisation of the YFP plasmid. The disruption of the plasma membrane integrity could promote the incorporation of the plasmid into the cellular machinery, leading to successful transfection and expression of the YFP gene.

The results align with studies that have explored the use of membrane-disrupting agents to enhance transfection efficiency (Aydin et al, 2015). Several research articles have highlighted the potential of poloxamers in promoting cellular uptake of genetic material. The study extends this knowledge by specifically demonstrating the efficacy of P188 in CHO-S cells, contributing to the growing body of evidence supporting the use of poloxamers in transfection studies. The link between increased fluorescence intensity and transfection efficiency can be attributed to the successful integration of the YFP plasmid into the cell. The P188-enhanced cellular uptake and internalisation of the plasmid potentially lead to more copies of the plasmid being available for transcription and translation, resulting in higher YFP expression levels and subsequently elevated fluorescence intensity.

To understand further the influence of P188 on transfection mechanisms, a following experiment was planned in order to assess the transfection efficiency both before and after P188 treatment, in conjunction with lipid-mediated chemical transfection methods. This approach could showcase a potential synergistic effects or additive benefits of combining P188 treatment with other transfection techniques, which can offer a more in-depth evaluation of optimised transfection protocols for enhanced gene delivery in CHO-S cells.

5.4.4.2 Transfection efficiency after P188 treatment pre and post lipid-mediated chemical transfection

The success of P188 preincubation in promoting transfection efficiency can be attributed to several potential mechanisms, although it's important to note that the exact mechanisms might vary depending on the specific cell types, experimental conditions, and the nature of the DNA/RNA being transfected. Some possible explanations for the enhancement shown in the data of transfection efficiency due to P188 preincubation could be related to multiple factors (Figure 7).

Poloxamers, like P188, are known for their ability to disrupt lipid membranes. Preincubation with P188 could increase the permeability of the plasma membrane, making it easier for nucleic acids (such as plasmid DNA or siRNA) to enter the cells. This effect could result in improved transfection efficiency (Deshpande, et al 2016). Successful transfection requires nucleic acids to escape from endosomes after internalisation. Poloxamers have been reported to facilitate endosomal escape by destabilising endosomal membranes, preventing nucleic acids from getting trapped in endosomes and improving their availability in the cytoplasm (Vasir and Labhasetwar, 2007). Poloxamers can even act as stabilisers, preventing aggregation of nucleic acids in the medium and thus maintaining their bioactivity. Preincubation with P188 might prevent the formation of aggregates and allow for better interaction between nucleic acids and the cells (Aydin et al., 2016). Poloxamers have been suggested to interact with cellular components and molecular machinery involved in endocytosis and intracellular trafficking. Preincubation with P188 might modulate these interactions, leading to enhanced uptake and trafficking of nucleic acids (Kaminskas, et al, 2008). Most importantly, P188 has been theorised to interact with the plasma membrane, effectively helping the healing process by preferentially adhering to the breakage and successfully releasing itself once the healing process is complete.

While these findings are promising, there are some limitations to consider: the exact mechanisms through which P188 exerts its influence on YFP expression remains unclear. Further studies could deepen the knowledge into the molecular interactions between

P188 and cellular components, making its impact on transfection efficiency clearer. Additionally, exploring time points beyond 48 hours could provide insights into the longer-term effects of P188 treatment and electroporation. Following the increase in YFP fluorescence intensity reported in the data with preincubation of P188 during chemical transfection, exploring the zeta potential between Xgene transfection reagent and P188 becomes a robust next step. Understanding their electrostatic interactions could elucidate mechanisms behind the observed enhancement in transfection efficiency. Assessing the zeta potential provides additional data into charge distribution and potential binding, aiding in optimising protocols and deepening the understanding of how P188 facilitates plasmid uptake or enhances transfection processes.

5.4.4.3 Zeta potential behaviour of Xgene in presence of surfactant P188:

The observed shift towards lower zeta potential values when P188 is added is consistent with the known behaviour of P188 as an amphiphilic surfactant. The hydrophilic and hydrophobic segments of P188 enable it to adsorb onto particle surfaces, thereby affecting their charge distribution. In the data, the interaction between P188 and Xgene led to a neutralisation or shielding of some of the surface charges, diminishing the overall positive charge density. This reduced electrostatic repulsion within Xgene particles, which is evidenced by the shift towards lower zeta potential values. Interestingly, the zeta potential distribution of pure P188 in water (Figure 8) showed a peak at approximately -7 mV, indicating that P188 carries a negative charge under the given experimental conditions. This discrepancy in charge sign between Xgene and P188 emphasises the significance of the interaction between the two components. The shift in zeta potential upon the introduction of P188 suggests a potential electrostatic complex formation or adsorption, resulting in the altered zeta potential distribution shown in the data).

The shift in zeta potential can have interesting implications for the molecular interactions and stability of the system. It implies changes in particle-to-particle interactions and the potential for altered aggregation behaviour due to the variation in surface charge

properties. The reduction in zeta potential upon interaction with P188 might impact the stability of Xgene colloids and influence their behaviour in various applications.

The statistical analysis of the zeta potential distributions has yielded further insight into the molecular interactions involving Xgene and its interaction with the 1:1 P188 surfactant. The pronounced shift in zeta potential values, accompanied by the calculated p-value of 0.000479126, highlights the robustness and significance of the observed change in surface charge properties. The observed change in zeta potential values, supported by such a low p-value, suggests a genuine alteration in particle-particle interactions driven by the interaction with P188. This alteration could potentially influence aggregation tendencies, stability, and other colloidal behaviours, as well as complex formation and interaction with the plasma membrane. Furthermore, the highly significant difference in means enhances the credibility of the hypothesis that the introduction of P188 causes a substantial change in the electrostatic properties of Xgene particles. This supports the notion that P188, with its amphiphilic nature, effectively interacts with Xgene and leads to a modification in the overall surface charge distribution.

The zeta potential analysis of figure 7, the mixture of Poloxamer and Xegene was conducted at the specific incubation temperature of 37°C. This has shown in the data the behaviour of Xgene in H2O and its interaction with the 1:1 P188 surfactant. The shift in zeta potential values at this elevated temperature indicates that temperature is not a critical factor influencing the molecular interactions within the system. The initial zeta potential distribution of Xgene in water at 37°C displayed a peak at 37 mV. This significant positive surface charge suggests strong electrostatic repulsion between particles, indicating colloidal stability.

In figure 8, The constant zeta potential of 10 mV regardless of the ratio of P188 to Xgene suggests that the electrostatic properties of the mixture remain consistent. This property is favourable for applications like transfection because it indicates that the mixture's surface charge does not fluctuate with different ratios, ensuring the stability and reliability of the formulation. The zeta potential of 10 mV for both P188 and Xgene mixtures is a promising result for stability and consistent electrostatic properties. The total count

differences between the two ratios may indicate variations in composition or particle size, which should be further investigated to assess their impact on transfection efficiency and overall suitability for the intended application.

5.5 Conclusion and future work:

This chapter aimed to investigate the effects of P188 on transfection efficiency and investigates the mechanisms of increased efficiency by also investigating how the poloxamer interacts with the chemical transfection reagent at a physical level. P188 exhibits membrane-stabilizing properties that are critical for repairing and stabilizing cell membranes following electroporation. It is though that, by integrating into damaged membrane regions, P188 facilitates rapid and efficient sealing of transient pores, thereby reducing cell death and enhancing cell survival post-transfection. It also promotes enhanced cellular recovery by mitigating electroporation-induced stress, decreasing apoptotic signalling, and providing protection against oxidative damage from reactive oxygen species (ROS), thus supporting more effective and timely cellular recovery.

This study of electroporation highlights the effectiveness of P188 in enhancing transfection efficiency in CHO-S cells. The substantial increase in transfection efficiency, as indicated by the higher percentage of YFP-expressing cells and the elevated mean fluorescence intensity, supports the role of P188 as a potential enhancer of gene delivery. Further research is needed to elucidate the underlying physics, chemical and biologic mechanisms and evaluate any potential cytotoxic effects will contribute to a more comprehensive understanding of P188's applicability in transfection protocols.

Using chemical transfection, the experiment demonstrated the effects of P188 treatment and electroporation on YFP fluorescent intensity. Preincubation with P188 and its presence during recovery appear to enhance YFP expression, with the most pronounced effect observed when P188 is present throughout the experiment. These findings contribute to the understanding of optimising transfection protocols for increased gene expression and highlight the potential of P188 as a transfection enhancer. The enhancement of transfection efficiency through P188 treatment could have exciting implications for both research and practical applications as transfection is an extremely common procedure in biotechnology. Improved gene delivery and expression are essential in fields such as gene therapy and biotechnology. The findings of this study could lead to more efficient and successful transfection protocols, potentially accelerating

the development of therapeutic interventions and advancing the understanding of cellular processes.

The zeta potential analysis of Xgene and its interaction with the 1:1 P188 surfactant has revealed significant insights into the molecular interactions within the system. The shift from 40 mV to 20 mV in zeta potential, accompanied by the inherent negative charge of P188, suggests poloxamer to transfection reagent interactions involving charge neutralisation or surface adsorption. Future research is needed to further investigate into the specific nature of the Xgene-P188 interaction, elucidate the impact of varying experimental parameters, and explore the practical implications of these findings in relevant applications. The fact that the zeta potential remains constant at different temperatures suggests that the mixture is suitable for use in physiological conditions. This stability at 37 degrees Celsius is particularly important for mammalian cell transfection applications, as it implies that the mixture is viable for use with cells incubated at this temperature.

Further research in this field could further elucidate the specific mechanisms by which P188 enhances transfection efficiency. This could involve investigating the role of P188 in improving membrane permeability, cellular uptake, or intracellular trafficking of genetic material. Comprehensive characterization of the complexes formed between P188, and the chemical transfection reagent can provide insights into their physical and chemical properties. Techniques such as NMR, X-ray crystallography, or other spectroscopic methods could be implemented to examine the nature of the interaction at the molecular level. Beyond zeta potential analysis, functional assays can be conducted to assess the practical consequences of the P188-X Gene interaction on transfection efficiency, cellular uptake, and gene expression.

6. Discussion of thesis main findings

6.1 Chapter 3 main findings

- Shear stress and apoptosis in mAb production:

The experiments conducted using baffled flasks as scaled-down models of bioreactors revealed critical insights into how shear stress influences apoptosis in CHO cells. The findings of the experiments using baffled flasks as scaled-down models of bioreactors demonstrated that the turbulent environment created by the baffles in the flasks led to an earlier onset of c-casp3 signal (Figure), a key marker of apoptosis. This early activation suggests that the cells are experiencing higher mechanical stress, which triggers the apoptotic pathways sooner than in smoother-walled flasks. These results are particularly significant when compared to the study by Zhan et al. (2020), which also linked high shear stress to increased apoptosis via pathways associated with ER stress, cytoskeleton reorganisation, and extracellular signalling. The earlier appearance of c-casp3 in cells exposed to higher turbulence underscores the direct impact of mechanical forces on cell viability—a key challenge in industrial bioprocessing. This finding is consistent with Grilo et al. (2019), who emphasised that apoptosis, driven by various stresses within bioreactors, is a major contributor to cell death, ultimately affecting culture longevity and product quality.

However, I aimed to offer detailed and controlled examination of the relationship between shear stress and apoptosis compared to what is typically observed in larger, less controlled bioreactor environments. The baffled flask model allows for the isolation of shear stress as a variable, making it possible to directly link the increased turbulence to the earlier onset of apoptosis, something that is more challenging to achieve in full-scale bioreactors where multiple variables are at play. This specificity in experimental design highlights the robustness of baffled flasks as a model system for studying the mechanical stresses that cells endure during bioprocessing.

The findings also align with the observations of Zhan et al. (2020), who reported that high shear stress not only promotes apoptosis but does so through multiple cellular pathways, including those related to oxidative stress and the unfolded protein response. My experiment adds to this by suggesting that the mechanical forces generated by turbulence in baffled flasks can initiate these stress responses earlier (Figure), leading to quicker activation of apoptosis. This earlier onset of cell death is particularly problematic in bioprocessing, as it can lead to reduced cell densities and lower overall productivity, reinforcing the need for strategies to mitigate shear stress in bioreactor environments.

- Role of surfactant P188 in mitigating shear-induced apoptosis:

In figure 5 and 7, I aimed to define the role of P188 in protecting CHO cells from shear-induced apoptosis in bioreactor environments. The findings revealed that the presence of P188 in culture media significantly delayed the onset of apoptosis, as indicated by the delayed appearance of the c-casp3 signal compared to P188-free media. This suggests that P188 plays a cytoprotective role, which is essential in environments where cells are subjected to high mechanical stress, such as in bioreactors. Peng et al. (2014) noted that shear protectants like P188 are crucial for maintaining cell viability during cell culture bioprocessing, especially under conditions of high turbulence and shear forces. However, the original research advances this understanding by providing empirical evidence that P188 can effectively delay the apoptotic process, thereby prolonging cell survival and potentially enhancing productivity.

The literature, such as the work by Peng et al. (2014), discusses the variability in the performance of different P188 lots, suggesting that maintaining a consistent and optimal concentration of P188 is critical for ensuring reliable bioprocess outcomes. In chapter two, DLS was used to quantify P188 and establish a calibration curve further contributes to this understanding by showing that a specific concentration of 0.15% w/w P188 yields outcomes that are more comparable to the standard Freestyle media than a lower

concentration of 0.1% w/w. This finding is particularly significant because it shows the importance of optimising P188 concentration to balance its protective effects with its impact on cell growth and productivity.

In figure 7 the delayed activation of caspase-3 in the presence of P188 observed in the original research aligns with the broader understanding of how surfactants can stabilise cellular membranes under stress. The cytoprotective effects of P188 are likely due to its ability to interact with the cell membrane, reducing the mechanical damage caused by shear forces. This mechanism is supported by the literature, including the study by Zhan et al. (2020), which linked mechanical stress to the activation of apoptosis through cellular pathways. I aimed to add to this narrative by showing that P188 can intervene in this process, delaying the stress-induced activation of apoptotic pathways, which is crucial for maintaining higher cell viability in industrial bioprocesses.

- Cell death mechanisms in bioprocess development and the role of surfactants:

One of the main contributions of the findings of chapter 2 is its detailed examination of how shear stress can activate different cell death mechanisms, particularly apoptosis, in CHO cells. The study's focus on the early activation of caspase-3 in response to shear stress aligns with the findings of Zhan et al. (2020), who noted that high shear stress stimulates apoptosis through various cellular pathways. However, the work in this thesis extends this understanding by demonstrating that shear stress does not act in isolation but is part of a broader context of environmental and mechanical factors that interact to influence cell fate. This is particularly relevant in bioreactor environments where cells are exposed to a range of stresses that can collectively impact viability and productivity.

The immunoblot in chapter 3 shows that the timing of caspase-3 activation varies depending on the type of surfactant present in the culture medium (Figure 7). Specifically, surfactants like P237, P407, and P338, which have higher molecular weights and greater polyethylene oxide (PEO) content, induce caspase-3 activation within 12 hours,

suggesting a quicker initiation of apoptosis. In contrast, cells grown in baffled flasks and exposed to P188, and PPG show caspase-3 activation after 24 hours, indicating a delayed apoptotic response. This variability suggests that surfactant molecular weight and composition significantly influence the rate of apoptosis. Surfactants with higher PEO content, such as P407 and P338, may not protect cells as effectively, leading to earlier apoptosis. This is consistent with findings from studies like those by Peng et al. (2014), which suggest that the protective efficiency of surfactants is closely linked to their molecular characteristics. Additionally, the presence of higher polypropylene oxide (PPO) content in surfactants like P407, despite their high molecular weight, also correlates with earlier caspase-3 activation, indicating that PPO-rich surfactants might interact differently with cellular membranes or intracellular components, thus accelerating apoptotic processes. These observations corroborate broader literature, which highlights the critical role of surfactant composition in modulating cellular stress responses and apoptosis (Grilo et al., 2019).

The findings of chapter 3 also point to the importance of evaluating different cell death pathways, as the study observed that apoptosis and autophagy are not isolated processes but can interact and influence each other under stress conditions. Han et al. (2011) discussed the mutual relationship between apoptosis and autophagy in programmed cell death. The results of c-casp3 and LC3 immunoblots suggest that in the context of bioprocessing, where cells are subjected to continuous stress, the balance between these pathways could be important for determining cell survival and productivity.

- Autophagy as a cellular response to shear stress and interaction with P188:

Braasch et al. (2021), found that modulating autophagy could enhance productivity in CHO cells. The signal in the LC3 immunoblot in chapter three shows the delayed onset of autophagy markers LC3-I and LC3-II in cells treated with various surfactants. Surfactants with higher molecular weights and different compositions, such as P407 and P338, tend to delay the appearance of LC3-II, a key marker of autophagosome formation.

This suggests that these surfactants may slow down the autophagic process, potentially leading to prolonged cellular stress and earlier onset of cell death. This observation contrasts with the function of P188, which was shown to protect cells more effectively by not delaying the autophagy process as significantly. The data shows that not all surfactants are equally beneficial in promoting autophagy, highlighting the need for careful selection of surfactants in bioprocess environments and how the levels of autophagy can be detrimental for cell viability in turbulent environments (Figure 8).

Han et al. (2011) discussed how nutrient supplementation can delay the onset of both apoptosis and autophagy, improving cell survival and productivity. Figure supports this view by demonstrating that specific surfactants can similarly modulate the autophagic process, delaying it in a way that may not always be beneficial for cell survival. The delayed conversion from LC3-I to LC3-II observed with certain surfactants suggests that these additives might interfere with the timely initiation and progression of autophagy, potentially compromising the cell's ability to cope with stress. The study observed that surfactants like P407 and P338, which delayed the autophagic process, also seemed to accelerate the onset of apoptosis, as indicated by the earlier activation of caspase 3. This finding suggests that when autophagy is impeded, cells may be more prone to undergo apoptosis, a relationship that has been noted in the literature but is further clarified by this research. Han et al. (2011) also noted the mutual relationship between autophagy and apoptosis, where the inhibition of one can led to the activation of the other.

The practical implications of chapter 3 findings are significant for the design of bioprocesses. Selecting surfactants that not only protect cells from shear stress but also promote a balanced autophagic response could be key to enhancing cell viability and productivity in bioreactor environments. The original research suggests that P188 might be a more effective surfactant for this purpose, as it does not delay autophagy as much as P407 or P338, thereby allowing cells to better manage stress and avoid premature apoptosis. This contrasts with some of the broader literature, which often focuses on the general benefits of autophagy modulation without considering the specific effects of different surfactants.

6.2 Chapter 4 main findings

- Micellization behaviour and surface activity as screening potential for P188 in pharmaceutical manufacturing:

Chapter 4 findings focus on the micellization behaviour and surface activity of P188, and how these changes influence its efficacy as a surfactant in bioprocessing environments. The observed alterations in micelle size and surface activity have significant implications for the stability and performance of P188 in maintaining cell viability under the conditions of bioreactors.

The DLS (Figure 4, Figure 5) results from the study showed that the addition of P407 to P188 at a 20% concentration caused a dramatic increase in micelle size, with an average radius 10 times larger than that of purified P188. This substantial shift in micellization behaviour highlights the sensitivity of P188's micelle formation to the presence of high molecular weight impurities. Compared to the literature, particularly the work of Chang et al. (2017), who demonstrated that P407 exhibits higher surface activity but lower cell protection than P188, the surface activity studies and the micelle size and intensity patterns of poloxamers in water and media indicate a mechanistic understanding of why this occurs. The larger micelles formed in the presence of P407 are likely less stable and more prone to disrupting cellular processes, as they fail to provide the consistent protection afforded by smaller, more uniform micelles of pure P188. This disruption likely results from the differing molecular weights and PEO content between the two poloxamers, as P407's higher molecular weight and lower PEO content lead to weaker chemical bonds in the micelles, causing them to become larger and less effective.

The dynamic surface tension analysis (Figure 6, 7) adds robustness to these findings, showing that the contaminated P188 exhibited significantly different surface tension dynamics compared to the purified P188. Specifically, the P188/P407 mixture demonstrated a higher initial surface activity, which gradually decreased over time, leading to less stable foam and potentially contributing to earlier cell death in culture. This behaviour contrasts with the performance of pure P188, which maintained more

consistent surface tension and foam stability, crucial for protecting cells in a bioreactor setting.

These results are particularly significant when compared to the findings of Safta et al. (2022), who noted that poloxamer variability, particularly in molecular weight distribution, can have a profound impact on cell viability and production outcomes in bioreactors. I aimed to provide a molecular explanation for these observations, demonstrating that even small amounts of higher molecular weight impurities like P407 (Figure 8) can drastically alter the micellization behaviour of P188, leading to less effective cell protection. The larger, less stable micelles formed in the presence of these impurities likely fail to adsorb efficiently to the cell membrane or gas-liquid interface, thereby diminishing the protective effects that are critical for maintaining cell viability under shear stress.

Moreover, the findings from Figure 4-7 suggest that the shift in micelle size and surface activity could serve as a reliable indicator of P188 contamination. This is in line with the work of Bareford et al. (2018), who emphasised the need for sensitive and rapid methods to detect impurities in P188, as these contaminants can compromise the surfactant's protective properties and lead to batch failures.

The implications of the findings in chapter 4 are important to enhance industrial bioprocessing. The ability to screen P188 batches for performance-affecting impurities using DLS and surface tension analysis could become a standard practice, much like the CMATH assay discussed by Narayanappa et al. (2019). While the CMATH assay provides a rapid biological assessment of poloxamer performance, it suggests that physical-chemical screening methods like DLS and surface tension analysis could complement these biological tests, providing a more comprehensive evaluation of batch quality. This combined approach would not only ensure that low-performance batches are identified and discarded but also enhance the overall reliability and efficiency of biopharmaceutical production processes. Furthermore, the original research highlights the broader potential of these screening techniques beyond just P188. The methods developed and validated in this thesis could be applied to other surfactants and excipients used in bioprocessing, offering a new level of quality control across various components

of cell culture media. This is particularly relevant as the biotechnology industry continues to push for higher product titres and more intensive bioprocessing conditions, where the quality and consistency of every component become increasingly critical.

6.3 Chapter 5 main findings

- P188 and plasma membrane resealing:

The ability of P188 to facilitate plasma membrane resealing following mechanical disruption, such as sonoporation and electroporation, could contribute to a significant advance in optimising transfection efficiency, particularly in the context of CHO-S cells. The integrity of the plasma membrane is critical for cell viability, and its disruption during transfection procedures often leads to cell death or diminished cellular function if not properly managed. P188's role in promoting membrane resealing is crucial in this regard, as it helps to maintain cell viability post-transfection, thereby improving the overall success of gene delivery.

The protective effects of P188 can be understood through its interaction with cellular membranes. Srikanth Sriadibhatla et al. (2006) have shown that Pluronics, including P188, promote membrane resealing and reduce cellular trauma after electroporation. This is particularly important because electroporation and sonoporation work by temporarily disrupting the cell membrane to allow the entry of genetic material. While this disruption is necessary for transfection, it also poses a risk to the cell, as prolonged membrane damage can lead to apoptosis or necrosis. P188 mitigates this risk by interacting with the lipid bilayer of the cell membrane, helping to patch and stabilise the membrane more quickly after disruption. This resealing process is critical not only for maintaining cell viability but also for ensuring that the transfected material is retained within the cell, which is a prerequisite for successful gene expression.

Further supporting this theory, Elena V. Batrakova and Alexander V. Kabanov (2008) showed that Pluronics can act as biological response modifiers by integrating into cellular membranes and altering cellular functions, such as mitochondrial respiration and ATP synthesis. This interaction with the membrane may be particularly beneficial in the context of transfection, where the energy demands of the cell are increased due to the stress of membrane disruption and the need to process the incoming genetic material. By stabilising the membrane and potentially supporting mitochondrial function, P188 could help cells to better cope with the stress of transfection, thereby enhancing cell survival and the efficiency of gene expression.

The ability of P188 to facilitate membrane resealing could have broader implications for its use in other forms of cellular therapy or drug delivery that involve mechanical disruption of the cell membrane. For instance, in therapies where cells are exposed to shear stress or other mechanical forces, the inclusion of P188 could help protect the cells from damage and improve their overall viability, thereby enhancing the therapeutic outcome.

- Potential impact of P188 on intracellular trafficking and gene expression:

The results in chapter 5 showed a marked improvement in the transfection efficiency of electroporated and chemically CHO-S cells when preincubated with P188 (Figure 7), leading to higher expression levels of the YFP plasmid. This suggests that P188 not only aids in the initial uptake of genetic material but also enhances the subsequent intracellular processes necessary for effective gene expression.

One of the key mechanisms by which P188 appears to exert its influence is through the modulation of intracellular trafficking pathways. Zhang et al. (2014) highlighted that amphiphilic block copolymers, such as P188, can assist nonviral vectors in overcoming later intracellular barriers by acting as biological response modifiers. The findings from the current study suggest that P188 might similarly modulate the interactions between the

transfected plasmid and the cell's intracellular machinery, promoting more efficient trafficking of the genetic material to the nucleus, where it can be transcribed.

The increased fluorescence intensity observed in cells treated with P188 (Figure 6) indicates successful transfection and subsequent expression of the YFP gene. This enhanced expression is likely due to P188's role in facilitating the intracellular transport of the plasmid. Once inside the cell, the plasmid must navigate a complex intracellular environment, avoiding degradation and successfully reaching the nucleus. P188's ability to interact with and stabilise cellular membranes could play an important role here, not just by protecting the plasmid during entry but also by assisting in its trafficking through the cytoplasm.

The findings of chapter 5 also resonate with the research of Batrakova and Kabanov (2008), who demonstrated that Pluronics can enhance gene expression by modulating cellular functions, including the activity of drug efflux transporters and signal transduction pathways. In the context of my experiments, P188 may be influencing similar pathways to ensure that once the YFP plasmid is internalised, it is not only preserved but also efficiently transported to the site of action within the cell.

Further analysis of the chemically transfected cells revealed that P188-treated cells exhibited a more robust expression of the YFP gene compared to controls (Figure 6,7). This finding suggests that P188 may have a dual role: first, in facilitating the physical entry of the plasmid into the cell, and second, in enhancing the cell's internal processes to support gene expression. This dual functionality could be attributed to P188's known effects on cellular energy metabolism, as described by Batrakova and Kabanov (2008). By stabilising mitochondrial function and enhancing ATP synthesis, P188 might provide the energy necessary for the cell to effectively process and express the transfected gene. The original research also pointed out that preincubation with P188 led to a more pronounced increase in transfection efficiency compared to post-transfection treatment. This observation suggests that P188's primary action occurs early in the transfection process, possibly by preparing the cell's intracellular environment to better accommodate and process the incoming genetic material. This is consistent with the idea that P188 can

modulate intracellular trafficking pathways, potentially by interacting with components of the cytoskeleton or endosomal membranes, thus facilitating the movement of the plasmid toward the nucleus.

- Synergistic effects of P188 with chemical transfection reagents:

In addition to these effects, the study's results on zeta potential changes (Figure 8) provide more information on how P188 might influence intracellular trafficking and gene expression. The shift towards lower zeta potential values of transfection reagent Xtreme Gene HD in the presence of P188 indicates a reduction in surface charge, which could lead to reduced aggregation and enhanced stability of the transfection complex. This change in zeta potential might also impact the interactions between the plasmid and intracellular membranes, making it easier for the plasmid to traverse the cytoplasm and reach the nucleus.

One of the most interesting findings of chapter 5 is the idea that P188's effects are not limited to its well-known roles in membrane resealing and cellular uptake. The research results indicate that P188 may also interact favourably with lipid-mediated transfection reagents, such as Xgene, to create a more conducive environment for gene delivery (Figure 8-10). The investigation of zeta potential behaviour further supports the theory of a synergistic interaction between polymer and lipid mediated transfection reagents. The observed shift in zeta potential towards lower values upon the introduction of P188 suggests that P188 can alter the electrostatic environment of the transfection complex. This shift could reduce electrostatic repulsion between particles in the transfection mixture, allowing for closer interaction between the plasmid and the lipid-based vector. The reduction in zeta potential may enhance the stability of the transfection complex, ensuring that the plasmid remains associated with the lipid vector until it reaches the target cells. This improved stability and reduced aggregation may result in a more efficient delivery of the genetic material, ultimately leading to higher transfection efficiency.

Batrakova and Kabanov (2008) reported the importance of surface charge in the behaviour of polymer-based delivery systems, noting that Pluronics can affect the zeta

potential of nanoparticles, thereby influencing their colloidal stability and interactions with cellular membranes. In figure 8, the interaction between P188 and Xgene led to a neutralisation or shielding of surface charges, reducing electrostatic repulsion and potentially stabilising the transfection complex. This protective effect could be particularly important in industrial applications where maintaining the viability and functionality of the transfection components is critical for achieving high efficiency and consistency. The reduced repulsion could facilitate closer interactions between the genetic material and the cell membrane, enhancing the likelihood of successful uptake. This modification could lead to more stable and efficient transfection systems, as well as improved outcomes in gene delivery applications.

7. Summary of results

This thesis has undertaken a detailed exploration into the molecular characteristics of P188 and its effects on the viability of Chinese Hamster Ovary (CHO) cells under mechanical stress, a topic of growing importance in biotechnological applications, particularly in bioreactor environments. The approach of this research, utilising a scaled-down model of bioreactors through baffled Erlenmeyer flasks, has allowed for the replication of sparging and shearing forces typically observed in larger-scale bioreactors. This model has been instrumental in showcasing the protective mechanisms offered by P188 in environments subjected to enhanced hydrodynamic forces.

The investigation revealed that the presence of P188 in cell culture under shear stress media significantly delays the appearance of cleaved c-casp3, a key marker of apoptosis. This finding is crucial as it indicates the potential of P188 in mitigating apoptosis, a common challenge in bioreactor-based cell cultures. The protective role of P188 is further underscored by the observation that other surfactants, with varying molecular weights and polyethylene oxide/polypropylene oxide (PEO/PPO) compositions, do not exhibit the same efficiency in delaying apoptotic processes, which can explain why common impurities can hinder the protective efficacy of P188. In addition to apoptosis, the study has shed light on the impact of P188 and other surfactants on the autophagic pathways within cells. The LC3 immunoblot data pointed towards a delayed autophagic response in the presence of surfactants richer in PEO, compared to other surfactants. This delay in the appearance of LC3-1 and LC3-2 signals suggests a possible modulation of the autophagy pathway by P188, providing a new perspective on how cell survival mechanisms can be influenced by surfactant composition. Cells exposed to mechanical stress, as simulated in the bioreactor model using baffled flasks, activate a range of molecular pathways to cope with the stress. The protective role of P188 observed in this study suggests that it can be integrated into the existing models of cell stress response. This integration is crucial for optimising cell culture conditions in biotechnological applications, particularly for enhancing the survival and productivity of cells in bioreactors.

The research also investigated the chemical and interfacial properties of P188, through experiments assessing micelle size and distribution, surface tension, and critical micelle concentration (CMC) in the presence of P188. These studies have been vital in describing the surfactant characteristics of different P188 batches, offering information into how impurities in P188 can alter its protective efficacy against shear stress in cell cultures. A significant finding from these experiments is the enhanced surface activity observed in P188 contaminated with impurities or mixed with P407, which is associated with lower cell viability over time, compared to purified P188. The ability to manipulate and understand these interactions has profound implications, ranging from improving cell culture techniques to enhancing the efficacy of biotechnological processes as it can potentially lead to a new process to screen out low performing batches of P188 without the use of time consuming and unreliable cell culturing.

The study has also made contributions to the field of gene therapy and biotechnology by demonstrating the effectiveness of P188 in enhancing transfection efficiency in CHO cells. The use of electroporation and chemical transfection methods revealed the synergistic effects of P188 treatment, highlighting its role as a potential enhancer of gene delivery in chemical transfection and electroporation. This aspect of the study is particularly relevant in the context of developing more efficient and successful transfection protocols, which are vital for therapeutic interventions and advancing cellular research.

The zeta potential analysis in chapter 3 further provided insights into the molecular interactions between the Xgene transfection reagent and P188, suggesting complex interactions that could be pivotal in improving transfection efficiency as well as understanding how molecule charges play a role in the efficacy of transfection reagent and plasma membranes. These findings also prove an essential need for further research to fully understand the mechanisms by which P188 enhances gene delivery and its mechanisms of interaction.

In summary, this thesis has provided a comprehensive understanding of the molecular characteristics of P188 and its impact on CHO cell viability under mechanical stress. The data can potentially have significant implications for biotechnological applications,

particularly in optimising cell culture conditions and enhancing gene delivery processes. While the data obtained offers valuable insights, it also opens up avenues for further research to explore the intricate mechanisms by which P188 and other surfactants influence cell survival pathways as well as other molecular interactions. Future studies could expand on understanding different types of cell death, such as necroptosis or ferroptosis, and delve deeper into the molecular interactions of P188 with other compounds in cell culture media.

8. Supplemental

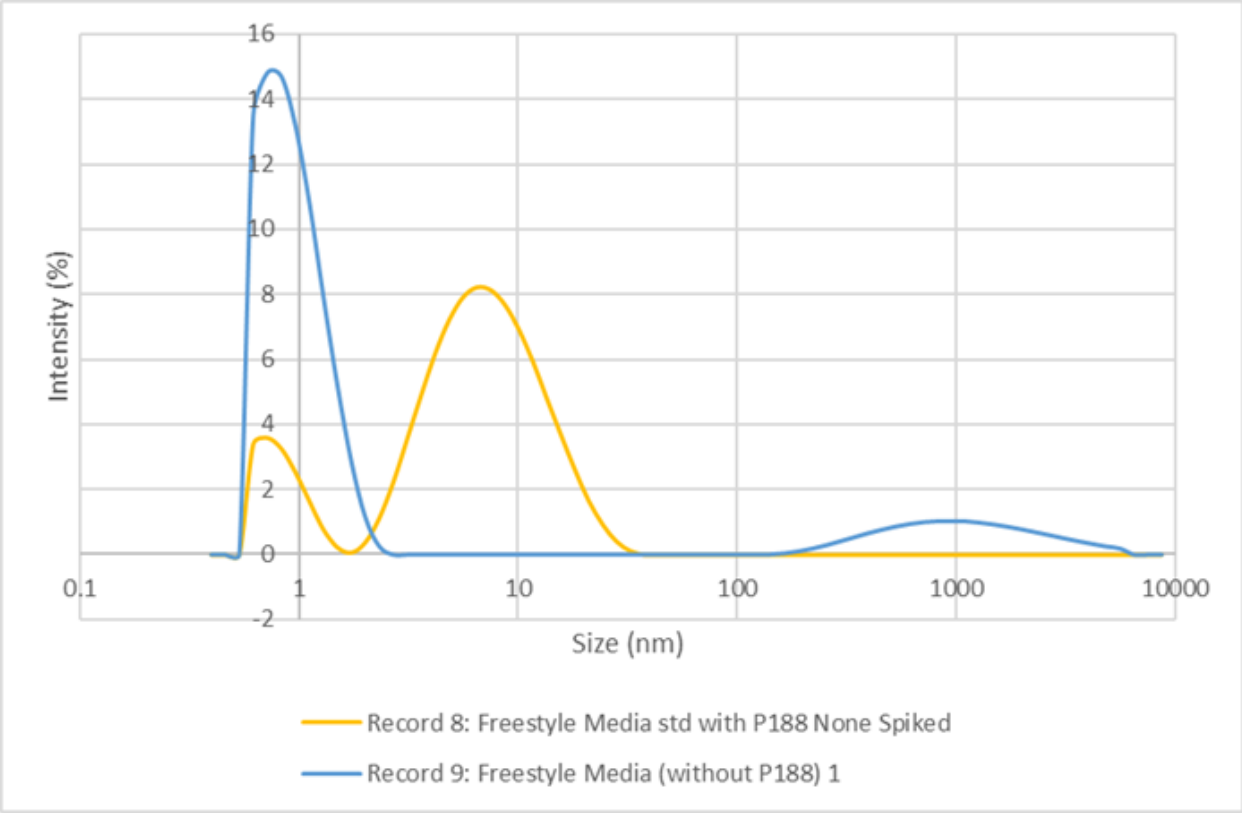


Figure1 : Analysis of Size Distribution using Dynamic Light Scattering: Contrasting Standard Freestyle Media with Inherent P188 and Custom-Formulated Media Devoid of P188.

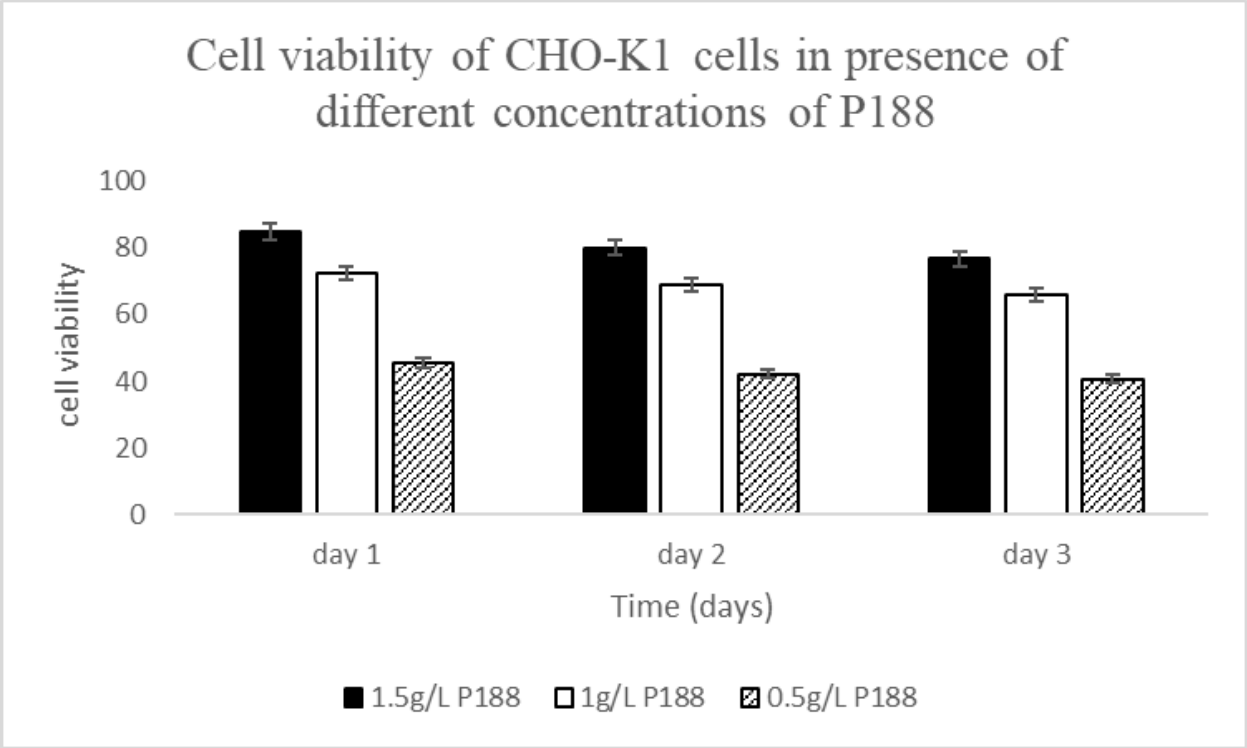


Figure 2: Effects of different concentrations of P188 on cell viability of CHO-K1 cells over three days culture

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